The EMBO Journal vol.7 no.4 pp.949-957, 1988

Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease

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Communicated by K.Beyreuther

The precursor of the Alzheimer's disease-specific amyloid A4 protein is an integral, glycosylated membrane protein which spans the bilayer once. The carboxy-terminal domain of 47 residues was located at the cytoplasmic site of the membrane. The three domains following the transient signal sequence of 17 residues face the opposite side of the membrane. The C-terminal 100 residues of the precursor comprising the amyloid A4 part and the cytoplasmic domain have a high tendency to aggregate, and proteinase K treatment results in peptides of the size of amyloid A4. This finding suggests that there is a precursor-product relationship between precursor and amyloid A4 and we conclude that besides proteolytic cleavage other events such as post-translational modification and membrane injury are primary events that precede the release of the small aggregating amyloid A4 subunit.

Key words: Alzheimer's disease/amyloid precursor/amyloid A4/amyloidogenicity/*in vitro* expression

Introduction

Alzheimer's disease (AD) is a human cerebral degenerative disorder characterized by gradual loss of memory, reasoning, orientation and judgement (Katzman, 1986). The impairment of memory and intellectual function is correlated with the appearance of amyloid depositions mainly in the hippocampus and associaton cortex of the brain (Roth *et al.*, 1966). The amyloid deposition is found in three locations: intracellularly as neurofibrillary tangles (NFT) in the cortex; extracellular amyloid deposits in the cortex are found as neuritic plaques that consist of NFT-containing neurites surrounding an amyloid plaque core (APC) and as cerebrovascular amyloid (ACA) in the walls of meningeal and intracortical blood vessels (Alzheimer, 1907; Scholz, 1938; Pantelakis, 1954). The major protein component isolated from NFT, APC and ACA is a small polypeptide of 4-4.5 kd, called A4 protein or β protein due to its M_r or a partial β pleated sheet structure respectively (Glenner and Wong, 1984; Masters *et al.*, 1985a,b; Kidd *et al.*, 1985). The brains of patients with Guamanian parkinsonism-dementia (Guamanian PD) have NFT and the brains of aged individuals with Down's syndrome (DS) (trisomy 21) have NFT, APC and ACA and also contain the A4 protein as the major amyloid component (Glenner and Wong, 1984; Masters *et al.*, 1985a; Beyreuther *et al.*, 1986; Guiroy *et al.*, 1987). Protein sequence analyses have shown that the A4 protein isolated from brains of AD, DS and Guamanian PD patients contain the same sequence (Guiroy *et al.*, 1987). The only difference found was a varying degree of N-terminal raggedness for NFT-A4 and APC-A4 (Masters *et al.*, 1985b; Beyreuther *et al.*, 1986; Guiroy *et al.*, 1987). The identical sequences suggest that the amyloid in AD, DS and Guamanian PD is derived from a common precursor protein and furthermore, that a common mechanism may underly the formation of amyloid in these conditions. Recently, this precursor has been characterized with respect to both nucleotide sequence and deduced amino acid sequence by isolation and analysis of a full-length cDNA encoding a primary translation product of 695 residues hereafter termed A4 precursor (PreA4) (Kang *et al.*, 1987). Northern blot hybridization reveals two transcripts of 3.4 and 3.2 kb in RNA preparations from fetal cortex indicating that more than one translation product is encoded by the PreA4 gene on the human chromosome 21 (Kang *et al.*, 1987). Recently the longer transcript was shown to be alternative splicing forms of the PreA4 gene containing a 168-bp or 225-bp insert after the second domain in the otherwise unaltered sequence described by Kang *et al.* (1987) and to encode a 751-residue or 770-residue PreA4 protein (Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988). We suggested that the 695-residue PreA4 protein is a membrane-spanning *N*-glycan protein that displays the typical structural features of cell surface receptors: an N-terminal signal sequence followed by three extracellular domains, a transmembrane region and a cytoplasmic domain of 47 residues. The amyloid subunit which we showed to be maximally either 42 or 43 residues long corresponds to residues 597-639 of the precursor sequence. About half of the amyloid A4 protein is therefore derived from the putative transmembrane region (residues 625-648 of PreA4). The N terminus of amyloid A4 protein is derived from the third extracellular domain that spans residues 290-624 of PreA4 and contains the two potential *N*-glycosylation sites of the precursor. During amyloidogenesis the C-terminal cleavage of the PreA4 has to occur within the membrane domain. Membrane damage is therefore a likely primary event which would have to precede the release of the small aggregating amyloid A4 subunit.

The PreA4 gene is expressed in brain and in peripheral tissues such as muscle and epithelial cells (Tanzi *et al.*, 1987; Goedert, 1987; Shivers *et al.*, 1988; Bahmanyar *et al.*, 1987; Zimmermann *et al.*, 1988). In contrast, the pathological hallmarks of AD, amyloid depositions in the form of NFT, APC and ACA, are strictly confined to the brain. Since the precursor-product relationship for PreA4

