Human amylin (hA), a 37-amino-acid polypeptide, is one of a number of peptides with the ability to form amyloid fibrils and cause disease. It is the main constituent of the pancreatic amyloid deposits associated with type 2 diabetes. Increasing interest in early assembly intermediates rather than the mature fibrils as the cytotoxic agent has led to this study in which the smallest hA oligomers have been captured by atomic force microscopy. These are 2.3 ± 1.9 nm in height, 23 ± 14 nm in length, and consist of an estimated 16 hA molecules. Oligomers first grow to a height of about 6 nm before they begin to significantly elongate into fibrils. Congo red inhibits elongation but not the growth in height of hA oligomers. Two distinct phases have thus been identified in hA fibrillogenesis: lateral growth of oligomers followed by longitudinal growth into mature fibrils. These observations suggest that mature fibrils are assembled directly via longitudinal growth of full-width oligomers, making assembly by lateral association of protofibrils appear less likely.

Amylin is one of a number of peptides with the ability to form amyloid deposits occurring in disease (1). It is the main constituent in the deposits that form in the islets of Langerhans of the pancreas in type 2 diabetes mellitus (2, 3). The deposits in this disease correlate with the depletion of islet β-cells and are considered a hallmark feature of disease progression. Amylin, a 37-amino-acid peptide, is produced in the islet β-cells and co-secreted with insulin. It belongs to a family of peptides (amylin, calcitonin, calcitonin-gene-related-peptide, and adrenomedulin), sharing to varying extents metabolic functions in the control of nutrient assimilation, storage, and disposal (4–10).

The propensity of amyloid proteins to form fibrils in vitro parallels their cytotoxicity to cultured cells (11–14). Amylin peptides derived from different species illustrate this point. Human amylin (hA) forms amyloid-like fibrils in vitro and is toxic to cultured pancreatic islet β-cells (15). In contrast, the rat amylin isoform (rA), despite having 84% amino acid sequence identity, does not form fibrils and is not cytotoxic. Mounting evidence suggests, however, that the fibrils themselves are not the toxic agent in the amyloid diseases but rather some intermediate structures (16–23). In the hA transgenic mice, for example, β-cell death and impaired insulin secretion occur before fibrillar deposits can be detected (23).

The polymorphic aspects of mature hA fibrils have been well characterized, but studies investigating the assembly process remain limited (24). Kayed et al. (25) described a partially folded population of hA molecules that, with its self-associated forms, may act as an early, soluble precursor of β-sheet and in amyloid fibril formation. This partially folded state has been characterized as adopting a higher content of secondary structure and as having fluctuating tertiary and quaternary aromatic interactions and solvent-exposed hydrophobic patches. Their data support a nucleation-dependent process with a distinctive lag time for hA fibril formation. Other researchers have also proposed a nucleation-dependent aggregation mechanism (26, 27), which differs from the former in that it postulates the existence of a reservoir, which maintains a constant concentration of soluble monomeric peptide. Padrick and Miranker (27) extended this proposal by suggesting that hA fibril formation occurs via a double-nucleation mechanism. This includes the formation of nuclei (primary nucleation) that then elongate followed by a further step, which is fibril-dependent (secondary nucleation). One of the proposed mechanisms by which this occurs is by “templated nucleation” using a non-growing surface of a fibril. This secondary nucleation may be insignificant initially but becomes important once fibrils accumulate.

