Ribozyme mediated degradation of β -amyloid peptide precursor mRNA in COS-7 cells

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ABSTRACT

Two sets of eucaryotic expression vectors encoding *trans*-acting hammerhead ribozymes and *trans*-acting hairpin ribozymes were constructed. In one set of vectors ribozyme RNA transcription was placed under the control of a mouse mammary tumor virus long terminal repeat (MMTV-LTR). In the other set ribozyme expression was controlled by a metallothionein IIA (Mt-IIA) promoter. Each ribozyme was directed to the first target sequence in the Alzheimer amyloid peptide precursor mRNA (β APP mRNA), 5'↓GUC↓3'. Ribozyme RNA transcribed from these vectors, which should cleave all six alternatively spliced forms of β APP mRNA as well as β APP pre-mRNA, was shown to cleave a β APP RNA substrate analog *in vitro*. Stably transfected COS-7 cell lines bearing both vector types were prepared. Steady-state levels of β APP mRNA were reduced 25–30% in cells containing either active or mutant hammerhead ribozyme vectors driven by the MMTV-LTR promoter grown in the presence of glucocorticoids. In cell lines bearing Mt-IIA driven ribozymes steady-state levels of β APP mRNA were reduced 67–80% in both hammerhead and hairpin ribozyme containing cell lines following promoter induction by glucocorticoids. These levels correlate with the appearance of low levels of induced ribozyme RNA. In contrast, steady-state α -actin mRNA and G3PDH mRNA levels in these cells remained constant. Western blotting of cell extracts revealed that all forms of β APP were correspondingly reduced. Neither the RNA nor protein decreases observed in ribozyme transfected cell lines were observed in stably transfected control cells bearing the vector alone. These results suggest that ribozyme-mediated degradation of β APP mRNA in COS-7 cells does not depend on ribozyme cleavage.

INTRODUCTION

The β -amyloid precursors (β APPs) derive from the gene encoding the β -peptide, the primary proteinaceous component of amyloid

deposited in the brains of Alzheimer disease (AD) victims (for reviews see 1–3). To date, six alternatively spliced variant β APP mRNAs have been identified. Genomic DNA sequence studies (4–6) have identified the boundaries of the 16 introns in β APP-695, the smallest known form of the message. Three longer forms, β APP-770, β APP-751 and β APP-714 point to the existence of at least 19 exons (7). In addition, another alternatively spliced mRNA variant, β APP-L, that is formed by skipping exon 15 of β APP-751 has been found recently in human T-lymphocytes, macrophages and brain microglia (8). Knowledge of β APP's function may suggest what factors affect its metabolism, and how abnormal metabolism of β APP may be related to AD. Some aspects of the function of β APP have been inferred from its primary structure. These include a cell adhesion molecule that directs neuronal growth and/or a receptor for a substance required for growth or survival (9,10). In addition, it has been shown that a secreted form of β APP-751 from rat pheochromocytoma (PC12) cells is associated with mitogenic stimulation of Swiss 3T3 cells (11). The function of this form of β APP was further clarified by its identification as protease nexin II, a previously characterized protease inhibitor (12–14). Therefore, β APP may play a role in protease inhibition *in vivo*. β APP must have at least one other important function in brain, because the predominant form, β APP-695, lacks the protease inhibitor domain. Ninomiya *et al.* have recently shown that a 5 aa peptide found in secreted β APP-695, RERMS, can stimulate the growth of A-1 fibroblasts (15,16). However, since this sequence is common to all forms of β APP the associated activity may not be unique to β APP-695.

A common strategy used to deduce the phenotype of a gene of unknown function is to examine the effect(s) its deletion produces in the cell or organism in question. Although reverse genetic approaches for developing null mutations have been useful in studying lower eucaryotes such as yeast and *Drosophila*, and higher eucaryotes such as mice (17) it cannot be applied to humans. Targeted message degradation by antisense DNA, antisense RNA or ribozymes however offers a simple selective approach to reduce a specific gene product (18,19). When successfully applied, this technique effectively introduces a null mutation into cells.