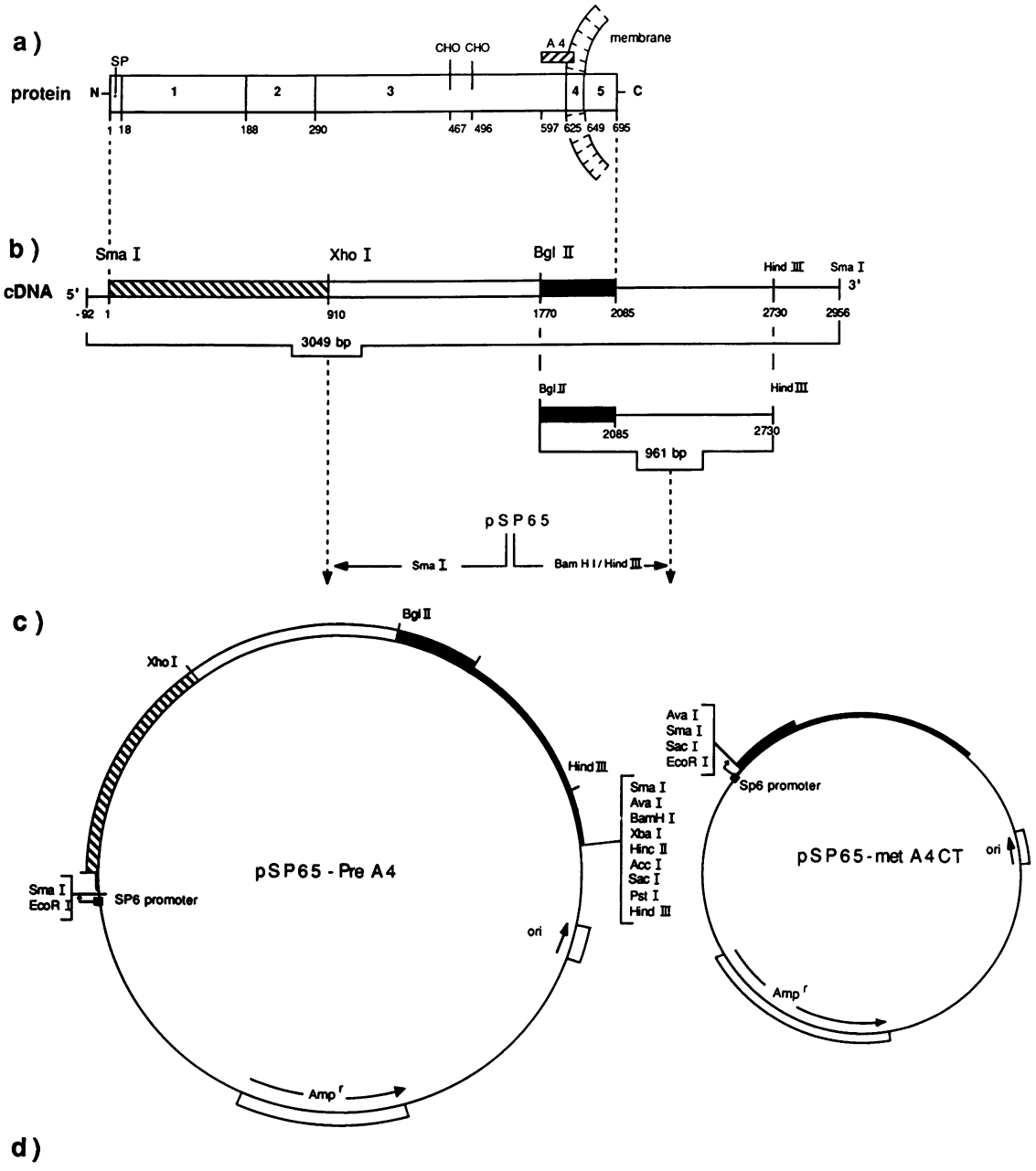

Fig. 1. Construction of pSP65−PreA4 and pSP65−metA4CT which code for the 695-residue amyloid precursor PreA4 protein and the 100-residue C-terminal part of the PreA4 protein respectively. **Panel a** is a schematic representation of open reading frame with the putative domain structure according to Kang *et al.* (1987). SP, signal sequence; 1−3, extracellular domains; CHO, *N*-glycosylation site; 4, transmembrane domain; 5, cytoplasmic domain. The shaded bar indicates the position of the amyloid A4 sequence. **Panel b** shows the full-length cDNA that encodes the 695-residue PreA4 represented in panel a. Restriction enzymes used and their positions in the cDNA sequence of Kang *et al.* (1987) are indicated. The coding sequence terminating at the *Xho*I site corresponds to the hatched part. The black bar indicates the cDNA used for construction of pSP65−metA4CT which encodes the C-terminal part of the precursor beginning with the amyloid sequence. **Panel c** shows the final pSP65 constructs used for *in vitro* transcription. Plasmid pSP65−PreA4 linearized with *Xho*I served as template for the 303-residue N-terminal fragment of the precursor. The *Bgl*II−*Hin*dIII cDNA fragment was inserted into *Bam*HI−*Hin*dIII-digested pSP65. The SP6 promoter (P) and direction of transcription are indicated. **Panel d** shows the 5′ coding sequence with the transcription intitiation nucleotide and the translation initiation codon of pSP65−metA4CT.

and amyloid A4 and the specific biochemical events leading to the release of the amyloidogenic peptide are not established, we have studied the *in vitro* expression of the full-length cDNA copy of the 695-residue PreA4. We have also analysed the transmembrane orientation and biogenesis of the *in vitro* translation product. We constructed, cloned and expressed DNA fragments that encode the putative signal peptide and two of the three putative extracellular domains of the PreA4 protein, as well as a DNA fragment that encodes the part of the precursor corresponding to the amyloidogenic fragment together with the putative transmembrane and cytoplasmic domains. Thus, we were able to identify and determine the size of the putative signal peptide and the aggregational properties of the part of the molecule that includes the amyloid sequence.

To determine whether the PreA4 protein is an integral membrane protein and to characterize its transmembrane orientation, we studied its biogenesis in a cell-free system programmed with SP6 transcripts of the full-length cDNA. Following this approach we have been able to demonstrate that the PreA4 protein encoded by the cDNA described by Kang *et al.* (1987) indeed represents an integral, glycosylated membrane protein which spans the bilayer once. The 695-residue PreA4 products are not resistant to proteinase digestion. However, we show that a shortened cell-free translation product comprising the amyloid A4 part and the cytoplasmic domain of the precursor does have a high tendency to aggregate. This finding suggests that there is a precursor–product relationship between the 695-residue PreA4 and the amyloid A4.

Results

In-vitro expression of PreA4

The pSP65–PreA4 construct (Figure 1) including the full-length cDNA copy of the 695-residue PreA4, was linearized with *Xba*I and transcribed using SP6 RNA polymerase. The resulting PreA4-mRNA (3083 bases) was capped by addition of the cap precursor m^7 GpppG to the transcription reaction (Konarska *et al.*, 1984).

