FEBS Letters 390 (1996) 124–128 FEBS 17272

Hypothesis

A mechanism for β-amyloid overproduction in Alzheimer's disease: precursor-independent generation of β-amyloid via antisense RNA-primed mRNA synthesis

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Received 19 April 1996; revised version received 7 June 1996

Abstract The overproduction of β-amyloid (A_β) appears to be a primary cause of Alzheimer's disease (AD). A_β can be generated by proteolytic cleavage of precursor protein ($_\beta$APP) at β- and γ-secretase sites in both disease and normal cells. There is, however, no evidence that proteolytic processing of $_\beta$APP in sporadic AD-affected tissues differs qualitatively or quantitatively from that occurring in normal cells, and additional pathways for the enhanced production of A_β in sporadic AD which constitutes the majority of all AD cases should be considered. The major factor limiting the production of A_β in normal cells is cleavage at the α-secretase site within the A_β sequence. But, whereas the intact $_\beta$APP is a substrate for cleavage at the α-secretase site, the immediate precursor of A_β, 12-kDa C-terminal $_\beta$APP fragment, is not susceptible to the α-secretase cleavage but it can be cleaved by γ-secretase thus generating A_β. Moreover, the γ-secretase cleavage is not the rate-limiting step in the production of A_β. Therefore, the increase in production of the 12-kDa C-terminal $_\beta$APP fragment may be an efficient way to overproduce A_β. A mechanism for the generation of the 12-kDa fragment independently of $_\beta$APP is proposed. It postulates an additional step of amplification of mRNA, namely the antisense RNA-mediated generation of a truncated mRNA encoding 12-kDa C-terminal fragment. Initiation of translation at the first AUG in the truncated mRNA results in a polypeptide that is cleaved by γ-secretase generating A_β. The proposed model makes several verifiable predictions and suggests new directions of experimentation that may lead to a better understanding of the mechanisms involved in AD.

Key words: Alzheimer's disease; $_\beta$-Amyloid precursor protein ($_\beta$APP); $_\beta$-Amyloid (A_β); $_\beta$-Amyloid overproduction; Antisense RNA; mRNA replication

1. Introduction

The accumulation of β-amyloid (A_β), a small 4-kDa segment in the C-terminal portion of β-amyloid precursor protein ($_\beta$APP), is associated with and considered by many to be the primary cause of Alzheimer's disease (AD) [1]. Although the accumulation of A_β might be due to its increased aggregation or decreased clearance, recent results from a number of investigators strongly suggest that the overproduction of A_β is, in fact, the major cause of its accumulation and, consequently, of AD. The notion of the causative role of A_β overproduction in AD is supported by several types of results: a number of mutations in the $_\beta$APP gene that underlie some forms of familial AD were found to increase the production of A_β [2–4]; cells from patients with a defect in the S182 gene, associated with the most common form of the early-onset AD, make abnormally high amounts of A_β [5]; Down syndrome, associated with trisomy 21 and, consequently, increased dosage of the $_\beta$APP gene which is located on chromosome 21, is characterized by increased levels of $_\beta$APP gene expression and by the early cerebral deposition of A_β [6,7]. The most convincing evidence for the notion that overproduction of A_β is sufficient to trigger AD comes from experiments with transgenic mice overexpressing full-length human $_\beta$APP, which generate high levels of A_β and develop AD-like neuropathology [8].

A_β can be produced by proteolysis of $_\beta$APP, a process that occurs constitutively in both normal and AD-affected tissues as well as in cultured cells [8–12], and its overproduction in AD is currently explained in terms of proteolytic processing of $_\beta$APP. $_\beta$APP proteolysis is effected by yet to be identified enzymes designated α-, β- and γ-secretases (Fig. 1) and occurs at Met^{596}/Asp^{597} (β-secretase; $_\beta APP_{695}$ numbering), at Lys^{612}/Leu^{613} (α-secretase) and between Val^{635} and Thr^{639} (γ-secretase). Cleavage by β-secretase generates an ectodomain fragment ending at Met^{596} and a 12-kDa C-terminal $_\beta$APP fragment containing A_β at its N-terminus (beginning with Asp^{597}). The production of A_β by proteolysis of $_\beta$APP requires cleavage by both β- and γ-secretases. Cleavage by α-secretase generates an ectodomain fragment ending at Lys^{612} and precludes formation of A_β because it occurs within the A_β sequence. Mutations within the $_\beta$APP gene that affect $_\beta$APP proteolytic pathway are responsible for overproduction of A_β and, consequently, for increased generation of A_β deposits in some types of familial AD. In such familial AD cases, muta-