Time-lapse atomic force microscopy (AFM) was used to trace hA fibril formation in real time, identifying a putative hA protofibril and observing that it grew bidirectionally with a speed of 1.1 nm/min under the conditions used (28). The protofibril was proposed to be a “building block” of the thicker higher-order mature fibrils (24, 28), which were thought to form by lateral association of protofibrils. However, such side-by-side association of protofibrils has never been directly observed. Mature fibrils with frayed ends, which would support the notion of a lateral association of protofibrils, were not observed. An open question therefore remains about the nature of the assembly mechanism of the mature higher-order hA fibrils. Specifically, do they assemble by lateral association of long preformed protofibrils, or do they form by longitudinal growth of full-width structures? Our new data point to the latter as the more likely scenario. We first illustrate how the pretreatment of lyophilized hA with 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) produces an initial solution of small oligomeric structures, which can be observed by AFM. By time-lapse
imaging, we were able to show that these oligomers first increase in height before elongating into mature fibrils. Congo red partially inhibits the height increase of the oligomers and fully inhibits elongation into fibrils, also supporting the notion of a two-step assembly process. Our observations thus reveal an important new aspect, which needs to be considered in the light of increasing evidence that some distinct oligomeric intermediates rather than the mature fibrils are the cytotoxic agents.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Preparation**—Lyophilized preparations of synthetic hA (lot numbers 514905, 538994) and rA (lot number 542554) were purchased from Bachem (Torrance, CA). Purity was checked by high pressure liquid chromatography and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Amylin stock solutions were prepared by weighing out the appropriate amount of lyophilized peptide and solubilizing it in HFIP (Fluka) for 1–2 days. The stock solutions of amylin in HFIP were prepared in the concentration range of 0.5–3.2 mM as per BCA assay. At the start of each experiment, amylin HFIP solutions were diluted into buffer, or the HFIP was evaporated off under a nitrogen flow and the amylin was dissolved in water.

**Atomic Force Microscopy**—For the visualization of hA oligomers at specified time points, a fresh solution was prepared (10 μM amylin, 10 mM Tris-HCl, pH 7.4, 1.25% HFIP) and added to freshly cleaved mica. After adsorption for 30–120 s, the sample was washed three times with water and imaged under water. For time-lapse AFM, 10 mM Tris-HCl, pH 7.4, alone was scanned initially to check for the presence of any contaminants. Freshly made amylin solutions were injected into the fluid cell to produce a final concentration of 5–10 μM (0–1.25% HFIP, 10 mM Tris-HCl, pH 7.4). Congo red was added to hA in water, just prior to the addition of buffer and injection into the AFM fluid cell (8 μM hA, 240 μM or 2.4 mM Congo red, 10 mM Tris-HCl, pH 7.4). Congo red solutions were freshly prepared in water and filtered three times. Images were obtained with a Nanoscope IIIA multimode scanning probe work station (Veeco, Santa Barbara, CA) operating in tapping mode using a silicon nitride cantilever with a spring constant of 0.32 N/m. All imaging was obtained with a Nanoscope IIIa multimode scanning probe work station (Veeco Nanoscope software version 5.12). In the case of time-lapse AFM experiments, fibril and oligomer lengths used to estimate the mass of the oligomers were measured using the sectioning tool (Veeco Nanoscope software version 5.12). Treating the hA oligomers as a partial sphere, the molecular volume was estimated by using the height and lengths, at half-maximal height as described previously (29). The number of monomers in the oligomers was deduced by dividing the volume of the hA oligomers by the calculated molecular volume for a hA monomer (29). Alternatively, the mass of the oligomers was estimated by dividing the height of the oligomers by 6.9 nm, multiplying by 20 kDa/nm and the length of the oligomer, measured at half-maximal height since the mass-per-length of 6.9 nm high fibrils was determined previously by scanning transmission electron microscopy to be 20 kDa/nm (24, 28).

**RESULTS**

**Hexafluoroisopropanol (HFIP) Dissolves Preformed Structures and Synchronizes Assembly of hA Oligomers and Fibrils**—To examine the early stages of hA fibril assembly, we had to assure ourselves that the stock solution, when diluted into water/buffer at the start of an experiment, did not contain preformed fibrils or assembly intermediates. When hA was dissolved in water and injected into the fluid cell of the AFM filled with 10 mM Tris-HCl, pH 7.4, structures of 90–500 nm in length and 7–20 nm in height appeared within 4–20 min (Fig. 1a, 4-min time point). The structures were polymorphic, representing a heterogeneous mixture of various lengths of fibrils and different sized oligomers. This suggested the presence of preformed structures in the lyophilized stock of hA. The time-lapse AFM experiment revealed that fibrils ranging from about 4 to 6 nm in height, started to grow out of these preformed fibril seeds within the next 20–30 min, elongating further over the next 3 h (Fig. 1a, 44-, 95-, and 237-min time points).