The *in vitro* transcripts obtained were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine resulting in efficient production of a 91.5-kd protein (Figure 2). The primary translation product is completely digested by trypsin (Figure 2, lane 2). Amyloid A4 formation was not found under these conditions. The same result was obtained with proteinase K (data not shown).

Membrane insertion

In order to study membrane insertion of PreA4, the *in vitro* translation was performed in the presence of dog pancreas microsomes. This also resulted in a protein of 91.5 kd. This suggests that removal of the putative signal sequence and the expected subsequent glycosylation of the precursor (Figure 1a) during translocation does not change the apparent molecular mass of the post-translationally modified PreA4.

Membrane insertion of the PreA4 was inferred from the observation that the translation product was partially resistant to protease added post-translationally (Figure 2, lane 4, and Figure 3). Under conditions which left the microsomal vesicles intact, proteinase K digestion resulted in mol. wt reduction of 4 kd. Trypsin digestion produced a shift of 2 kd and chymotrypsin produced only a small effect (Figure 3).

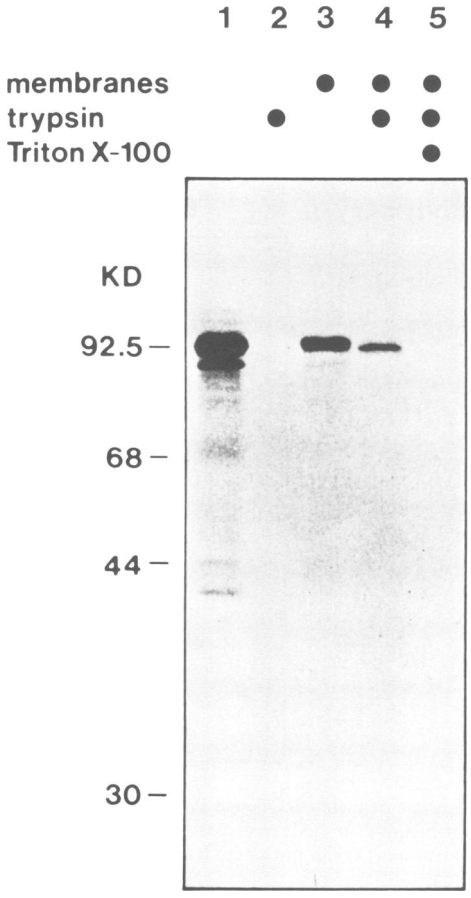

Fig. 2. Size and membrane insertion of PreA4. mRNA encoding the 695-residue PreA4 was transcribed from pSP65–PreA4, translated in the rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analysed as described in Materials and methods. **Lane 1** shows *in vitro* translation in the absence of dog pancreas membranes; **lane 2**, as lane 1 but with post-translational trypsin treatment; **lane 3**, as lane 1 but in the presence of membranes; **lane 4**, as lane 3 and with post-translational trypsin treatment; **lane 5**, as lane 4 but with Triton X-100 addition prior to trypsin treatment.

Protease treatment in the presence of the non-ionic detergent Triton X-100 led to complete degradation of the precursor synthesized in the presence of microsomal membranes (Figure 2, lane 5). The shift in molecular mass after trypsin or proteinase K digestion is important with respect to membrane insertion and transmembrane orientation of PreA4, since the sequence of the protein reveals only a single putative transmembrane sequence which is followed by a 47-residue domain at the C-terminal site. Given that proteases are sterically hindered by the membrane, trypsin is expected to cleave C-terminal to Arg 672 and to shorten the protein by 22 residues, whereas proteinase K could cut after Ile 656 and thus release 39 of the 47 residues of the cytoplasmic domain (Figure 1a). Since the remaining 638 residues of the precursor are located N-terminal to the putative transmembrane sequence, the removable 4 kd is expected to correspond to the putative cytoplasmic domain of the precursor. The data presented in Figures 2 and 3 are compatible with the assumption that the protein spans the bilayer only once. The amino terminus residing in the lumen of the vesicles is protected from proteolysis. The carboxy terminus is exposed to the protease at the vesicle surface,

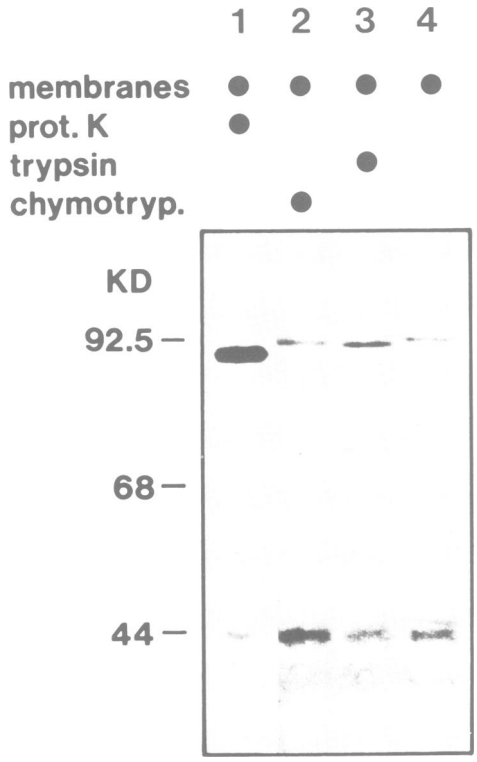

Fig. 3. Topology of membrane-bound PreA4. Procedures were the same as for Figure 2 in the presence of membranes. **Lanes 1–3**, post-translational treatment with proteases; **lane 4**, control without protease addition.