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Trans-acting ribozymes based upon the hammerhead or hairpin RNA motifs have been used to reduce or eliminate specific gene products *in vivo*. For example, Dropulic *et al.* have shown by inserting a hammerhead ribozyme targeted to U5 RNA into the *nef* gene of HIV-1 that viral infectivity was greatly reduced (20). Delivery of this ribozyme to human T cells *via* a defective amphotropic vector, was found to partially protect them from HIV-1 infection. Similarly, retroviral vectors containing a hairpin ribozyme, under the control of either a β -actin or a pol III promoter have been shown to inhibit expression in diverse strains of HIV-1 in HeLa cells (21). In addition, Hullier *et al.* have shown that delivering ribozymes targeted to α -lactalbumin mRNA by a T7-vaccinia virus led to efficient reduction of this message in C127I mouse cells (22). Finally, Xing *et al.* have shown that a hammerhead ribozyme targeted to a sequence in the S segment of Lymphocytic Choriomeningitis Virus (LCMV) effectively reduced viral RNA in NIH 3T3 cells (23). These studies suggest that ribozymes expressed during viral infection can function in cells. Recent studies also suggest that ribozymes can also function under normal cellular conditions. Scanlon *et al.* have shown that a ribozyme targeted to *fos* mRNA selectively reduced *fos* and *fos*-controlled gene expression *in vivo* (24), while Snyder *et al.* have shown that transient transfection of a DNA/RNA hybrid ribozyme targeted to the *bcr-abl* splice junction reduced levels of the *bcr-abl* fusion protein, p210^{bcr-abl} in a cell line derived from a patient with chronic myelogenous leukemia (25). Finally, stably transformed HeLa cells bearing a hammerhead ribozyme targeted to the 5' region of human O⁶-methylguanine-DNA methyl transferase (MGMT) were shown to lack both MGMT protein and message (26).

These results encouraged us to attempt to eliminate endogenous β APP mRNAs from cells by inducibly expressed β APP-specific ribozymes. We report here the activity of three of these vectors displayed in stably transfected COS-7 cells under a variety of conditions.

MATERIALS AND METHODS

Ribozyme constructs and *in vitro* ribozyme cleavage reactions

pMEP4-Rz1 and pMEP4-Rz2 vectors were prepared by cloning double-stranded cDNA cassettes containing the conserved bases of the hammerhead or hairpin ribozyme, respectively flanked by sequences complementary to the target site of β APP mRNA into the *Bam*HI/*Hind*III sites of the eucaryotic expression vector pMEP4 (Invitrogen Corp.). RNAs were transcribed from double-stranded cDNA cassettes containing the ribozyme flanked by a T7 RNA polymerase promoter or from pGEM-7z(f) (Promega Corp.) procaryotic transcription vectors containing these cDNA cassettes. Ribozyme cleavage reactions were performed as described previously (27,28). Ribozyme vectors pMAMneo-Ribozyme and pMAMneo-Dead have been described previously (27).

Analysis of β APP mRNA levels

Total RNA was isolated from *ca.* 1×10^7 COS-7 cells by the single-step TRISOLV procedure according to the manufacturer's specifications (BioTex Laboratories, Inc.). Quantitative slot blot hybridization of 20 μ g of total RNA from each sample was performed essentially as described in (29) using deoxyoligonucleotide probes for β APP, G3PDH, ribozyme and α -actin mRNA respectively. Each probe was 27 b in length, had a GC content of 50% and nearly identical T_m s. Northern blots

hybridized and washed under identical conditions produce single bands of the appropriate size for each message (29).

Tagged RT-PCR detection of ribozyme RNA

Total RNA (10 μ g) isolated from each cell line was reverse transcribed into single strand cDNA with 20 U of mouse mammary tumor virus reverse transcriptase, MMTV-RT, using a primer containing 20 bases complementary to the SV40 polyadenylation site in pMEP4 and an additional 21 base tag corresponding to the conserved sequence of a class III bacteriophage T7 RNA polymerase promoter (italics), 5'TCCC-TATAGTGAGTCGATTATTATTGTCAGCTTATAATGGT3'. Single-stranded cDNA was then amplified by PCR for 30 cycles consisting of a 1 min denaturation step at 94°C, a 2 min annealing step at 55°C and a 2 min extension step at 72°C with a 5', 21 base primer corresponding to the *Hind*III site (italics) and stem I of the hammerhead ribozyme, 5'AAGCTTCATTCTGCTG-ATGAG3', or the *Hind*III site (italics) and stems I and II of the hairpin ribozyme, 5'AAGCTTATTCTGAGAATTCAA3' and a 3', 21 base primer complementary to the T7 RNA polymerase promoter, 5'TAATACGACTCACTATAGGGA3'. Following amplification, the 193 bp (hammerhead) or the 204 bp (hairpin) reaction products were resolved by electrophoresis through 2% agarose. The identity of the products was confirmed by Southern blotting using 27 base ³²P-labeled deoxyoligonucleotide probes, 5'CATTCTGCTGATGAGTCCGTGAGGACG3' (hammerhead) or 5'ACCACAACGTGTGTTTCTCTGGTTGAA3' (hairpin), that were complementary to conserved core sequences of each ribozyme.