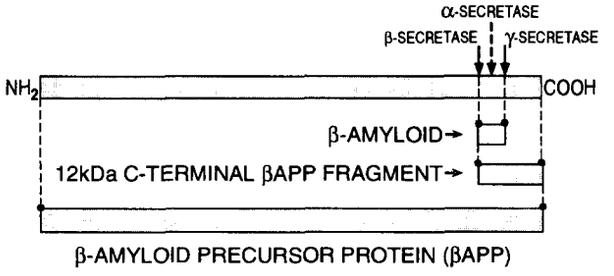

Fig. 1. In-scale schematic diagram of the $_\beta$APP and its proteolytic processing pathways. In the amyloidogenic pathway (solid vertical arrows), cleavage at the β-secretase site generates 12 kDa C-terminal $_\beta$APP fragment, the immediate precursor of A_β; cleavage of the 12 kDa fragment at the γ-secretase site results in A_β. In the non-amyloidogenic pathway (dashed arrow), cleavage at the α-secretase site precludes formation of A_β because it occurs within the A_β segment. See details in text.

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PII S0014-5793(96)00663-1

tion-mediated enhancement or alteration of proteolytic cleavage of βAPP is sufficient for an accelerated cerebral deposition of A_β and there is no need to invoke an additional mechanism beside the mutations. For example, the naturally occurring Flemish mutation (Ala^{616} to Gly^{616}) as well as the genetically engineered mutation Phe^{614} to Pro^{614} interfere with the cleavage of βAPP at the α-secretase site and, consequently, increase the production of A_β [3,13]. Probably the best example is proteolytic processing of βAPP containing the mutation Lys^{595}-Met^{596} to Asp^{595}-Leu^{596}, seen in Swedish familial AD, where the enhanced production of A_β is due to the increased efficiency of cleavage at the mutated β-secretase site [14] and the consequent unsusceptibility (see below) of the resulting 12-kDa fragment to α-secretase cleavage [15,16]. The A_β production pathway in Swedish familial AD, however, is qualitatively different from that responsible for the production of A_β from wild-type βAPP in that Leu^{596}/Asp^{597} cleavage occurs in a different cellular compartment [14,15] and possibly by a different enzyme [15], and therefore is not relevant to A_β production in sporadic, i.e. involving wild-type APP, AD where there is no evidence that proteolytic pathway leading to the production of A_β differs qualitatively or quantitatively from that occurring in normal cells. Therefore, since proteolytic processing alone cannot account for the increased production of A_β in sporadic AD, an additional A_β production pathway may be employed in this type of AD, which constitutes the majority of all AD cases. How could this new mechanism operate? To answer this question, one should consider factors which may influence the production of A_β.

The major factor that dramatically limits the generation of A_β in normal cells is cleavage of βAPP at the α-secretase site which precludes the formation of A_β. Can this cleavage be avoided? The answer is affirmative: whereas the intact βAPP is a substrate for cleavage at the α-secretase site, the 12-kDa C-terminal βAPP fragment containing A_β at its N-terminus is not susceptible to the α-secretase cleavage. What is the relationship between the 12-kDa C-terminal fragment of βAPP and A_β? The amount of the 12-kDa C-terminal βAPP fragment correlates with the amount of released A_β [17,18], indicative of the precursor-product relationship. Direct evidence that the 12kDa C-terminal βAPP fragment is the immediate precursor of A_β and can be further cleaved by γ-secretase, thus generating A_β, comes from work of Dyrks and co-workers, who transfected cells with a 5′-truncated βAPP gene [16,19]. RNA transcribed from transfected DNA was translated starting at Met^{596} and resulting in an extensive overproduction of the 12-kDa C-terminal βAPP fragment with [19] or without [16] signal sequence. In both cases, this fragment was rapidly cleaved by γ-secretase to generate A_β, which was subsequently secreted. Importantly, the 12-kDa C-terminal βAPP fragment, expressed in transfected cells independently of βAPP, was not cleaved at the α-secretase site [16,20]. This observation is consistent with results obtained by Haass and co-workers, who demonstrated that cleavage of βAPP at the β-secretase site appears to prevent cleavage at the α-secretase site [15]. The same experiments [15,16,20] also lead to another important conclusion, namely that cleavage at the γ-secretase site is not the rate-limiting step in the production of A_β. The facts that the 12-kDa C-terminal βAPP fragment, the immediate precursor of A_β, is not susceptible to cleavage at the α-secretase site and that cleavage at the γ-secretase site is not the rate-limiting step in the generation of A_β suggest that the increase in production of the 12-kDa C-terminal βAPP fragment may be an efficient way to overproduce A_β. The most effective manner to achieve this would be generation of the 12-kDa fragment independently of βAPP. How could such a process be accomplished?