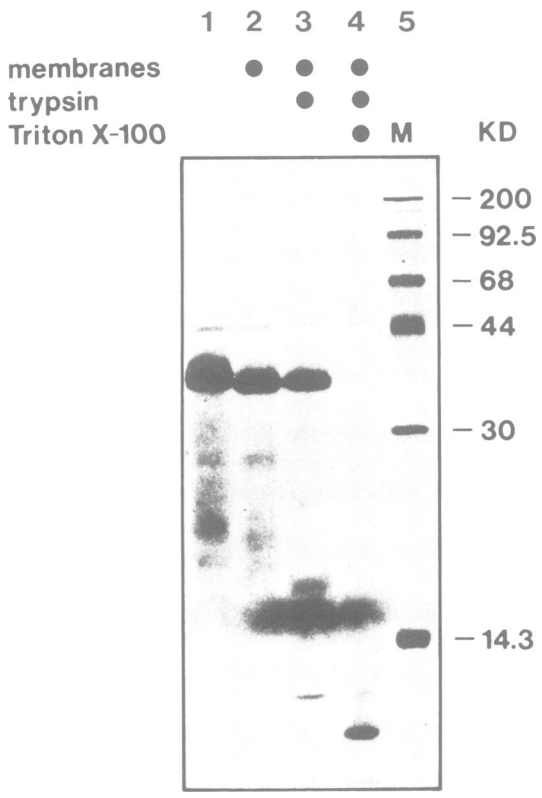

Fig. 4. Evidence for signal sequence cleavage of PreA4 *in vitro*. Fluorogram of *in vitro* translation products of transcripts obtained from *Xho*I-digested pSP65–PreA4 using the reticulocyte system. **Lane 1**, translation in the absence of membranes; **lanes 2–4**, translation in the presence of dog pancreas microsomal membranes, **lane 3** with post-translational trypsin treatment and **lane 4** proteolysis in the presence of Triton X-100; **M**, lane with molecular mass marker proteins.

which corresponds to the cytoplasmic site of the membrane.

Further evidence for insertion of the protein into membrane was obtained with the sedimentation assay for integral membrane proteins (Fujiki *et al.*, 1982). Only the translation product obtained in the presence of microsomes could be selectively sedimented with the sodium carbonate technique (data not shown).

Identification of the signal sequence

Because glycosylation and signal peptide cleavage do not result in alteration of the molecular mass of the precursor (Figure 2), we constructed *Xho*I mRNA which encodes the signal peptide and the two N-terminal domains of the precursor. The entire *N*-glycan domain, the transmembrane domain and the cytoplasmic domain are not included. Translation of this template should show the signal peptide removal and the size of the signal peptide. Figure 4 shows that addition of membranes led to the removal of a peptide of ~1.5 kd which would correspond to a signal sequence of <20 residues. Removal of the signal sequence confers entire protease reistance, indicating that the product was translocated across the microsomal membrane (Figure 4, lane 3).

To determine the signal peptidase cleavage site, we sequenced *in vitro* synthesized and [^{35}S]Met-labelled protein. Sequencing of translation products obtained in the presence of the signal peptidase-containing microsomal membranes revealed methionine residues at positions 19, 26 and 28 (Figure 5). This result places the signal peptidase cleavage site C terminal to alanine 17 of the PreA4 sequence. A signal peptide of 17 residues is in agreement with the above mentioned mol. wt shift of 1.5 kd. We could show that this cleavage is membrane dependent since 30 degradation cycles of the primary translation product (translated in the absence of membranes) only gave rise to a weak signal in the first degradation step which could be due either to an N-terminal methionine residue or to non-specific release of radioactivity (data not shown).

The data presented here demonstrate that PreA4 has the expected transient signal sequence. The signal peptide is shown to correspond to the 17 N-terminal residues and signal peptidase cleavage occurs during translocation, which results in a mature precursor of 678 residues with an N-terminal leucine residue.

N-glycosylation of PreA4

We have used a competitive inhibitor of glycosylation, benzoyl-Asn-Leu-Thr-*N*-methylamide (Lau *et al.*, 1983), to provide a protein that will not become glycosylated *in vitro* in the presence of membranes. This is expected to result in a decrease in mobility on SDS gels of 1.5 kd per carbohydrate chain. Control experiments were carried out with the non-competitive tripeptide benzoyl-Asn-Leu-alloThr-*N*-methylamide, which should not interfere with *N*-glycosylation. In the presence of the inhibitor, the mol. wt of the precursor was diminished by 2.0 kd (Figure 6). In the presence of the non-competitive tripeptide the mol. wt of the precursor was unaltered. Combining this result and the signal peptide release, we conclude that there are one or two carbohydrate moieties added post-translationally. If we take into account the inherent error for the determination of mol.

