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A SYSTEM FOR STUDYING THE EFFECT(S) OF FAMILIAL ALZHEIMER DISEASE MUTATIONS ON THE PROCESSING OF THE ß-AMYLOID PEPTIDE PRECURSOR

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Summary: Three different point mutations have been observed in some familial Alzheimer's disease pedigrees at a unique valine, Val_{717}, near the carboxyl end of the ß Amyloid Peptide Precursor (ßAPP). The effects of these mutations on the processing and cellular functions of ßAPP can best be determined in the absence of the normal form(s) of the protein. We have used targeted mRNA degradation by a trans-acting hammerhead ribozyme to cleave and inactivate ßAPP expression *in vitro*. The consensus ribozyme cleavage site, 5'GUC↓X3, matches the Val_{717} nucleotide sequence in ßAPP mRNA. Introduction of FAD point mutations which change Val_{717} decrease the rate of ribozyme cleavage by more than three orders of magnitude. Thus, ribozyme targeting of this site should allow the study of protein processing *in vivo*. Furthermore, a ribozyme targeted to mutant ßAPP mRNA (Val_{717}→Ile) cleaved the mutant sequence 300-fold faster than the normal sequence. This suggests that ribozymes might lower mutant ßAPP mRNA levels in FAD cells.

One of the hallmarks of Alzheimer's disease is the formation of senile neuritic plaques (1). The major proteinaceous component of these plaques is the ß or A4 peptide, a 42 amino acid fragment derived from a larger precursor protein (ßAPP) by an unknown process (2). The gene encoding ßAPP has been localized to the long arm of chromosome 21 (3) near a locus genetically linked to familial Alzheimer disease (FAD) (4). Recent studies suggesting a nonallelic heterogeneity in FAD (5) indicate that one or more defects in the ßAPP gene may be responsible for some fraction of the disease, and indeed, several point mutations near one of the putative ß-peptide cleavage sites have been found (6, 7, 8). In order to better study the effects of these mutations on the production of ß-peptide it would be helpful to be able to analyze the processing and other biochemical and phenotypic properties of ßAPP mutant proteins in the absence of a wild-type ßAPP background. Selective mRNA degradation based on a single nucleotide difference has been demonstrated for *ras* proto-oncogenes through the use of targeting agents known as ribozymes (9, 10). We have used this technique to examine the ability of a trans-acting ribozyme targeted to ßAPP mRNA to selectively cleave model ßAPP substrate mRNAs containing point mutations in

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the ribozyme consensus cleavage site. We further demonstrate that the cleavage kinetics of the wild-type substrate are not drastically altered, even in the presence of excess mutant substrate mRNA.

Materials and Methods

Construction of ßAPP Ribozymes.
A double-stranded cDNA cassette, ßAPP-141, containing a class III T7 RNA polymerase promoter fused to bases 133-154 of ßAPP mRNA (11) was synthesized and purified as previously described (12). A similar cassette, APPrbz-141, which contained the conserved bases of the hammerhead ribozyme (13) and the complementary bases of ßAPP mRNA, was also constructed. The addition of *SalI* and *XhoI* flanking sequences allowed forced cloning into the multiple cloning site of the eucaryotic expression vector pMAMneo (Clontech). The resulting plasmid, was linearized with *XhoI* and used to produce either a 46 base or 116 base RNA by *in vitro* transcription (12). Cassettes containing the various FAD mutations were prepared similarly.

RNA Transcription Reactions and Ribozyme Reactions.
RNA was generated by run-off transcription with T7 RNA polymerase in the presence of [α-^{32}P]CTP from linearized plasmid DNA or cDNA oligonucleotide cassettes. Ribozyme reactions were performed as previously described (12). The kinetics of ribozyme cleavage were measured at 37°C in a total of 25 µl total volume under single turnover conditions (14, 15) using ~ 10 nM labeled ßAPP substrate mRNA and 10-160 nM unlabeled ribozyme RNA. Five microliter aliquots of the reaction mixture were withdrawn at various times following the addition of $MgCl_2$. Reactions were stopped by addition of 2.5 µl of PAGE loading buffer (10M urea, 50 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole). Samples were stored at -100°C until they were electrophoresed through 10% (19:1) acrylamide, 7M urea gels. Cleavage products were detected by autoradiography and quantified by scanning densitiometry (16). The fraction of substrate remaining, Frac S_t^*, plotted as a function of time for each model substrate mRNA was used to determine the apparent first order rate constant for the reaction.