2. The model

In βAPP mRNA, the A_β-coding segment is preceded immediately by the in-frame AUG codon at position 1786 (nucleotide residues are numbered from the start of the βAPP coding region as in [21]) corresponding to the Met^{596}. The initiation of translation at this position would produce the 12-kDa C-terminal βAPP fragment independently of βAPP. It is noteworthy that the AUG_{1786} is situated within a nucleotide context optimal for initiation of translation, i.e. an A in position −3 and a G in position +4 relative to the A of the AUG codon [22]. In fact, AUG_{1786} is one of only two AUG codons within the entire βAPP mRNA which are located within an optimal translation initiation context. Such favorable location of AUG_{1786} was the basis for an earlier proposal that the 12-kDa C-terminal fragment may be generated independently from βAPP by *internal* initiation of translation of the intact βAPP mRNA at the AUG_{1786} [23], but this has been subsequently ruled out by the elegant experiments of Citron and co-workers [24]. *There is, however, another possibility of utilization of the AUG_{1786} as a translation initiation codon, namely the generation of a 5′-truncated βAPP mRNA in which the AUG_{1786} becomes the first AUG codon.* A possible mechanism for the generation of such a truncated βAPP mRNA is suggested by recent results from the author's laboratory with globin mRNA in erythroid precursor cells [25].

Recently, we obtained evidence that, in erythroid cells, globin mRNA can be produced by RNA-dependent RNA synthesis via antisense RNA intermediates and that this process may also occur in other cell types with other mRNA species. Elucidation of the structure of antisense globin RNA suggested a possible mechanism [25] illustrated in Fig. 2. Syn-

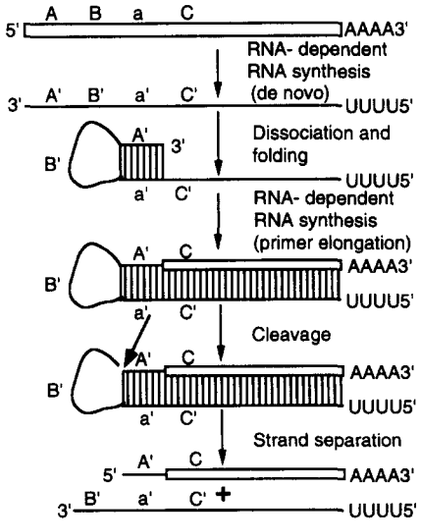

Fig. 2. Postulated mechanism of cellular mRNA replication. Box, sense strand; line, antisense strand. A′ and a′, complementary elements within an antisense strand. Note that the A′ element is strictly 3′-terminal. See details in text.

