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Growth Factor-Mediated Stabilization of Amyloid Precursor Protein mRNA Is Mediated by a Conserved 29-Nucleotide Sequence in the 3′-Untranslated Region

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Abstract: Using a cell-free translation system, we previously demonstrated that the turnover and translation of amyloid precursor protein (APP) mRNA was regulated by a 29-nucleotide instability element, located 200 nucleotides downstream from the stop codon. Here we have examined the regulatory role of this element in primary human capillary endothelial cells under different nutritional conditions. Optimal proliferation required a growth medium (endothelial cell growth medium) supplemented with epidermal, basic fibroblast, insulin-like, and vascular endothelial growth factors. In vitro transcribed mRNAs with the 5′-untranslated region (UTR) and coding region of β-globin and the entire 3′-UTR of APP 751 were transfected into cells cultured in endothelial cell growth medium. Wild-type globin-APP mRNA containing an intact APP 3′-UTR and mutant globin-APP mRNA containing a mutated 29-nucleotide element decayed with identical half-lives ($t_{1/2}$ = 60 min). Removal of all supplemental growth factors from the culture medium significantly accelerated the decay of transfected wild-type mRNA ($t_{1/2}$ = 10 min), but caused only a moderate decrease in the half-life of transfected mutant mRNA ($t_{1/2}$ = 40 min). We therefore conclude that the 29-nucleotide 3′-UTR element is an mRNA destabilizer whose function can be inhibited by inclusion of the aforementioned mixture of growth factors in the culture medium. **Key Words:** Amyloid protein precursor—3′-Untranslated region—mRNA decay—Growth factor—Capillary endothelial cells.
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The deposition of β-amyloid peptide (βA4), around the cerebral vasculature and neurons, is a pathological hallmark of Alzheimer's disease (AD), hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D), and Down's syndrome (DS) (Selkoe, 1993; Castano et al., 1996). This 39–42 amino acid peptide is derived from the aberrant processing (Citron et al., 1992) of a larger, membrane-associated glycoprotein, the β-amyloid precursor protein (βAPP). All mutations that cause the inherited forms of AD, HCHWA-D, and DS act by a common mechanism of increasing βA4 deposition.

In the vast majority of AD cases that are late-onset and sporadic, the etiology of the disease remains poorly understood. Overexpression of different isoforms of βAPP in transfected cells or transgenic animals alters its normal processing, resulting in the deposition of amyloidogenic fragments (Fukuchi et al., 1992; Yoshikawa et al., 1992). In postmortem AD brain, increases in the proportions of different APP mRNA isoforms have been reported. Neuronal cells showed moderate, but significant, disease-associated increases in the proportion of Kunitz-type protease inhibitor (KPI)-containing APP 751 and APP 770 mRNA isoforms (Johnson et al., 1990; Johnston et al., 1996). In skin fibroblasts from DS patients, the proportions of KPI-containing isoforms were elevated significantly at a very young age (mean age, 5 years), but not so in the aged DS group (Urakami et al., 1996). Thus, in sporadic AD and in DS, the early overexpression of specific APP isoforms could initiate and/or propagate pathological cascades.

Overexpression of APP and βA4 deposition can be accelerated by growth factors and proinflammatory cytokines. The coexpression of transforming growth factor-β1 (TGF-β1) in transgenic mice expressing human βAPP accelerated the overexpression of APP and βA4 deposition in cerebral blood vessels and meninges (Wyss-Coray et al., 1997). βA4 also induced production and secretion of interferon-γ and interleukin-1β (IL-1β) in human vascular endothelial cells (Suo et al., 1998).

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Abbreviations used: βA4, β-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; bFGF, basic fibroblast growth factor; DS, Down's syndrome; EBM, endothelial cell basal medium; EGF, epidermal growth factor; EGM, endothelial cell growth medium; FBS, fetal bovine serum; GAP-43, growth-associated protein-43; Gl-APP, globin-APP; Gl-APPmut, mutated globin-APP; Gl-APPwt, wild-type globin-APP; HCHWA-D, hereditary cerebral hemorrhage with amyloidosis, Dutch type; HUVEC, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; IL-1, interleukin-1; KPI, Kunitz-type protease inhibitor; TGF-β, transforming growth factor-β; UTR, untranslated region; VEGF, vascular endothelial growth factor.