Results

To date, all but one of the mutations observed in ßAPP have been located within exons 16-18 of the gene. In the case of FAD, two distinct mutation sites, one at Met_{670} (17) and one at Val_{717} (6, 7, 8) which flank the amino and carboxyl terminal sequence of the ß-peptide have been observed in seven different FAD pedigrees, Figure 1. The location of these mutations near the putative ß-peptide cleavage sites has prompted speculation that they may alter the rate or specificity of proteolytic processing (18). This in turn, may result in an increased accumulation of ß-peptide. To specifically examine the effects of FAD mutations upon the processing of ßAPP, either endogenous wild type ßAPP must be eliminated or mutant ßAPPs must be "tagged"; however, even slight modifications of the peptide backbone may itself affect processing.

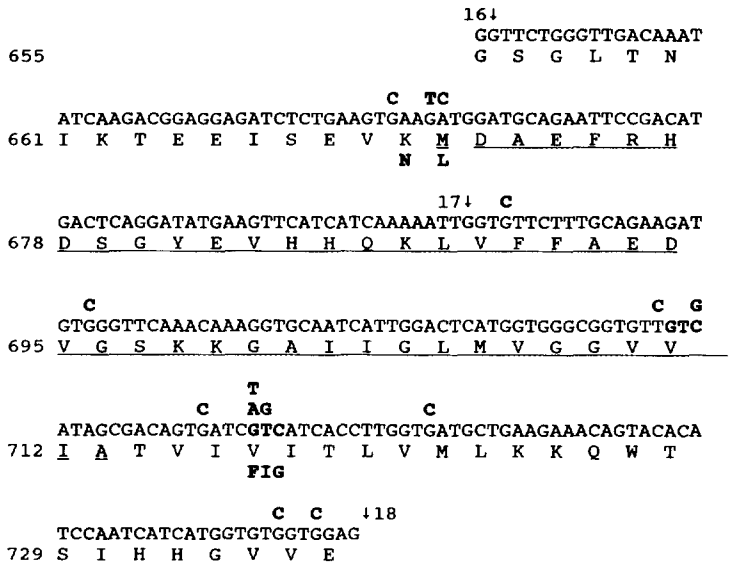

Figure 1. Partial nucleic acid and protein sequence of ßAPP surrounding the region of the A4 or ß-peptide (underlined). ßAPP intron-exon junctions 16-18 (26) are marked with arrows. Known point mutations are shown in bold-face type above the sequence. The resulting amino acid substitutions are shown in bold type below the sequence. Conservative valine mutations at positions 668, 689, 695, 710, 715, 721, 734 and 735 (numbers on left) confer susceptibility to cleavage by a trans-acting ribozyme, while mutations at positions 711 and 717 confer resistance.

The Val_{717} codon in normal ßAPP mRNA offers a point of attack for ribozymes. The wild-type codon sequence, GUC, corresponds to the consensus cleavage site found in the hammerhead catalytic motif of many pathogenic plant viroid and virusoid RNAs (19). Mutations in this consensus cleavage site are known to affect catalytic activity (13). FAD mutations at Val_{717} alter a GTC codon to TTC, ATC or GGC, respectively. We have previously shown that a trans-acting hammerhead ribozyme targeted to the first 5'GUC↓X3' sequence in ßAPP mRNA, Figure 2A, cleaved an [α^{35}S]CTP labeled model ßAPP substrate in a site-specific fashion *in vitro* (12). Here we show that the cleavage reaction can be forced to completion, Figure 2B, lane 4, in the presence of $MgCl_2$. Thus, one of the criteria necessary to study FAD mutant ßAPPs can be fulfilled by ribozymes *i.e.* ribozymes can specifically degrade wild-type ßAPP mRNA.