To render the starting preparation more homogeneous, we initially dissolved lyophilized hA in HFIP before injection into the buffer-filled fluid cell of the AFM. With this pretreatment, the initial oligomers that appeared were much smaller in height, i.e. ranging from 0.7 to 3.6 nm, in comparison with those that formed when the peptide was dissolved in water (Fig. 1b, 18-min time point). The oligomers were initially 9–65 nm in length and elongated into mature fibrils over the next 30–60 min (Fig. 1b, 35- and 48-min time points). In comparison with the polymorphic fibrils formed from hA stock directly dissolved in water (Fig. 1a), following pretreatment with HFIP, fibrils were generally shorter and more numerous (Fig. 1b, 27-min time point). As expected, rA did not form fibrils under the same conditions, and no oligomeric structures were observed even after 3.5 h of imaging (data not shown). In all of the following experiments, we therefore dissolved hA initially in HFIP, as this enabled the smallest structure intermediates to be imaged in a more reproducible fashion.

**Growth of Oligomer Precedes Fibril Elongation**—Two kinds of experiments were performed to capture the early phases of hA fibril assembly. First, we let assembly begin in a drop of freshly diluted hA on a mica surface. Oligomers and fibrils were free to assemble in the bulk of the solution and adsorb to the mica at any time. Assembly intermediates were trapped at 30, 60, or 120 s by removing the bulk solution and washing the mica surface with water to remove all non-adsorbed structures and soluble hA. The mica was then imaged under water by tapping mode AFM (Fig. 2). After 30 s, small round oligomeric structures were predominant that had an average height of 1.9 nm and length of 23 ± 14 nm (n = 166) (Fig. 2a). Oligomers were also observed in the samples that had been incubated for 60 s; however, their height was 4.6 ± 2.1 nm, and their length was 47 ± 28 nm (n = 160) (Fig. 2b). The number of oligomers increased with the time of adsorption, and short fibrils were observed occasionally after 60 s incubation. In samples that had been allowed 120 s for assembly and adsorp-
tion, typical hA fibrils were formed (Fig. 2c). Measurements of their heights and lengths were 10.6 ± 7.8 nm and 203 ± 170.0 nm, respectively (n = 198). The height and length measurements obtained from all three time points were significantly different from each other (p < 0.0001).

This first series of experiments shows that a significant increase in oligomer size occurs before fibrils visibly elongate. This initial enlargement is evident as a doubling of height and length of the oligomer. The height roughly doubles again upon elongation to fibrils. This is distinct from a scenario where protofibrils elongate and subsequently associate laterally to form thicker fibrils (24, 28).

Time-lapse AFM Quantifies Oligomer Growth—To confirm that the oligomers grew in height before elongating into fibrils, a second kind of experiment was performed. We used time-lapse AFM to watch hA fibrils form in real time. For this, the fluid cell of the AFM was first filled with 10 mM Tris-HCl, pH 7.4, and the mica surface was examined for the presence of any contaminants. An equal volume of freshly diluted hA was then injected into the fluid cell, and the appearance and growth of hA structures on the mica surface were monitored by repeated scanning. Oligomers with heights between 0.7 and 3.6 nm formed on the mica surface within 4–10 min and elongated into fibrils during the next 40–50 min (Fig. 3a). When the height versus length was plotted for individual structures under this assembly regime, it became evident that significant height increases occurred early on in most cases, often before a substantial increase in length (Fig. 3, a and b). When all the heights and lengths from the time-lapse AFM experiments were graphed together, a pattern emerged whereby the oligomers increased in height first, and only when they reached about 6 nm in height did they start to significantly elongate (Fig. 3c). This pattern only emerged clearly when averaging many structures and was less obvious when studying individ-
ual structures (Fig. 3b), probably due to the time required to record each picture frame (4 min).