Thus, βA4 acting in concert with proinflammatory cytokines could trigger a self-propagating cycle of chronic βAPP overexpression and deposition of βA4, with neurodegenerative consequences. Factors that control both APP expression and processing are therefore of critical significance in AD pathogenesis.

Previous work from our laboratory identified a conserved 29-nucleotide sequence, ~200 nucleotides downstream from the APP stop codon that functioned as an APP mRNA-destabilizing element in a rabbit reticulocyte lysate cell-free translation system (Rajagopalan et al., 1998). We hypothesized that this instability element could similarly regulate APP mRNA decay in intact cells. Preliminary studies with the widely used rat pheochromocytoma (PC12) and human neuroblastoma (SH-SY5Y) cell lines revealed that endogenous APP mRNA was constitutively stable ($t_{1/2} > 12$ h; unpublished observations). This likely reflects dysregulated mRNA decay, as is often seen in tumor lines (Ross et al., 1991). For example, labile mRNAs coding for cytokines, growth factors, and protooncogenes that typically decayed with half-lives of 15–30 min, were six- to 10-fold more stable ($t_{1/2} > 3$ h) in transformed cell lines (Ross et al., 1991).

Human umbilical vein endothelial cells (HUVEC) have the advantage of being primary cells that transcribe KPI-containing APP 751 and APP 770 mRNA isoforms (Haass et al., 1992) that are elevated in AD and DS (Johnson et al., 1990; Johnston et al., 1996; Urakami et al., 1996). Further, the lysosomal processing of βAPP in these cells has been shown to generate potentially amyloidogenic, β-peptide-bearing fragments (Haass et al., 1992). Goldgaber et al. (1989) demonstrated that in HUVEC, growth factors could up-regulate the APP promoter and thereby increase steady-state APP mRNA levels. However, the short half-life (4 h) of the APP transcript in the absence of growth factors (Goldgaber et al., 1989) suggested that regulation may also occur posttranscriptionally at the level of APP mRNA decay. HUVEC therefore provided a good vascular endothelial cell model to examine the regulation of APP expression and its processing.

In this study, we have examined the decay of endogenous APP mRNA and the role of the 29-nucleotide instability element in regulating the decay of transfected, chimeric, globin-APP (Gl-APP) mRNAs. Chimeric mRNAs consisted of the globin 5′-untranslated region (5′-UTR) and coding regions fused to full-length APP 751 3′-UTR containing either a wild-type (Gl-APPwt) or mutated (Gl-APPmut) 29-nucleotide instability element. Our data demonstrate that in rapidly dividing cells cultured in endothelial cell growth medium (EGM; see Experimental Procedures), APP mRNAs (wild-type and mutated) were equally stable. When all supplemental growth factors were removed, but proliferation maintained, APP mRNAs containing an intact 29-nucleotide, 3′-UTR element were rapidly destabilized. These data demonstrate for the first time that APP mRNA decay is *cis*-sequence-specific and can be modulated by the extracellular environment.

EXPERIMENTAL PROCEDURES

Cell culture

HUVEC (obtained from Clonetics, San Diego, CA, U.S.A.) were cultured on BIOCOAT Collagen I-coated six- or 12-well plates (Becton–Dickinson, Franklin Lakes, NJ, U.S.A.). In accordance with the manufacturer's protocol for optimal growth, cells were cultured in a growth medium (EGM-2) containing epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and 5% fetal bovine serum (FBS). To transfer to a growth factor-lacking medium, cells were first washed two or three times in endothelial cell basal medium (EBM-2) before being cultured in EBM-2 supplemented with 1, 2.5, or 5% FBS. Where indicated, cells were trypsinized by strictly following the manufacturer's protocol. Fifth-passage HUVEC were used in all experiments.