To examine whether a ribozyme can distinguish wild-type ßAPP mRNA from substrates which recapitulate the FAD point mutations observed at Val_{717} of the ßAPP gene we have determined the cleavage rate of various model RNA substrates under single turnover conditions. Under these conditions, the rate of ribozyme cleavage is determined solely by the chemical step of the reaction and not ribozyme formation (16). Since the

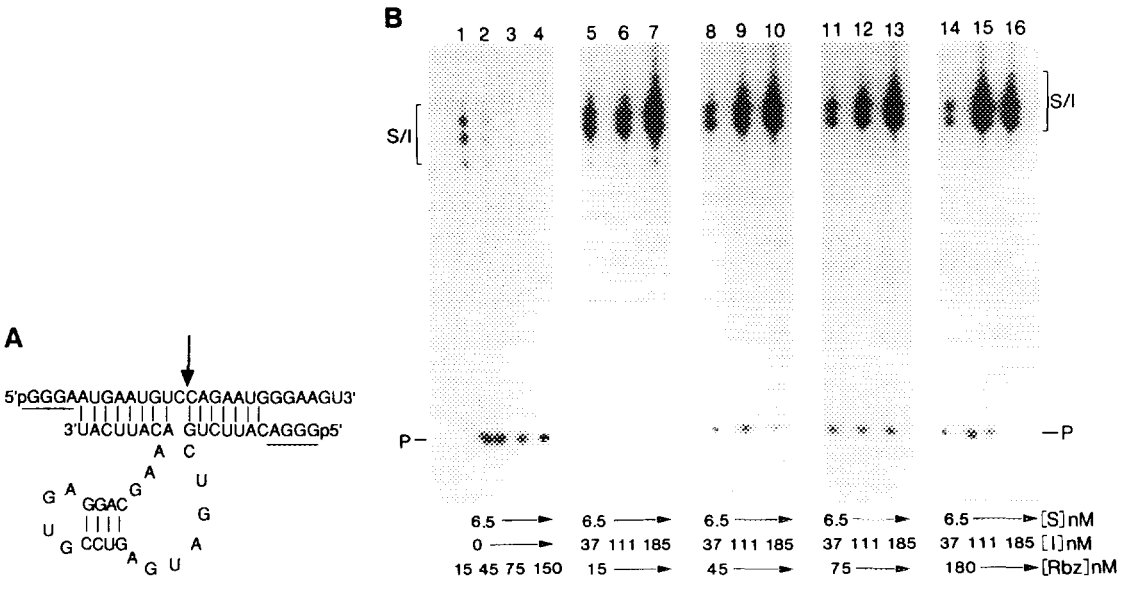

Figure 2. A. Secondary structure model of the APPrbz-141 ribozyme RNA interacting with its target, ßAPP mRNA. Vector derived sequences are underlined. Substrate cleavage at the hammerhead consensus cut site (arrow) produces a pair of 13 base products. B. *In vitro* transcription of the ßAPP-141 cDNA substrate cassette produces a 26 base unit length RNA as well as one and two base longer forms (S) that are extended at the 3' end (lane 1). Cleavage of (S) RNA by ribozyme APPrbz-141 RNA (R) occurs in a metal ion dependent, site-specific (lanes 2-4) manner *in vitro*. The effect of excess mutant ßAPP substrate mRNA upon the cleavage of wild type ßAPP substrate mRNA was measured by cleaving wild-type ßAPP substrate mRNA (S) in the presence or absence of an increasing amount of the non-cleaving U_{12}→G substrate RNA (I) at various APPrbz-141 RNA concentrations, as indicated.