In conclusion, both kinds of experiments clearly demonstrate that hA oligomers grow in height before they visibly elongate into fibrils. On average, the height increases from an initial 2 nm to about 6 nm before significant elongation starts (Fig. 3c). A further increase in height to 8–10 nm occurs concomitantly with elongation.

Congo Red Allows hA Oligomer Growth but Inhibits Elongation into Fibrils—Congo red is known to interfere with hA fibril formation (30). In light of our data, which have distinguished an initial phase of oligomer growth from a secondary phase of elongation into fibrils, it was of interest to determine at what stage Congo red intervened. For this, we used time-lapse AFM. Again, without the compound, hA formed oligomers within 4–10 min after injection, which elongated into mature fibrils covering much of the mica surface during the next 40–50 min (Fig. 4a). The fibrils that formed after 40–50 min were on average 11.5 ± 4.7 nm in height and 224.9 ± 168.7 nm in length (n = 227) (Fig. 4c). In contrast, hA, to which a 30× molar excess of Congo red had been added, prior to the addition of buffer and injection into the fluid cell of the AFM, formed oligomers within 4–10 min on the mica surface, which did not elongate over time (Fig. 4, b and e). After 90 min of imaging, the oligomers grew to an average height of 3.74 ± 1.1 nm and length of 50.6 ± 26.5 nm (n = 348) (Fig. 4d). Only very few fibrils formed in the presence of the compound. Congo red on its own, at equivalent concentrations, did not produce any precipitates on the mica surface that could have been misinterpreted as hA oligomers even after extensive imaging (data not shown).

It appears that Congo red completely prevents the elongation of the oligomers into fibrils. However, in the presence of the inhibitor, the oligomers were still capable of increasing laterally to a height of 3.74 nm, in comparison with the oligomers formed on a mica surface after 30 s, which had a height of only 2.3 nm. These data lend further support to our novel finding that hA fibrillogenesis follows two distinct phases: oligomer growth and elongation into fibrils.

The Number of hA Molecules in the Oligomers Can Be Estimated from AFM Data—Dimensions recorded by AFM can be used to crudely estimate the mass of protein assemblies or oligomers (29), and therefore, the number of protein molecules per structure. We used the height and length data recorded from hA oligomers to calculate their molecular volume (29). The number of hA molecules was deduced by dividing the volume for the oligomers by the calculated volume for an hA monomer. Using the data from the time-lapse AFM experiments (Figs. 1b and 4a), oligomers of <4 nm in height had an estimated mass of 330 ± 140 hA monomers (n = 29). The smallest oligomers, which had a height <2 nm, were estimated...
to contain $67 \pm 28.5$ $(n = 5)$ hA molecules. Alternatively, using the data from the timed adsorption experiments (Fig. 2a), oligomers with heights of approximately $<2$ nm or $2–4$ nm were composed of $19 \pm 10$ $(n = 60)$ and $63 \pm 36$ $(n = 22)$ hA molecules, respectively. The latter set of estimates was considered more reliable as it was based on larger particle numbers but was probably still inaccurate because the protein density used to calculate the mass was for globular proteins. Therefore, an alternative estimate was also made using the mass density derived from previous scanning transmission electron microscopy mass-per-length measurements. The predominant hA fibril type with a 25 nm periodicity had a mass-per-length value of $20$ kDa/nm and a height of $6.9$ nm (24, 28). Assuming that hA oligomers had a packing density similar to this fibril, we estimated the oligomer mass by dividing the height of the oligomers by $6.9$ nm and multiplying by the length of the oligomer and the mass-per-length value of $20$ kDa/nm. Applying this to the data from the timed adsorption experiments, oligomers $<2$ nm or $2–4$ nm in height were estimated to contain $16 \pm 4.6$ and $40 \pm 14.5$ hA molecules, respectively. We consider these as the most accurate estimates for the molecular size of hA oligomers at this stage.