thesis of antisense RNA is initiated within the poly(A) region of mRNA, presumably by a mechanism similar to that employed in viral systems [26]; consequently, it contains a poly(U) stretch at the 5′ end. At the 3′ end, antisense RNA contains two strongly complementary elements within the segment corresponding to the 5′-untranslated region of globin mRNA. This complementarity within the antisense strand is highly conserved during evolution [27] and extends not only to the presence of complementary elements but also to their position, the complementary elements being always located within the segment of antisense strand corresponding to the 5′-untranslated region of globin mRNA, one always being strictly 3′-terminal [27]. The strong evolutionary conservation suggested an underlying function, namely the priming of the synthesis of a sense RNA strand by the 3′-terminal element of an antisense strand. The strict 3′-terminal location of one of the complementary elements appears to be an absolute requirement, the addition of only one 'noncomplementary' nucleotide to the 3′ terminus of the antisense globin strand strongly inhibiting its self-priming capacity [27]. The initial product of such a self-priming process is a chimeric antisense-sense molecule in the form of a hairpin loop (Fig. 2). The separation of strands appears to involve cleavage at the 3′ end of the loop that generates 5′-truncated mRNA molecules. Since a full-size mRNA molecule can be repeatedly used as a template in such a process, it can direct the synthesis of many truncated mRNA molecules; the subsequent translation of this RNA would result in enhanced production of a polypeptide. Potentially, this process, which may have evolved to enhance expression of specific genes, may be involved in diseases, such as AD, associated with the overproduction of specific proteins.

Because self-priming by globin antisense RNA occurs within the segment corresponding to the 5′ untranslated region of mRNA, the resulting sense strand is truncated within its 5′ untranslated region but retains an intact coding capacity. Recently, we identified antisense counterparts, putative intermediates in RNA-dependent RNA synthesis, for a number of spliced mRNA species in mammalian cells, an indication that this phenomenon may be quite widespread. Principally, in non-globin systems, self-priming might occur within a portion of an antisense molecule corresponding to the coding region of an mRNA thus generating sense strand with 5′-truncated coding region. In such a case, provided the first available AUG is in-frame, translation would result in the C-terminal portion of an original polypeptide. If the $_{\beta}$APP mRNA were subject to such a replication process and if truncation occurred so that the first available AUG is the AUG_{1786}, translation would result in the 12-kDa C-terminal fragment of $_{\beta}$APP.

For a 5′-truncated $_{\beta}$APP mRNA with the first AUG codon in position 1786 to be produced by the mechanism described above, the antisense $_{\beta}$APP RNA should be able to self-prime the synthesis of a sense-strand in a manner illustrated in Fig. 2, starting upstream from and in the vicinity of the AUG_{1786}. For this to occur, the antisense strand would have to contain two topologically compatible complementary elements, one strictly 3′-terminal and another corresponding to a segment of $_{\beta}$APP mRNA upstream of the AUG_{1786}. Is this requirement satisfied in a complement of $_{\beta}$APP mRNA? An answer to this question could be obtained in a model experiment of a type carried out with globin mRNA [27]. In such an experiment, an mRNA of interest serves as a template for synthesis of cDNA (the antisense strand) and is subsequently removed by RNase H activity usually present in preparations of reverse transcriptase. If complementary elements were present within the antisense strand, if one of them were 3′-terminal and if they were topologically compatible, self-priming of sense strand synthesis would occur. The result would be an antisense strand (cDNA) extended at the 3′ end by a segment of a sense strand in a hairpin-like structure. Moreover, provided that the 5′ terminus of an mRNA were defined, the junction between the antisense and sense components would indicate the site of self-priming.

In fact, such an experiment was actually carried out with $_{\beta}$APP mRNA. In 1988, when several $_{\beta}$APP cDNAs had already been obtained but the sequence of the gene and its flanking regions were not yet known, a new $_{\beta}$APP cDNA was reported [28]. It differed from the others in that it contained, in addition to a known complement of $_{\beta}$APP mRNA, an over 70-nucleotide-long segment at the 3′ end. When the 5′ end of the $_{\beta}$APP gene and its flanking regions were sequenced [29], no genomic sequence corresponding to the additional 3′ segment of the new cDNA could be seen. On the other hand, it was found that the non-coded segment of the new cDNA was identical to a segment of $_{\beta}$APP mRNA within the coding region of the molecule about 2 kb downstream from its 5′ terminus which overlaps in part with the A_{β} coding region [30]. This unusual $_{\beta}$APP cDNA can be analyzed in terms of our model for RNA-dependent RNA synthesis (Fig. 2). It consists of an antisense molecule (cDNA), extended by a sense-strand fragment identical to the segment of $_{\beta}$APP mRNA immediately downstream from residue 1728. It can thus be viewed as a chimeric antisense-sense molecule in which a hairpin loop connects a segment of the antisense portion with a complementary sense extension, which corresponds to a segment of the coding region of $_{\beta}$APP mRNA. Such an extension could have been triggered, upon completion of cDNA synthesis [27], by complementarity of the 3′-terminal segment of $_{\beta}$APP antisense strand with a topologically compatible internal fragment of the same molecule corresponding to a segment of $_{\beta}$APP mRNA immediately upstream of nucleotide 1728.