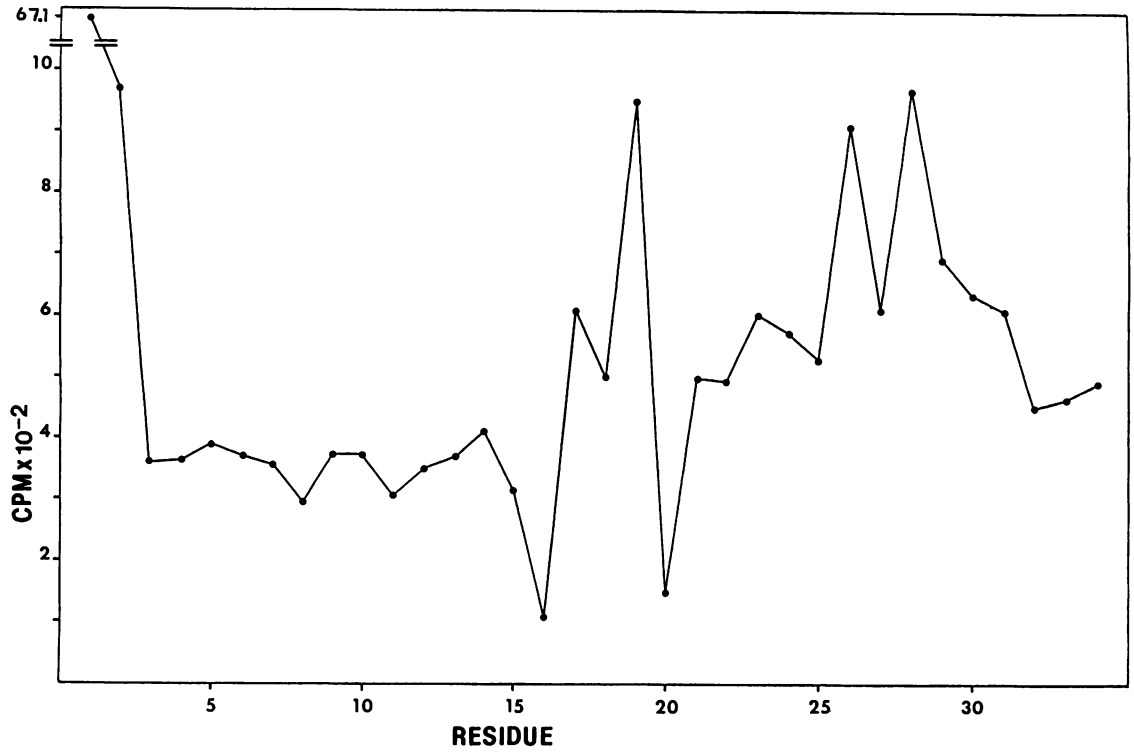

Fig. 5. Radio sequence analysis of translation products obtained in the presence of membranes allowed the determination of the signal peptidase cleavage site for PreA4. *In vitro* translation was as described for Figure 2, lane 3 and sequencing as given in Materials and methods. Radioactivity peaks at degradation steps 19, 26 and 28 are in agreement with signal peptidase cleavage after residue 17 of the precursor sequence. Sequencing (30 cycles) of *in vitro* translation products obtained in the absence of membranes does not lead to radioactivity release after the first two steps. The radioactivity released in degradation steps 1 and 2 was similar in control experiments performed in the absence of PreA4 mRNA (data not shown). The values given for degradation steps 16 and 20 correspond to ~30% of the total due to incomplete sampler transfer to the fraction collector.

wt on SDS−PAGE and the number of glycosylation acceptor sites predicted from the cDNA sequence (Figure 1a), it is likely that both *N*-glycosylation sites of the PreA4 are used.

Expression of the amyloid sequence

The full-length precursor synthesized *in vitro* does not aggregate (Figure 2). Also, protease treatment did not lead to aggregation. One of the reasons for this failure might have been the experimental problem of adequate membrane distortion, a prerequisite for exposure of the amyloid part to proteolytic action. In order to examine this point further, we used a construct of the PreA4 that has the amyloid sequence at the N terminus, ends with the natural C terminus and has no signal sequence for membrane insertion. Such a construct (pSP65−metA4CT) would not require membrane damage for proteolytic attack, its folding would not be influenced by the lipid bilayer and its aggregation would not be sterically hindered by additional N-terminal sequences. In the context of the β-pleated sheet structure of amyloid (Eanes and Glenner, 1968; Kirschner *et al.*, 1986), it should be noted that the PreA4 sequence corresponding to amyloid A4 includes two regions with a predicted tendency for β-sheet formation (Figure 7a), which will, however, adopt an α-helical conformation when inserted into membranes (Kyte and Doolittle, 1982; Rao and Argos, 1986). Without membranes, the β-sheet confirmation required for amyloid formation is expected to dominate and therefore promote aggregation. Figure 7c shows that the deletion of the N-terminal part of PreA4 up to the methionine residue immediately preceding the N-terminal Asp residue of the amyloid A4 sequence leads

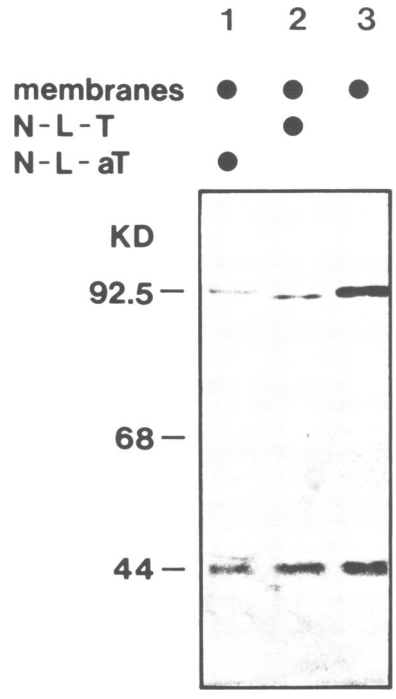

Fig. 6. Evidence for *N*-linked glycosylation of PreA4 *in vitro*. PreA4 mRNA was translated *in vitro* in the presence of dog pancreas microsomes (**lane 3**). Translation was performed in the presence of the competitive inhibitor tripeptide bz-Asn-Leu-Thr-meNH$_2$ (N-L-T) of *N*-linked glycosylation (**lane 2**) and the non-inhibiting tripeptide bz-Asn-Leu-alloThr-meNH$_2$ (N-L-aT) (**lane 1**).