Genomic Southern analysis

Genomic DNA was isolated from *ca.* 1×10^7 stably transfected COS-7 cells by differential precipitation (30). The presence of vector DNA sequences was determined by PCR amplification using one of the two 5' primers from the RT-PCR assay and a 3' primer, 5'TTTATTGTCAGCTTATAATGGT3', that amplifies a 173 bp (hammerhead) or 184 bp (hairpin) fragment spanning the multiple cloning site in the vector. Southern blotting with the 27 base deoxyoligonucleotide probes used in the RT-PCR assay (above) was used to confirm the identity of the band.

Western blotting

Total cellular proteins were isolated from *ca.* 1×10^7 COS-7 cells and quantified according to previously described methods (31). Western blots of total cellular extracts were performed as described in (29) using polyclonal antibodies directed toward N-terminal, C-terminal and KPI domain peptides within β APP.

COS-7 cell culture

COS-7 cells obtained from the American Type Culture Collection were grown and subcultured as described in the supplier's instructions. Briefly, cells were grown and passaged in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, 5% fetal calf serum, 2 mM glutamine and antibiotics. Cells were maintained at 37°C in a 94% air–6% CO₂ atmosphere and were refed and subcultured weekly. Stably transfected COS-7 cell lines were prepared using lipofectin (32) and were selected for their resistance to geneticin (400 μ g/ml) or hygromycin (250 μ g/ml) depending on the vector used. All experiments carried out using these cells were performed in the constant presence of the antibiotic. Promoter activity studies were performed in RPMI 1640 media in which endogenous levels of

Figure 1. Ribozyme expression vectors pMAMneo-Ribozyme (A) pMEP4-Rz1 (B) and pMEP4-Rz2 (C). Ribozyme encoded RNA is enclosed in each box. Bold-face italic G and A represent sites within the ribozyme that inactivate cleavage when they are mutated to an A and G, respectively (27,36). The target sequence and cleavage site at position 141 of β APP mRNA is shown above each ribozyme.

glucocorticoids in the serum had been reduced by filtration through charcoal (Sigma Chemical Co.). Briefly, subconfluent cells grown in RPMI 1640 were washed and then grown for 48 h in charcoal filtered media and subsequently harvested.

RESULTS

Eucaryotic ribozyme expression vectors

The first naturally occurring cleavage site in β APP mRNA, (5'GUC↓X3') for a *trans*-acting hammerhead ribozyme and (5'Y↓GUC3') for a *trans*-acting hairpin ribozyme, occurs at position 141 (33). We chose the sequences surrounding this site to construct various RNA expression cassettes and cDNA plasmid expression vectors. RNAs transcribed from these cassettes should, in the presence of β APP mRNA, fold into cleavage-competent ribozymes. Two primary considerations were used in selecting this site. First, previous studies have shown that the 5' ends of some mRNAs react more readily with antisense agents than those targeted to interior coding regions (34). Second, all of the known variant β APP mRNAs arise from alternative splicing of exons 6 and beyond. Since the sequences surrounding position 141 in β APP mRNA are entirely contained in exon 2 of the gene, ribozymes targeted to this site should cleave and inactivate all of the known alternatively spliced β APP variant mRNAs as well as the pre-mRNA. Two sets of ribozyme expression plasmids were constructed. The first set was based upon the vector pMAMneo (Clontech) (Figure 1A). Expression of ribozyme RNA *in vivo* then, depends on transcription from the glucocorticoid responsive mouse mammary tumor virus long terminal repeat (MMTVLTR). The second set of vectors that were prepared were based on the vector pMEP4 (Invitrogen) (Figure 1B, C). In this case, cDNA cassettes bearing either a hammerhead or hairpin ribozymes were inserted into the *Hind*III/*Bam*HI sites of pMEP4 generating the eucaryotic episomal expression vectors pMEP-Rz1 and pMEP-Rz2 respectively. Because they contain the Epstein Barr virus (EBV) origin of replication, Ori P, and nuclear antigen EBNA-1, these vectors can be maintained extrachromosomally and produce high levels of recombinant insert. An inducible metallothionein IIA (MtIIA) promoter upstream of the cDNA cassette drives ribozyme RNA transcription *in vivo* (35).