**Fig. 4.** Congo red inhibits the growth of hA oligomers into fibrils. a and b, time-lapse AFM experiments for $8 \mu M$ hA (a), $10$ mM Tris-HCl, pH 7.5, and $8 \mu M$ hA plus $2.4$ mM Congo red, $10$ mM Tris-HCl, pH 7.5 (b). c and d, the heights and lengths of the oligomers and fibrils formed for hA (c) and hA (d) in the presence of $2.4$ mM Congo red at $\sim 50$ and $90$ min, respectively. e, a zoom of the oligomers formed in the hA plus Congo red time-lapse experiment shown in b (framed in the 90-min time point).
Amylin has been known for some time to be the principal molecular constituent of the amyloid deposits that form in the pancreas of patients with type 2 diabetes. Yet the mechanism of amyloid formation by amylin and its role in disease progression have remained poorly understood. Increasing evidence points to oligomeric intermediates rather than the mature fibrils as the cytotoxic agents (18, 31, 32). Our in vitro assembly experiments with synthetic amylin reveal, for the first time, two distinct phases of fibril formation: oligomer growth followed by fibril elongation. The smallest oligomers visualized may contain as few as 16 hA molecules. They have a height of about 2 nm and a length of 23 nm, and they grow to about 6 nm in height before visibly elongating into fibrils.

Based on these data and the data of others, we propose a model for the assembly of hA fibrils (Fig. 5) (25). Monomeric, soluble hA exists in a predominantly random coil conformation in solution (25, 33). On acquiring secondary structure (a mixture of α- and β-structure), amylin begins to assemble into fibrils (33). Initially, oligomeric structures form, and upon reaching a critical size, they act as nuclei for fibril elongation. Although this critical size appears to be about 6 nm in height, oligomers may also grow larger concomitantly with elongation, leading to fibrils 8–10 nm high and giving rise to the previously observed fibril polymorphisms.

Time-lapse AFM represents a powerful tool to directly follow the growth of individual fibrils (28). The original time-lapse AFM work performed by Goldsbury et al. (28) revealed growth of both the 2.4-nm protofibril and a variety of thicker fibrils. The latter were the first to appear, whereas the protofibrils only appeared after a lag phase of approximately 2 h, when hA concentrations were likely to be significantly reduced. Protofibrils did not feature in our present experiments, which focused on the first 2 h only. We can confirm, therefore, that the thicker fibrils represent the primary product, which, in turn, implies that substantial growth of hA oligomers or nuclei precedes fibril assembly.

We have been able to image hA oligomers by time-lapse AFM because of the use of HFIP, which was able to dissolve fibril seeds in stock solutions. We estimated the mass of the smallest hA oligomers, observed by AFM, to be composed of 16 monomers. This size of the smallest hA oligomers is similar to mass estimates for other amyloid oligomers reported in the literature. For example, a fibril aggregation unit was described in the case of recombinant amyloidogenic light chain variable domain, SMA, and was reported to consist of 20–24 monomers (34). Further, in the case of amyloid β-peptide, a micelle spherocylinder was described consisting of 30 monomers (35), and in the case of phosphoglycerate kinase (PGK), an oligomer was reported consisting of 10 monomers (36). The smallest oligomers that we were able to image averaged 2.3 nm in height. Interestingly, this height is close to the 2.4 nm height of the previously described hA protofibrils (28). This remarkable consistency suggests that the 2.4 nm protofibril, which has so far only been observed as a secondary phenomenon by AFM, i.e., when fibril growth was confined to a mica surface, in fact represents the thinnest possible hA fibril. As outlined above, hA oligomers tend to grow to form full-width nuclei, giving rise to thicker fibrils as the primary product of hA assembly.