primary sequence within the base-pairing stems of the ribozyme should not drastically affect activity (20), the FAD mutations were introduced into the backbone of a model substrate encompassing bases 133 to 154 of ßAPP mRNA. The results of these experiments are shown in Table 1. Product formation from cleavage of the wild-type substrate RNA exhibited first order kinetics with a $t_{1/2}$ of 9 min. This demonstrates that both RNAs can associate and form a competent cleavage complex. Of the substrate mutations shown here, only the G_{11}→A mutant exhibited any cleavage, and that activity is nearly three orders of magnitude less than wild-type. That the difference in the cleavage rates of the wild-type and mutant substrate RNAs was due to the loss of a single base-pair and not due to some defect in the mutant substrate RNAs was determined by measuring the rate of cleavage of the same substrate RNAs with a mutant APPrbz-141 RNA which restored the lost base-pair. As shown for the G_{11}→A mutant, wild-type cleavage activity was restored when base pairing was restored. These data indicate that a hammerhead ribozyme targeted to ßAPP mRNA can degrade wild-type but not FAD mutant RNAs *in vitro*.

Table 1.

Effect of Point Mutations Near the Hammerhead Ribozyme Consensus Cleavage Site on the Cleavage Activity of Model ßAPP-141 mRNA Substrate in vitro

Mutation	Cleavage Sequence[a]	Amino Acid[b]	Activity [c] k_{obs} (hr^{-1})	Relative	Decrease
Wild Type	-GUC↓C- || | CA- G	Val	4.62	100	0
G_{11}→A	-AUC↓C | | CA- G	Ile	0.0115	0.25	400
G_{11}→A	-AUC↓C || | UA- G	Ile	3.80	83	NA[e]
G_{11}→U	-UUC↓C | | CA- G	Phe	ND[d]	-	>11,000
U_{12}→G	-GGC↓C | | CA- G	Gly	ND[d]	-	>11,000
C_{13}→G	-GUG↓C || | CA- G	Val	ND[d]	-	>11,000

(a) ßAPP-141 mRNA substrate sequence for hammerhead ribozyme (Figure 2A) shown as top strand. Mutations are highlighted.
(b) In frame translation of mutated GUC codon results in new amino acid.
(c) Apparent first order rate constants, k_{obs}, were determined from semilogarithmic plots of kinetic data at 37°C in 10mM $MgCl_2$ with a [Ribozyme]/[Substrate]=50. Relative activity values are compared to wild type ribozyme. The fold decrease in activity was calculated as $100 x k_{wild\ type}/k_{obs\ mutant}$.
(d) ND, none detected. We are easily able to quantitate as little as 1% substrate cleavage. Over a 16 hr reaction, 1% cleavage results in a k_{obs} of 0.00042.
(e) NA, not applicable.

Finally, we examined whether the mutant RNA substrates might affect the cleavage kinetics of wild-type ßAPP mRNA, Figure 2B. In this experiment, increasing quantities of uncleavable U_{12}→G mutant RNA (Table 1) was added to reactions containing the wild-type substrate over a wide range of APPrbz-141 RNA concentrations. The results clearly indicate that cleavage is unaffected by up to a 30-fold excess of the mutant substrate, when the

APPrbz-141 RNA concentration was five-fold greater than the wild type substrate (lanes 8-16). When the APPrbz-141 concentration was twice that of the wild-type substrate a slight effect is observed (compare lane 1 to lanes 5, 6 and 7); however it appears to be independent of the concentration of mutant substrate RNA. Thus, within the complex milieu of cells harboring both mutant and wild-type ßAPP mRNAs a trans-acting ribozyme targeted to one should not be greatly affected by the presence of the other.