In vitro cleavage properties of β APP hammerhead and hairpin ribozymes

Previous studies of a hammerhead ribozyme targeted to β APP mRNA have shown both model and full-length β APP mRNA substrates cleaved in a metal-ion dependent, site-specific fashion under single-turnover conditions using a 3–15 fold molar excess of ribozyme (27,28,36). Introducing point mutations into the conserved core residues of the ribozyme decreased the apparent first-order rate constant, k_{obs} , of short model substrates by a factor of 11,000. Under similar conditions cleavage of full-length β APP-751 mRNA could not be detected. This is in keeping with a general 20-fold lowering of the cleavage rate between short and long substrate RNAs with the active ribozyme (36). When the catalytic strand of the β APP-141 hairpin ribozyme was tested for its ability to cleave β APP mRNA model substrates under single-turnover conditions with a 15-fold excess of ribozyme RNA, it was found to be much less efficient than its hammerhead counterpart (Figure 2, compare lane 2 with lanes 3 and 4). In fact, the cleavage activity of the hairpin ribozyme was only slightly greater than that of the catalytically inactivated mutant hammerhead ribozyme (unpublished observations). These data

Figure 2. Comparison of *in vitro* cleavage efficiencies of pGEM-Rz1 and pGEM-Rz2 RNA. *In vitro* transcription of the β APP-141 cDNA substrate cassette in the presence of [α - 32 P]CTP produces transcripts (S) of 25–28 bases in length (27,28,36). Substrate cleavage at the consensus hammerhead cut site in the presence of the catalytic strand of the hammerhead ribozyme, pGEM-Rz1, (lane 2) results in a labeled 5' product (HHP) of 13 bases and an unlabeled 3' product of 12–15 bases that mirrors the microheterogeneity of the substrate RNA. Substrate cleavage at the consensus hairpin cut site in the presence of the catalytic strand of the hairpin ribozyme, pGEM-Rz2 RNA, (lanes 3 and 4) results in an unlabeled 5' product of 10 bases and a labeled 3' product (HPP) of 15–18 bases reflecting the microheterogeneity of the substrate RNA. Lane 1 contains the β APP-141 substrate RNA in the absence of the catalytic strand of either ribozyme. Lanes 3 and 4 show 5 $\times$ and 1 $\times$ loadings of β APP-141 substrate RNA cleaved by pGEM-Rz2 RNA.

indicate that the two strands of the β APP-141 hairpin ribozyme do not readily form a cleavage-competent structure *in vitro*.

Expression of the β APP gene in ribozyme transfected cells

Our initial studies were carried out with a set of vectors based upon the plasmid pMAMneo (Clontech) (Figure 1A). Stably transfected cell lines bearing the parent vector, pMAMneo, pMAMneo-Ribozyme, or an inactive mutant ribozyme pMAMneo-Dead (27) were selected and maintained on serum containing growth media supplemented with 400 μ g/ml geneticin, G₄₁₈. The presence of vector DNA in these cells was confirmed by genomic Southern blotting, using probes specific to the neomycin transferase gene and the ribozyme. Quantitative slot blot hybridization of total RNA (29) was used to determine the steady-state levels of β APP mRNA in each cell line. The results depicted in Figure 3 reveal that β APP mRNA levels in both pMAMneo-Ribozyme and pMAMneo-Dead cell lines were slightly reduced (20–25%) following induction by 10 μ M dexamethasone. In contrast, cells harboring the vector alone were unaffected.