In our first set of experiments, hA oligomers predominantly increased in height with increasing incubation periods. This was confirmed in the second set of experiments using time-lapse AFM, which clearly showed that fibrils started elongating only after oligomers had grown to a height of ~6 nm. Our results support a mechanism by which fibrils grow directly from hA oligomers or nuclei, which already have the full thickness, rather than by lateral association of protofibrils. We have seen that oligomeric species first increase in height on the mica surface, probably by the stacking of the smallest oligomers. This may actually be the first visualization of a secondary nucleation mechanism hypothesized by Padrick and Miranker (27). The lateral association of small oligomers prior to their longitudinal growth may possibly describe their results. Notwithstanding that our experiments were performed on a mica
surface that might constrain the assembly of hA fibrils, we believe that the same two-step mechanism probably occurs in solution. It would explain why the 2.4 nm protofibril is seldom observed on its own except when growth is confined to a surface and the monomeric amylin concentration is depleted (28). Furthermore, oligomers reaching slightly variable heights prior to elongation may explain the different hA fibril polymorphisms that had been described previously (Fig. 5) (24, 26). This way, an oligomer that grows to 6.9 nm in height before it elongates could give rise to the prominent 25-nm-repeat left-handed coiled fibril, thought previously to assemble by coiling two protofibrils around each other. Oligomers that grow larger in height than this could give rise to mature fibrils consisting of three or more protofibrils.

One might argue that the initial increase in the height of the hA oligomer represents an elongation in the direction of the fibril axis. In this scenario, the oligomer would absorb so that the fibril axis is perpendicular rather than parallel to the mica surface; thus, when the oligomer elongates, its height increases. When the oligomer becomes too long, it tips over, and as it lays down on the mica surface, it elongates further. We consider this interpretation unlikely as it would mean that the growth of one of the ends of the fibril is blocked by the initial adsorption to the mica surface. This is clearly not the case because the fibrils observed in the present study generally grew bidirectionally. In fact, the same bidirectional growth was also observed previously for hA protofibrils on mica (28). One might also expect that the end of the fibril, which was initially adsorbed to the mica, would be deformed; however, there is no evidence of this. Thus, we believe that our two-stage model is the most plausible in that oligomers first laterally grow before they start elongating.

We have further demonstrated that time-lapse AFM can be used to assay the inhibitory effect of selected compounds on fibril formation. Congo red appeared to stabilize the hA oligomers, blocking both their lateral and longitudinal growth into mature fibrils, with inhibition of the former process occurring only in part. The inhibitory effect of Congo red is generally consistent with the literature (13, 30, 37, 38), but not when it comes to details of the mechanism. Podlisy et al. (37) reported that Congo red stabilized amyloid-β monomers before they formed oligomers. Also, Kim et al. (38) reported that at high concentrations of Congo red, the dimeric immunoglobulin light chain variable domain, SMA, favored protein unfolding over aggregation (37). Our observation that Congo red stabilizes hA at the oligomer level is particularly interesting in light of the current view that distinct amyloid oligomers rather than the polymorphic fibrils are the toxic agent. The situation may, however, be more complex as Congo red has also been reported to inhibit hA cytotoxicity and membrane activity (13, 30, 39, 40).

AFM is unique in its ability to image dynamic processes at the nanometer scale and in real time under physiological buffer conditions. One perceived drawback is that fibril growth may be constrained by the mica surface. It should be noted, however, that in vivo environments also contain many surfaces such as the cell membrane. There may actually be an advantage that interactions with a surface result in the stabilization and/or accumulation of structures that are not easily detected in bulk solution, as is the case for the 2.4-nm hA protofibril (28). Extending from this, direct visualization of the two-step process by which hA oligomers grow into fibrils has been achieved by trapping the oligomers on a surface and directly visualizing their growth and elongation by time-lapse AFM.