As shown in Fig. 3a, such complementary elements are indeed present within the antisense $_{\beta}$APP strand. Because one of the elements must be strictly 3′-terminal to initiate the extension [27], the self-priming ability of the antisense strand depends critically on the 5′-terminal sequence of $_{\beta}$APP mRNA. There are at least five known 5′ ends of $_{\beta}$APP mRNA in normal brain [29]. The corresponding 3′-ends of antisense strands are marked by dots in Fig. 3a. Only one of these, i.e. −149 in Fig. 3, would allow effective self-priming to yield the chimeric antisense-sense molecule – with other ends the 3′ complementary element would be either non-terminal or form an unstable hybrid. In normal subjects the predominant transcription start site is at residue −146 and whether it shifts in AD patients to residue −149 or elsewhere remains to be determined. Cleavage at the 3′ end of a hairpin loop (Fig. 3b) of the chimeric antisense-sense $_{\beta}$APP molecule would generate a severely 5′-truncated sense $_{\beta}$APP RNA molecule in which the first AUG codon, AUG_{1786} corresponding to Met^{596}, is in frame with and immediately precedes the A_{β} coding region. Translation of the 5′-truncated $_{\beta}$APP mRNA would result, therefore, in the 12-kDa C-terminal portion of

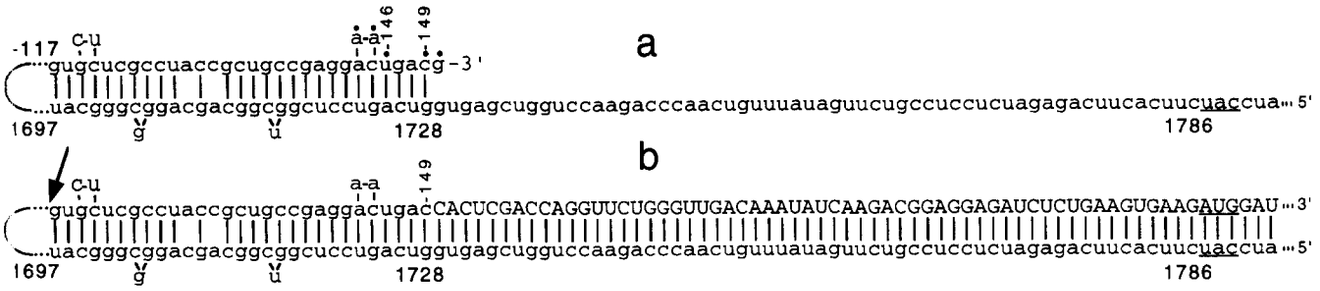

Fig. 3. 3′-terminal and internal complementary elements of the βAPP antisense strand. (a) Folded antisense βAPP RNA strand; (b) self-primed synthesis of a 5′-truncated βAPP mRNA. Numbers indicate positions of corresponding residues in βAPP mRNA. Nucleotide residues are numbered as in [21]; the nucleotide sequence upstream from residue −146 is from [29]. Positions corresponding to transcription start sites of βAPP mRNA are marked by dots. Lower-case letters, antisense strand; upper-case letters, sense strand. Arrow indicates postulated cleavage site at the 3′ end of a hairpin loop. Underlined: translation initiation codon AUG_{1786} and its complement.

βAPP containing Met^{596} followed by $A\beta$ at its N-terminus. This polypeptide could be processed further by two known enzymes to generate $A\beta$: N-terminal methionine aminopeptidase [31], which removes N-terminal methionine from a majority of newly synthesized eukaryotic proteins, and γ-secretase. Indeed, as was mentioned above, the 12-kDa C-terminal βAPP fragment, translated in transfected cells from Met^{596} without a leader sequence, is cleaved proteolytically at the γ-secretase site to generate $A\beta$, which is subsequently secreted [16].