In order to obtain more active ribozyme-producing cell lines, a hammerhead catalytic RNA expression cassette (pMEP4-Rz1)

Figure 3. A. Quantitative slot blot hybridization analysis of total RNA isolated from COS-7 cells transfected with pMAMneo, pMAMneo-Ribozyme and pMAMneo-Dead. Total RNA was isolated from two different cell pellets of each stably transfected cell line. Duplicate 20 μ g samples of each preparation were subjected to three separate slot blot hybridization experiments using 32 P-labeled, 27 base deoxyoligonucleotide probes that detect all the alternatively spliced β APP mRNAs or α -actin (29). Hybridization intensities were measured by scanning densitometry. β APP message levels for each sample were normalized to α -actin mRNA. The relative β APP level in pMAMneo cells was arbitrarily set to 100 percent. Shaded bars, glucocorticoid-depleted medium; solid bars, glucocorticoid-depleted medium plus 10 μ M dexamethasone.

Figure 4. Quantitative slot blot hybridization analysis of total RNA isolated from COS-7 cells transfected with pMEP4-Rz1, pMEP4-Rz2, or pMEP4. Total RNA was isolated from five different cell pellets of each stably transfected cell line. Duplicate 20 μ g samples of each preparation were subjected to at least four separate slot blot hybridization experiments. Hybridization intensities were measured by scanning densitometry. β APP message levels for each sample were normalized to α -actin mRNA. The relative β APP level in pMEP4 cells was arbitrarily set to 100 percent. Shaded bars, glucocorticoid-depleted medium; solid bars, complete medium.

and a hairpin catalytic RNA expression cassette (pMEP4-Rz2) were cloned into the expression vector pMEP4 (Figure 1B). Both of these vectors and pMEP4 alone were transfected into COS-7 cells. Stable cell lines were selected and maintained on serum-containing media supplemented with 250 μ g/ml hygromycin. Cells were shown to contain vector DNA by PCR amplifying genomic DNA using a set of primers that specifically recognize the cloned ribozyme cassette. Steady-state β APP mRNA levels (Figure 4) were significantly reduced (67–80%) in hammerhead

Figure 5. Western blot analysis of β APP levels in glucocorticoid induced COS-7 cells transfected with pMEP4-Rz2 (lane 1) pMEP4-Rz1 (lanes 2 and 3), or pMEP4 (lanes 4) and control lanes showing the patterns of untransfected COS-7 cells (lane 5), purified rat brain β APP (lane 6) and partially purified recombinant β APP-751 from baculovirus infected cells (lane 7). 10 μ g of protein from each cell line was electrophoresed through 10% SDS-polyacrylamide gels, transferred to nitrocellulose and stained with polyclonal antibodies to either the N-terminal (panel A), KPI (panel B) or C-terminal domains of β APP (panel C), respectively.

ribozyme cell lines containing serum-derived levels of glucocorticoids. This reduction did not occur in cell lines bearing the pMEP4 plasmid alone, nor in cells grown in charcoal-stripped media, which depletes the endogenous levels of glucocorticoids (37). Pleiotropic effects cannot explain this apparent decrease, since α -actin mRNA levels in ribozyme-containing cell lines were relatively unaffected. Similar decreases were also observed in cells harboring pMEP4-Rz2, the hairpin ribozyme.

Corresponding decreases in steady-state β APP levels were observed in Western blots of extracts from the pMEP-Rz containing cell lines. Figure 5 shows β APP protein patterns in total cell homogenates from various serum induced cell lines. Three different rabbit polyclonal antibodies directed to three different regions of β APP, N-terminal (panel A), Kunitz protease inhibitor (KPI) (panel B) and C-terminal (panel C) were used to probe Western blots of these extracts (31). In each instance, (lanes 1–3) the extracts from ribozyme-containing cells had less total β APP than that from cells containing the pMEP4 vector alone (lane 4). In contrast, α -actin protein levels from ribozyme and control cell lines were the same (unpublished observations). The three antibodies, coupled with two β APP standards, a purified preparation of β APP from rat brain (31) and a partially purified preparation of β APP-751 from baculovirus infected Sf9 cells (38), allowed us to identify some of the β APP isoforms in COS-7 cells. Rat brain β APP contains predominantly the post-translationally modified 695 isoforms as is apparent from the lack