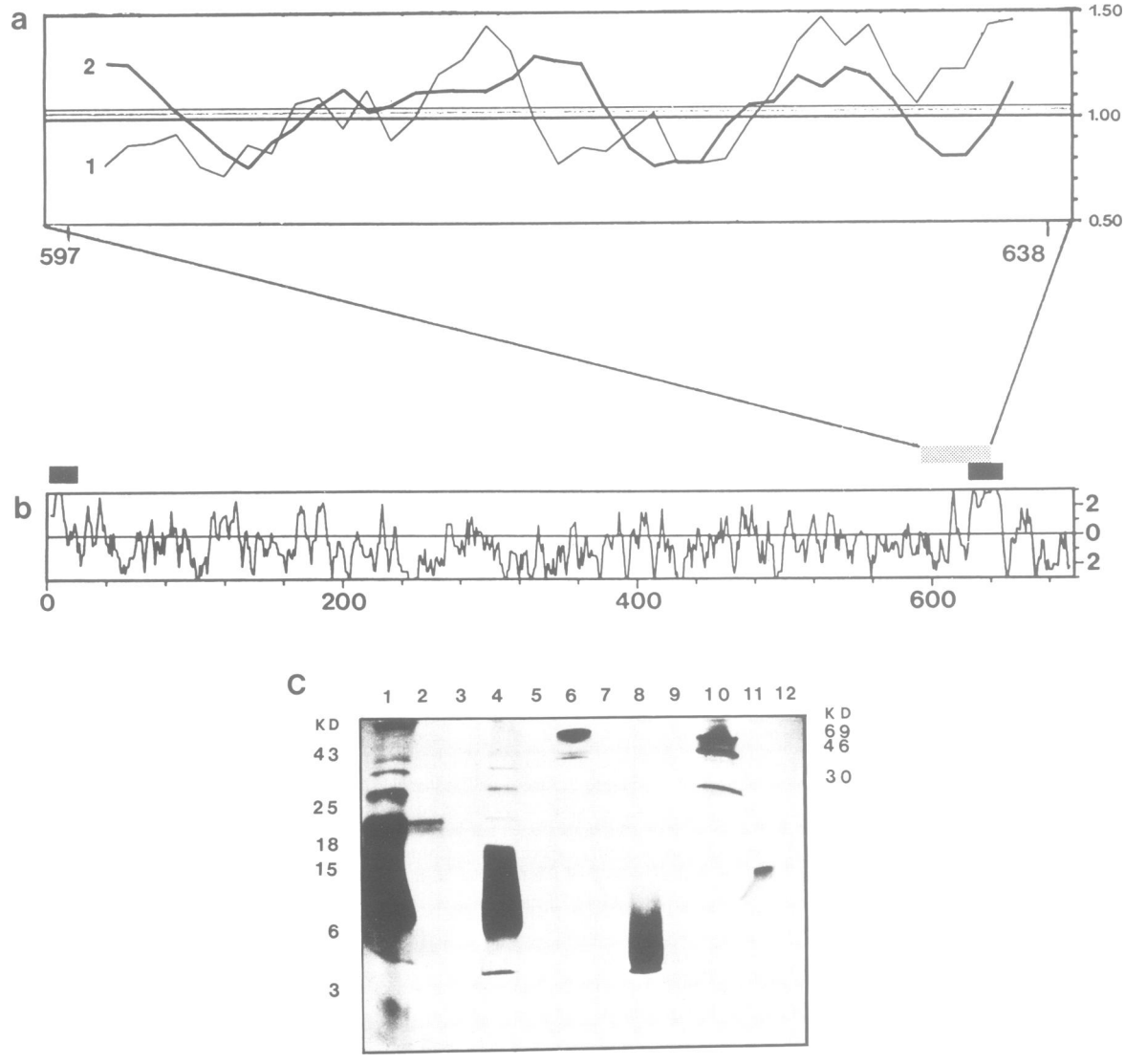

Fig. 7. Expression of an aggregating PreA4 fragment and predicted secondary structure and hydrophilicity profile of the amyloid A4 and PreA4 sequence. The amino acid sequence of the 42-residue amyloid A4 was analysed by computer for secondary structure **a** and the 695-residue PreA4 protein for hydrophilicity (window size is 9 residues, plotted with a 1-residue interval) **b** according to the procedure of Chou and Fasman (1978) and Hopp and Woods (1981) respectively. Signal peptide and transmembrane domain are indicated as black bars and the amyloid A4 sequence as stippled bar in **b**. Fluorogram after transcription and translation of pSP65−metA4CT which encodes a protein that begins with the methionine residue preceding the amyloid sequence and ends with the natural C terminus of the A4 precursor (596−695 in Figure 1a) **c**. Translation of pSP65−metA4CT in the reticulocyte system results in a protein which has a high tendency to aggregare (**c lane 1**). Without exogenous mRNA only aminoacyl tRNAs are labelled (**c lanes 2**) and show up as bands around 20 kd which disappear upon treatment with RNase A (**c lane 5**). RNase A treatment does not alter the pattern of the translation product obtained from pSP65−metA4CT mRNA (**c lane 4**). In **c lane 6** the *in vitro* synthesized A4 precursor is shown. The latter is sensitive to proteinase K treatment (**c lane 7**). Amyloid A4 protein is not generated. In contrast, the same proteinase K treatment of the translation product from pSP65−metA4CT mRNA yields A4-like proteins (**c lane 8**). RNase A and proteinase K digestion of translations performed in the absence of exogenous mRNA was done as control (**c lane 9**). Translation of pSP65−metA4CT mRNA was also possible in the wheat germ system and reveals a product of ~15 kd (**c lane 11**). The latter is absent in the corresponding control experiment performed without exogenous mRNA (**c lane 12**). **c lane 10** are radiolabelled and lane 3 are cold marker proteins respectively. All translations were carried out in the absence of microsomes. Post-translational treatment with proteinase-free RNase A was done in the experiments shown in c lanes 4−9 and with proteinase K in c lanes 7−9 (0.1 mg/ml, 10 min, 37°C). Translations were done in the reticulocyte system (c lanes 1, 2, 4−9) and in the wheat germ system (c lanes 11 and 12).

to aggregation without the addition of protease. The smear seen between 10 and 18 kd could be due to overloading but also due to high globin amounts in the reticulocyte system used. Interestingly, the aggregation is not inhibited by the presence of the complete C-terminal part of PreA4 (Figure 1a and 7c). Limited proteolysis with proteinase K results in a fragment that comigrates on SDS gels with amyloid A4 (Figure 7c, lane 8). Treatment with protease-free RNase A to digest amino acid tRNAs labelled with [^{35}S]methionine abolished the radioactive double bands seen in translations without mRNA addition (Figure 7c, lane 2) and has no effect on the other bands (Figure 7c, lane 4).

Expression of pSP65−metA4CT in a wheat germ system

In vitro translation of full-length PreA4 mRNA in the reticulosyte lysate is very efficient and allowed us to study the biogenesis of the protein under cell-free conditions. In

wheat germ, we expected to corroborate the *in vitro* studies with the animal system and to obtain the same translation products. All constructs expressed in the animal system were therefore also studied in the plant system. Only in the case of the pSP65−metA4CT template, a single product of 15 kd was synthesized (Figure 7c, lane 11) and aggregation was not encountered. Since translation in the plant system in contrast to the reticulocyte system is far less efficient for all templates, it is not unexpected that a low amount of aggregates would not be detected or that the amount of the 15-kd protein was not sufficient for aggregation.