3. Predictions and conclusions

The model for antisense RNA-mediated generation of a 5′-truncated βAPP mRNA encoding the 12-kDa C-terminal βAPP fragment, a process postulated to be activated or enhanced in AD-affected tissues, allows several verifiable predictions. These include the following for sporadic AD-affected brain tissues: (i) the presence of the antisense βAPP RNA, some of it specifically truncated at the 3′ terminus; (ii) shift of the transcriptional start site for βAPP mRNA to position −149 or downstream from position −143; (iii) the occurrence of a 5′-truncated βAPP mRNA encoding the 12-kDa C-terminal fragment of βAPP; (iv) the presence of RNA-dependent RNA polymerase activity.

Another prediction of the proposed model is the extensive synthesis of the 12-kDa C-terminal βAPP fragment as a primary translational product in $A\beta$-overproducing cells, a phenomenon that should be seen in a pulse-chase experiment. Currently, however, this prediction cannot be verified for lack of an experimental model system for sporadic AD.

It should be noted that two technical aspects could complicate the investigation of the proposed mechanism. Firstly, $A\beta$-overproducing cells are likely to be the first victims of AD neuropathology triggered by increased deposition of $A\beta$. Therefore, in post-mortem samples from patients with advanced AD, $A\beta$-overproducing cells may be poorly represented. A possible solution to this potential problem is to analyze a sufficient number of samples from 'normal' subjects, old enough to have relatively high probability of developing AD. Secondly, by analogy with globin mRNA replication in erythroid precursor cells ([25]; V. Volloch, unpublished results), 5′-truncated βAPP mRNA encoding the 12-kDa βAPP fragment may be heavily modified and consequently hybridize poorly with specific probes at high stringency conditions. Therefore, an initial search for βAPP antisense RNA appears to be the most efficient way to pursue this proposal.

In summary, the overproduction of $A\beta$ appears to be the primary cause of AD. In some cases it is caused by mutations within the βAPP gene that affect βAPP proteolytic pathway. In such familial AD cases, mutation-mediated enhancement or alteration of proteolytic cleavage is sufficient for the increased production of $A\beta$ and there is no need to invoke additional mechanisms beside the mutations. The best example to illustrate this point is the Swedish mutation where the mechanism proposed here cannot operate because Met^{596} is replaced by Leu. But neither the proposed nor any other additional mechanism is needed in this case; the mutation alone is sufficient. Indeed, the enhanced production of $A\beta$ in Swedish familial AD is due to the increased efficiency of cleavage at the mutated β-secretase site [14] and the consequent unsusceptibility of the resulting 12-kDa fragment to the α-secretase cleavage [15,16,20]. The $A\beta$ production pathway in Swedish familial AD, however, is qualitatively different from and therefore not relevant to $A\beta$ production in sporadic AD [15]. On the other hand, there is no evidence that proteolytic processing of βAPP leading to the production of $A\beta$ in sporadic AD differs qualitatively or quantitatively from that occurring in normal tissues, indicating that an additional $A\beta$ production pathway may be employed. A mechanism is described in this paper for the βAPP-independent generation and overproduction of the 12-kDa C-terminal fragment of βAPP, the immediate precursor of the $A\beta$. It proposes the occurrence of antisense RNA-mediated generation of 5′-truncated βAPP mRNA followed by initiation of translation at the AUG_{1786} immediately preceding the $A\beta$-coding region. It requires a shift of the transcriptional start site of βAPP mRNA and induction of RNA-dependent RNA synthesis machinery in response to an undefined stimulus and is postulated to be activated or enhanced in sporadic AD in the age-dependent and tissue-specific manner. In this model two factors contribute to the increased production of $A\beta$: (i) the proposed mechanism for generation of 5′-truncated βAPP mRNA leads to its overproduction; and (ii) in contrast to the intact βAPP, where cleavage at the the α-secretase site precludes formation of $A\beta$, 12-kDa C-terminal βAPP fragment, the primary translation product of the proposed mechanism, is not a subject to α-secretase cleavage, but is cleaved by γ-secretase. This cleavage generates $A\beta$ which is subsequently secreted. The proposed model makes several verifiable predictions and suggests

new directions of experimentation that may lead to a better understanding of the mechanisms involved in AD.

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