Figure 6. (A) Tagged RT-PCR assay of total RNA isolated from COS-7 cell lines transfected with pMEP4 (lanes 1–3) or pMEP4-Rz1 (lanes 4–7). Negative control lanes C1–C3 representing tagged RT-PCR of total RNA isolated from COS-7 pMEP4-Rz1 cells minus one of the three amplification primers and positive controls lanes R and D of *in vitro* transcribed pMEP4-Rz1 RNA and pMEP4-Rz1 DNA lanes 6 and 7 bracket the assay. The 193 bp product (P193) is indicated. (B) β APP, G3PDH and ribozyme RNA levels in various COS-7 transfected cell lines. Total RNA (20 μ g) isolated from pMEP4 ($n = 4$), pMEP4-Rz1 ($n = 4$) and pMEP4-Rz2 ($n = 4$) cells were subject to slot blot hybridization using 27 base deoxyoligonucleotides that detect all the alternatively spliced β APP mRNAs, α -actin, ribozyme and G3PDH mRNAs as described in Figure 3. Hybridization intensities were measured by scanning densitometry. β APP, G3PDH and ribozyme message levels for each sample were normalized to α -actin mRNA. Relative molar amounts of each species are compared.

of staining with an antibody targeted to the KPI domain (panel B, lane 6). This species is a minor component of COS-7 cells, but is significantly reduced in ribozyme-containing cell lines. The other β APP species that can be identified in COS-7 cells are those forms that possess the KPI domain. By comparing the staining patterns of the C-terminal antibody (panel C) with those of the KPI antibody (panel B) it is apparent that the two major β APP species in COS-7 cells contain the KPI domain. Based upon the migration of baculovirus-derived β APP-751 (lane 7) we conclude

Figure 7. Growth rates of COS-7 transfected cell lines in serum-containing media. (A) COS-7 cells containing pMEP4, and two different clones of pMEP4-Rz1 or (B) pMEP4 and pMEP4-Rz2 were plated in duplicate in serum-containing media at an equal cell density. Cell counts were taken at the indicated times. The mean cells/ml $\times 10^4$ for each cell line are shown.

that the faster of these β APP isoforms is β APP-751, whereas the other must be β APP-770 and/or a post-translationally modified form of β APP-751. These designations are in agreement with the relative amounts of the individual β APP mRNA isoforms found in COS-7 cells as well as pulse-chase labeling studies of β APP processing (unpublished observations).

In both types of ribozyme-containing cell lines (lanes 1–3) the faster migrating β APP-751 isoform was significantly reduced relative to that observed in pMEP4 control lines. Interestingly however, cell lines containing the hairpin ribozyme, pMEP-Rz2, showed a slightly different distribution of β APP-751 isoforms as compared to either the pMEP4 or pMEP-Rz1 cell lines. In this case, the slower moving form of β APP-751 was totally absent. The exact cause of this difference has yet to be determined. It appears to be due to one or more changes in post-

translational processing since the immature, faster migrating β APP species is still present.

In order to confirm that reduction in β APP steady-state levels in each of our ribozyme cell lines was due specifically to the transcription of ribozyme RNA we employed a tagged reverse transcriptase-PCR assay (RT-PCR) (39) to measure expression of ribozyme RNA. In this method, non-intron containing ribozyme RNA is distinguished from contaminating vector DNA by first strand cDNA synthesis using a primer whose 5' end contains a 20 base tag sequence corresponding to the bacteriophage T7 class III promoter. Amplifying the cDNA with a primer complementary to this tag ensures the detection of mRNA. The results shown in Figure 6 demonstrate that ribozyme mRNA was expressed in each of the pMEP-Rz transfected cell lines (lanes 4–7). By contrast, expression was not observed when one of the three PCR amplicons was absent from the reaction (lanes C1–C3), nor was amplification detected in cells bearing pMEP4 alone (lanes 1–3). Having shown that ribozyme RNA is transcribed in cells harboring the pMEP4-Rz constructs we used quantitative slot blot hybridization to measure its steady-state level relative to two control messages. Figure 6B. shows the level of β APP, G3PDH and ribozyme RNA in three COS-7 cell lines grown in serum containing media. The level of each message was normalized to α -actin mRNA which was constant in each of the lines. As shown previously (Figure 4) β APP mRNA levels in ribozyme containing cell lines were reduced 67–80% relative to levels in control cells bearing the vector alone. This reduction correlated with the expression of low levels of ribozyme RNA, *ca.* 5–7 fold less than β APP mRNA in control cells. The specificity of this reciprocal relationship is seen in the fact that steady-state G3PDH levels in each of these cell lines were not significantly different.