The apparent relative translational efficiencies might be obscured by the difference in the stabilities of the translation products in the two systems. Other possible explanations are differences in the sequences surrounding the AUG initiation codons in the various constructs or factors such as mRNA stability or salt concentrations which also effect translational efficiencies.

Discussion

In this study we have taken several approaches in an attempt to understand the transmembrane orientation and biogenesis of the 695-residue amyloid A4 precursor of Alzheimer's disease. Our results indicate that the mature precursor is an integral membrane protein of M_r of 91 500 that spans the lipid bilayer once. The N-terminal part of the molecule up to the single transmembrane domain is *in vitro* translocated to the lumen of the microsomal membrane vesicles which is the *in vivo* equivalent of either the lumen of cytosomes or the extracellular space. The sequence serving as membrane anchor was localized to the C-terminal part of the molecule. The size of the cytoplasmic domain exposed on the surface of the membrane vesicles used in this study is ~4 kd. This is in full agreement with the postulated size of the putative cytoplasmic domain of the amyloid precursor (Figure 1a).

The experimentally determined M_r of 91 500 determined for the *in vitro* synthesized proteins in the presence and absence of membranes is substantially higher than the theoretical value calculated from the cDNA-derived protein sequence for the PreA4 (78.6 kd) (Kang *et al.*, 1987). An explanation for this unexpected electrophoretic mobility on SDS gels would be reduced SDS binding of the very acidic second extracellular domain that includes 45 negatively charged Asp and Glu residues in the PreA4 sequence 188−290 (Kang *et al.*, 1987). *In vitro* synthesis of the 303 N-terminal residues of PreA4 which contains this negatively charged domain resulted in a protein of M_r of 39 000 instead of the calculated 33 700. This mass difference amounts to 15%. Since the same difference between calculated and experimentally determined molecular mass was found for the entire precursor, the postulated reduction in SDS binding may be responsible for this abnormal migration in the gel.

To determine the size of the signal peptide we constructed the *Xho*I RNA which does not encode the *N*-glycan domain. This domain could have been expected to interfere with the reduction in molecular mass expected for signal peptide removal. Translation in the presence of microsomal vesicles containing signal peptidase activity results in a mol. wt shift of 1.5 kd. Subsequent radiosequence analysis allowed us to identify the signal peptidase cleavage site after residue 17. Cleavage occurs in agreement with the known specificity of signal peptidase after an Ala-Arg-Ala sequence (von Heijne,G., 1984). The resulting mature PreA4 begins with Leu 18 of the sequence reported by Kang *et al.* (1987).

The PreA4 sequence includes two *N*-glycosylation sites in the third domain at positions 467−469 and 496−498 and an *N*-glycosylation-negative Asn-Pro-Thr sequence located in the putative cytoplasmic domain. Translation of the entire precursor in the presence of membranes could be expected to lead to the removal of the 17-residue-long signal peptide and the addition of two carbohydrate chains of ~3 kd due to *N*-glycosylation. Competitive inhibition of carbohydrate transfer with *N*-glycosylation-acceptor-tripeptides showed a 2-kd mol. wt change, a value compatible with one or, more likely, two carbohydrate moieties being added. Since the removal of the 17 residues of the signal peptide did result in a mol. wt reduction of 1.5 kd and not the expected 2 kd, the 2-kd difference obtained in the presence of the glycosylation inhibitor is also likely to be an underestimation. This leads us to conclude that two sites are used.

The question whether the 695-residue PreA4 defined by Kang *et al.* (1987) is related to amyloid A4 in a precursor−product relationship was studied by two different approaches. First, we analysed the proteolysis of the two forms of *in vitro* synthesized PreA4, the membrane-inserted form and the primary translation product. In the presence of membranes, only the putative cytoplasmic domain was cleaved and no alteration of the aggregational properties of the shortened proteins was seen by SDS−PAGE. Complete digestion of the membrane-bound precursor occurred after addition of the non-ionic detergent Triton X-100. The same was found for the precursor synthesized without membranes in the absence of Triton X-100. This unexpected finding could indicate that either a specific protease is required or that a modification of the protein occurs which targets the proteolytic release of the amyloid sequence from the precursor. The synthesis of an aggregating PreA4 fragment of 10−18 kd which begins with the methionine residue followed by the amyloid sequence, the transmembrane domain and the cytoplasmic domain, proves that aggregation is an inherent property of the amyloid sequence. This is in agreement with our finding of a high β-pleated sheet potential for this region (Figure 7a). The functioning of the methionine codon preceding the amyloid A4 sequence as a translation initiation codon in the *in vitro* system does not support the idea of translational reinitiation suggested by Breimer and Denny (1987), since the corresponding construct included a cap site upstream of the methionine codon. We have also been unable to identify the corresponding 15-kd protein in brain homogenates of post-mortem tissues from patients with Alzheimer's disease (unpublished data).

The aggregational properties of the truncated molecule establishes a precursor−product relationship between PreA4 and amyloid. However, details of the factors required for the release of the aggregating principle (the amyloid sequence) remain to be established. From the studies described above, we predict that a proteolytic cleavage, which generates the N terminus of the amyloid A4, to be a crucial step for amyloid formation. Furthermore, membrane association of the precursor is expected to interfere with the process of aggregation. Incorrect or failure of membrane insertion is another possible event relevant for amyloidogenesis. At present, we assume that the generation of the

C terminus of the amyloid A4 peptide is not essential for aggregation and does not require a specific protease. This is based on the proteinase K treatment of the shortened aggregating translation product which gave rise to a band on SDS gels corresponding to the size of the amyloid A4 peptide (Figure 7c).