To insure that the results obtained were applicable to the ßAPP sequence surrounding Val_{717}, a ribozyme targeted to bases 2209-2225 was constructed to cleave ßAPP mRNA bearing the Val_{717}→Ile_{717} mutation. We then examined whether this ribozyme could discriminate between itself and the wild-type ßAPP mRNA bearing the Val_{717} GUC codon. The results of this experiment are presented in Table 2. It is again apparent that the single base mismatched wild-type substrate was not readily cleaved, while the target FAD Ile_{717} ßAPP mRNA was cleaved. However, the rate of cleavage of this ribozyme was reduced approximately ten-fold as compared to the two other matched ßAPP-141 ribozymes (Table 1). This is almost certainly due to the varying abilities of these RNAs to form a cleavage competent structure (data not shown).

Table 2.

Effect of Point Mutations on the <u>in vitro</u> Cleavage Activity of a Ribozyme Targeted to FAD ßAPP-Ile_{717} mRNA

Mutation	Cleavage Sequence[a]	Amino Acid [b]	Activity [c] k_{obs} (hr^{-1})	Relative	Decrease
FAD G→A	-AUC↓C || | UA- G	Ile_{717}	0.6	100	0
Wild Type	-GUC↓C- | | UA- G	Val_{717}	0.002	0.35	285

(a) A trans-acting hammerhead ribozyme targeted to bases 2209-2225 of ßAPP mRNA and designed to specifically cleave the Familial Alzheimer Disease ßAPP-Ile_{717} mRNA (Figure 1) was assessed as in Table 1 with 21 base model substrates containing the mutated AUC codon or wild-type GUC codon.
(b,c) Defined in Table 1.

Discussion

Our data clearly show that a trans-acting hammerhead ribozyme targeted to the first 5'GUC↓X3' in ßAPP mRNA cleaves short model substrates in a metal-ion dependent, site-specific fashion *in vitro* **(Figures 2B)**. Point mutations within the conserved substrate sequence inhibit the rate of cleavage by at least 3-4 orders of magnitude **(Table 1)**. All point mutations are not equivalent however, but can be divided into two classes based on cleavage activity. One class, typified by the FAD Val_{717} mutation G_{11}→A, exhibit a low but demonstrable cleavage activity *in vitro*. The other class, which includes the FAD Val_{717} mutation G_{11}→U and U_{12}→G shows no activity even in the presence of a large molar excess of RNA containing the conserved ribozyme core sequence. The sequence requirements for ßAPP RNA cleavage determined here are consistent with those observed with other hammerhead ribozymes, with one exception. Perriman *et al*, but not Ruffner *et al* was unable to detect cleavage of a G_{11}→A substrate RNA, even when base-pairing was restored (13, 21). However it should be noted that this study was performed with near equimolar quantities of substrate and ribozyme RNA. Fedor *et al* have shown that RNA conformation can profoundly affect the formation of a competent hammerhead complex (22). Our experimental protocol which uses an excess of ribozyme RNA insures that if a competent cleavage structure can form, it will. Finally, neither the specificity nor the rate of cleavage of the wild-type substrate is affected by the presence of a 30 fold excess of mutant, inhibitor RNA, even when it is in excess of ribozyme RNA. These results agree with other specificity studies showing that ribozyme cleavage activity is not drastically altered by the presence of large amounts of non-homologous RNA (12, 23). Thus, the ability of the two different ribozymes to discriminate between messages that differ by a single nucleotide indicate that the FAD mutant proteins, Val_{717}→Phe, Val_{717}→Gly and Val_{717}→Ile can be studied in the absence of wild-type ßAPP in cultured cells by targeting a trans-acting hammerhead ribozyme to that site. Such studies will afford a better understanding of the role ßAPP plays in cellular metabolism *in vivo*. Furthermore, by constructing ßAPP expression vectors that combine a conservative Val, C→G mutation at either amino acids 711 or 717, **(Figure 1)** that inactivates ribozyme cleavage, with the mutations found in HCHWADT (24) or Schizophrenia patients (25) or the Arg/Met_{670}→Asn/Leu FAD mutant (17) the effect(s) of these mutations on ßAPP processing and other cellular processes can also be studied. In the future, potential gene therapies based on targeted ßAPP mRNA degradation by hammerhead ribozymes **(Table 2)** may also be possible.

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