β APP has been hypothesized to be involved in cell growth (see Introduction). If this is true, elimination or reduction of β APP may result in more slowly growing cells. To examine this question, the growth rate of our COS-7 ribozyme-containing cell lines were measured under serum-induced conditions and compared to cells bearing the vector alone. As shown in Figure 7, neither ribozyme construct had a significant effect upon the rate of growth of these cells.

DISCUSSION

Catalytic RNAs have been found in a large number of diverse organisms. Examples have been found in plants, animals, eubacteria, viruses and fungi (40–44). At present, there are seven types of catalytic RNA motifs known; they include the self-splicing group I introns, the self-splicing group II introns, the RNase P pre-tRNA processing enzyme, the self-cleaving hammerhead structure, the self-cleaving hairpin structure, the self-cleaving hepatitis δ motif and the *Neurospora* VS RNA self-cleaving motif. The hammerhead and hairpin ribozymes are the smallest known self-cleaving agents, requiring 19 and 50 bases respectively for efficient cleavage in *trans* (45,46). Because they are significantly smaller and better characterized, we have focused our efforts on developing *trans*-acting hammerhead and hairpin ribozymes as site-specific *in vivo* β APP mRNA degradation reagents.

The results presented in this paper demonstrate that β APP steady-state levels can be reduced *in vivo* by *trans*-acting ribozymes targeted to β APP mRNA. β APP levels were shown to correlate with the presence of ribozyme RNA in cells and could

be modulated by glucocorticoids. Reduction of β APP in these cells was also specific as judged from the unchanged levels of α -actin mRNA and G3PDH mRNA in quantitative slot blot experiments and by unchanged SDS-PAGE protein patterns of Coomassie stained one dimensional gels of total cell homogenates from various cell lines (unpublished observations). These results confirm the predictions of *in vitro* specificity studies (27,28).

Under *in vitro* conditions synthetic ribozymes can be made to cleave any appropriate mRNA target. By modulating the reaction temperature, ribozyme/substrate ratio, divalent cation type and concentration and the size of the target mRNA, ribozyme cleavage can be optimized. *In vivo*, however, ribozyme-mediated degradation of a particular mRNA depends upon ribozyme expression levels, ribozyme activity, ribozyme and target mRNA decay rates as well as mRNA higher-order interactions and mRNA interactions with other cell components. We have shown *in vitro* under single-turnover conditions (37°C, 10 mM MgCl₂, ribozyme/substrate = 15) the β APP hammerhead ribozyme cleaved small model substrates readily. In contrast, the mutated version of this ribozyme cleaved small model substrates poorly and did not cleave full-length β APP mRNA transcripts at all (27,36). Therefore, the fact that the reduction of β APP mRNA was the same for cell lines bearing the active (pMAMneo-Ribozyme) and mutant (pMAMneo-Dead) hammerhead ribozymes (Figure 3) suggests that while degradation of β APP mRNA in these cells depends on ribozyme expression it does not depend on ribozyme cleavage. This hypothesis was corroborated in the pMEP-Rz1 and pMEP-Rz2 cell line studies. Again, the similarity in β APP reduction in the hammerhead (pMEP-Rz1) and hairpin (pMEP-Rz2) cell lines was not related to the *in vitro* cleavage activities of the two ribozymes. Overexpression of hairpin ribozyme RNA cannot account for this; the steady-state levels of both hammerhead and hairpin ribozymes measured under serum-induced conditions were the same (Figure 6B). In addition, since ribozyme RNA levels were 5–7-fold lower than β APP mRNA levels, hairpin ribozyme cleavage in these cells should be negligible, barring the presence of a *trans*-acting factor to modulate hairpin activity. However, because this effect was also observed using a different expression vector and a different type of inactive ribozyme the existence of such a factor is unlikely.

In order for the *in vivo* activity of the ribozymes used in these studies to be unrelated to their *in vitro* activity, ribozyme cleavage of β APP mRNA must be slower than some other degradation step. We have shown that ribozyme cleavage *in vitro* is slowed simply by increasing the length of the β APP substrate mRNA (36). This is most likely due to the formation of higher-order RNA which masks the ribozyme binding site since full-length β APP mRNA does not compete with the short model substrate for ribozyme binding *in vitro* (unpublished observations). Therefore, higher-order RNA structure may inhibit β APP mRNA cleavage *in vivo* by interfering with ribozyme/substrate hybridization. This agrees with recent transient transfection studies using ribozymes targeted to Pit-1 mRNA (47).