From our studies, we conclude that there is not a simple PreA4–amyloid relationship. Protease treatment of the full-length *in vitro* translation products results in complete breakdown and not in amyloid A4 formation. In view of our finding that the precursor fragment beginning with the amyloid A4 sequence does aggregate, we conclude that besides proteolytic cleavage other events, such as post-translational modification, are important. Furthermore, our results are consistent with the possibility that amyloid formation may arise as a consequence of membrane damage, and from precursor molecules that are not or are incorrectly integrated into membranes. Amyloid formation is therefore a secondary event that may represent the selective neuronal cell death in Alzheimer's disease. However, if amyloid deposition at clinical target sites interferes with plasticity, amyloid formation may then be considered a primary event for development and the progression of Alzheimer's disease.

Materials and methods

Cloning procedures

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Maniatis *et al.* (1982).

Construction of pSP65–PreA4 and mutant templates

The strategy used for construction of the pSP65–PreA4 vector is presented in Figure 1. The *Sma*I fragment of the PreA4 cDNA corresponding to the entire coding sequence of the 695-residue precursor, 92 bp of 5′ and 871 bp of 3′ untranslated sequences were cloned into the unique *Sma*I site of the polylinker of pSP65. The resulting plasmid is termed pSP65–PreA4 and has 25 nucleotides between the transcription start site and the *Sma*I site of pSP65. Truncated mRNA encoding residues 1–303 (*Xho*I RNA) was obtained as a result of cleaving pSP65–PreA4 with *Xho*I and subsequent transcription. The final mRNA construct encodes the signal sequence and two of the three putative extracellular domains of PreA4. The putative two *N*-glycosylation sites at positions 467–469 and 496–498 of the *N*-glycan domain (residues 290–624) are not included in *Xho*I mRNA. Construct pSP65–metA4CT was obtained by cloning the 961-bp *Bgl*II–*Hin*dIII fragment of the A4 cDNA clone into pSP65. The resulting mRNA construct has 989 nucleotides and includes methionine codon 596 of PreA4 as initiation codon, the entire amyloid A4 sequence (codons 597–639/640 of the precursor) and the entire C-terminal domain (Figure 1b and d).

In vitro transcription

See Kanarska *et al.* (1984). DNA templates (100 mg/ml) were transcribed in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, RNasin (1 U/ml), 100 mg/ml BSA, 500 mM each ATP, CTP and UTP, 50 mM GTP and 500 mM m^7 G(5′)ppp(5′)G. Typically, 1 U of SP6 RNA polymerse was added per mg DNA template for a 1-h synthesis at 40°C. DTT and rNTPs stocks were prepared with diethylpyrocarbonate-treated water. The components of the transcription reaction were mixed at room temperature to prevent precipitation of DNA.

Following RNA synthesis the DNA template was removed by the addition of RNase-free DNase and after a 10-min incubation at 37°C the reaction mixtures were phenol:chloroform extracted after addition of NaOAc (pH 5.5) to 0.3 M. The RNA was precipitated with ethanol and washed with 70% ethanol.

In vitro translation

See Bause *et al.* (1986). Translation of mRNA in both a cell-free rabbit reticulocyte lysate and a wheat germ system in the absence and presence of dog pancreas microsomes followed the procedures as described in the supplier's manuals (Amersham International and NEN, FRG). Typically the reactions were carried out for 60 min in the presence of 60 mCi of [^{35}S]methionine and 0.5–1.0 mg mRNA.

Analysis of *in vitro* translation products by SDS–PAGE was performed according to Laemmli (1970), except that after electrophoresis the gel was incubated with autoradiography enhancer (EN^{3}HANCE, Du Pont) for detecting radiolabelled compounds.

Assay for protein insertion into microsomal membranes

See Scheele (1983). Proteolytic degradation of translation products was carried out at 0°C in the presence or absence of 1% Triton X-100 under the following conditions:

Protease	Final concentration	Incubation time
Trypsin	0.5 mg/ml	30 min
Chymotrypsin	0.5 mg/ml	15 min
Proteinase K	0.1 mg/ml	10 min

Proteolysis was terminated by the addition of phenylmethylsulphonyl fluoride (final concentration 1 mM) or Trasylol (final concentration 1 mg/ml) and incubation for 15 min on ice.

Membrane sedimentation and carbonate extraction

See Fujiki *et al.* (1982). To selectively isolate integral membrane proteins the translation products (20 ml) obtained in the presence of microsomal membranes were diluted with 100 ml ice-cold 0.1 M Na_2CO_3 pH 11 and incubated at 0°C for 15 min. Then the samples were layered over a sucrose cushion (50 ml 0.25 M sucrose–0.1 M Na_2CO_3 pH 11) and centrifuged for 10 min at 30 p.s.i. in a Beckman airfuge using the A-100/30 rotor and cellulose propionate centrifuge tubes. After centrifugation the entire supernatant including the sucrose cushion was removed and precipitated with an equal volume of 20% trichloroacetic acid. The pellet was resuspended in 10 ml 2% SDS. Samples used for protein sequence analysis were dialysed against 0.01 M NH_4HCO_3 for 36 h using membranes with a cut-off value of 50 000.

Protein sequence analysis

NH_2-terminal sequence analysis was performed on an Applied Biosystems model 470A gas/liquid phase sequencer. For radiosequencing the PTH amino acids were collected, dried and dissolved in 0.15 ml of methanol. After addition of 10 ml of Quickszint (Zinsser) the radioactivity released at each cycle was determined in a Kontron liquid scintillation counter.

Assay for N-glycosylation

See Tillmann *et al.* (1987). To analyse the amount of *N*-glycosylation the translation is performed in the presence of ether 200 mM of the tripeptide *N*-glycosylation acceptor Bz-Asn-Leu-Thr-MeNH_2 or the non-acceptor peptide Bz-Asn-Leu-*a*Thr-MeNH_2.

Acknowledgements

We thank Ernst Bause for the *N*-glycosylation inhibitors. Financial support from the Deutsche Forschungsgemeinschaft through SFB 74, the BMFT of Germany, the Thyssen Stiftung and the Fonds der chemischen Industrie is gratefully acknowledged. C.L.M. is supported by the National Health and Medical Research Council of Australia.

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Received on December 24, 1987; revised on January 27, 1988