All of the studies presented here used glucocorticoids to induce ribozyme mRNA expression. The Mt-IIA promoter in the pMEP-Rz constructs can also be induced by heavy metal ions such as Cd²⁺, Cu²⁺ and Zn²⁺. In general, the glucocorticoid responsive element (GRE) in the Mt-IIA promoter is less active than the metal responsive elements (MREs) (48). However, heavy-metal ions can also have other effects on cells, such as inducing a heat-shock response (unpublished observations). Such effects may

oppose or counteract the action of the ribozyme. We are currently determining whether heavy-metal ion induction is feasible in this system.

The effect of lowering β APP levels in stably transfected COS-7 cells differs from previously reported antisense (AS) studies using immortalized human fibroblasts (49). In this study transfected human A-1 fibroblasts bearing an AS-RNA expression plasmid that contained a 1.1 kb *Eco*RI fragment from the 3' end of β APP, including the β -peptide and part of the 3' untranslated region, grew poorly relative to *untransfected* parent cells. The antisense transfected cells expressed low levels of β APP mRNA and β APP and the inhibitory effects of the construct could be repressed by the addition of growth medium containing a secreted form of β APP. However, whether these effects are related to β APP expression, or due to a pleiotropic response to the antisense vector is currently unknown.

More recently, LeBlanc *et al.* have investigated the effect an AS- β APP construct had upon human neuroblastoma cells (50). In this case a 1.0 kb cDNA insert corresponding to the 3' end of β APP including part of the membrane spanning region of β APP, but not the β -peptide was cloned in front of a constitutive RSV LTR promoter in the episomal vector pREP3. Steady-state levels of β APP mRNA and β APP in AS- β APP cells were both significantly reduced as compared to either the parental, *untransfected* cell line or vector-alone controls. Furthermore AS- β APP cells, but not vector-alone cells, exhibited a 30% reduction in cell proliferation, reduced cell adhesion and a two to four-fold increase in the number of neurite bearing cells. However, although β APP mRNA was significantly reduced, AS- β APP RNA could not be detected in the antisense-transfected cells.

In our pMEP4-Rz cell lines the observed 67–80% decrease in β APP mRNA and β APP expression was not associated with slower growth rates. This raises the question, to what extent is β APP involved in cell growth regulation? Three possibilities present themselves. First, the postulated role of β APP in cell growth may be an artifact of the antisense vectors expression. Second, the dependence of β APP on cell growth may be cell-type specific. If this is true, COS-7 cells may be one of the cell types that does not require β APP for adequate growth in cell culture. Transfection of our ribozyme constructs into other cell lines will allow us to answer this question. A third possibility is that normal stimulation of cell proliferation can be achieved by low levels of β APP. Although the ribozymes described here appear to lower the level of β APP by a greater fraction than the previously reported antisense vectors, the fibroblast and neuroblastoma cell lines used in those studies may express lower absolute levels of β APP. Therefore, low residual levels of β APP in our pMEP4-Rz cell lines may be sufficient to sustain normal growth. This possibility is currently being tested by constructing COS-7 cell lines in which β APP is completely ablated and also by transfecting the pMEP4-Rz plasmids into cell lines containing low endogenous levels of β APP.

Our ultimate goal is to determine the normal cellular function of β APP in mammalian brain. The strategy chosen, eliminating message expression using targeted ribozymes, is analogous to gene inactivation or gene ablation methods. Recent gene ablation studies of APPI, a *Drosophila* protein that is distantly related to β APP (51) have shown that flies bearing APPI null mutations, *appl*⁻, exhibit subtle behavioral defects but are otherwise normal (52). This defect could be partially rescued by incorporation of a human β APP transgene. Thus, the β APP gene may also encode a luxury or redundant function in mammals

which will only become apparent in a transgenic system. The results we have obtained in cultured COS-7 cells suggest that β APP mRNA reduction by ribozymes is possible *in vivo*. This should enable us to construct a transgenic model based on these constructs in the future.

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