Plasmid construction

The construction of plasmids pT7APP751wtT90 and pT7APP751mutT90 has been described previously (Rajagopalan et al., 1998). The 5′-UTR and coding region of β-globin were PCR-amplified from the plasmid pSP6β-globinT90 (Rajagopalan et al., 1998). Primers were designed to create a *Not*I restriction site at the 5′ end and a *Bgl*II site at the 3′ end. The PCR product was then digested with *Not*I and *Bgl*II and ligated into *Not*I/*Bgl*II-digested pT7APP751wtT90 to produce pT7Globin-APP-I.

Full-length APP wild-type and mutated 3′-UTRs were PCR-amplified from pT7APP751wtT90 and pT7APP751mutT90, respectively. Primers were designed to create a *Bgl*II site at the 5′ end and an *Sph*I site at the 3′ end. Each PCR product was then digested with *Bgl*II and *Sph*I and ligated into *Bgl*II/*Sph*I-digested pT7Globin-APP-I to produce pT7Globin-APP3′UTRwtT90 and pT7Globin-APP3′UTRmutT90, respectively (see Fig. 3). Linearization of the plasmids with *Hin*dIII and transcription with T7 polymerase yielded polyadenylated (A_{90}) chimeric RNAs with the 5′-UTR and coding region of β-globin, followed by the 3′-UTR of APP containing either an intact (wild-type) or mutated 29-nucleotide 3′-UTR element.

mRNA synthesis and transfection

Capped, polyadenylated mRNAs were synthesized in vitro, purified, and evaluated for their integrity as previously described (Rajagopalan et al., 1998). Particle-mediated transfer of mRNAs into adherent HUVEC was performed using the Accell gene gun (Rajagopalan and Malter, 1996).

Northern analysis

As stated previously, HUVEC were seeded at identical densities and cultured in either six- or 12-well collagen-coated plates. Each well represented either a distinct culture condition or an individual time point. In experiments examining either the steady-state levels or decay rates of endogenous APP mRNA, cells were lysed by addition of 1 ml of TRI reagent (Molecular Research Center Inc., Cincinnati, OH, U.S.A.) to each well. The entire content of each well was then transferred to a 1.5-ml Eppendorf tube and snap-frozen in an ethanol bath at −80°C. After completion of an individual experiment, total RNA was quantitatively isolated from all conditions and analyzed by northern blotting and phosphorimaging as previously described (Rajagopalan and Malter, 1994). APP-specific signals were normalized to those of actin before being graphed.

RNA dot blots

At the indicated time points, transfected cells were trypsinized and washed twice with HEPES-buffered saline solution before being lysed in 1 ml of TRI reagent. Total RNA pellets from each time point were dissolved and denatured (70°C water bath, 15 min) in 3 μl of sample buffer (30 μl of deionized formamide, 2.4 μl of 25× MOPS, 10.8 μl of 37% formaldehyde, 16.8 μl of diethyl pyrocarbonate-treated water), before being dotted onto a MAGNA nylon transfer membrane (Micron Separations Inc., Westborough, MA, U.S.A.). Samples were allowed to air-dry and then were briefly immersed in 10× saline–sodium citrate buffer and auto UV-cross-linked in a Stratalinker (Stratagene, La Jolla, CA, U.S.A.). Transfected, chimeric Gl-APP mRNAs were detected by hybridization to random-primed globin cDNA probes (Rajagopalan and Malter, 1994). Gl-APP-specific signals were then normalized to those of actin and plotted versus time.

RESULTS

Modulation of APP mRNA steady-state levels by growth factors and serum

Previous studies have shown that the steady-state level of endogenous APP mRNA could be up-regulated in HUVEC by IL-1 (α and β), phorbol 12-myristate 13-acetate, or bFGF (Goldgaber et al., 1989). However, the underlying mechanism for these effects was not determined. We therefore investigated if APP mRNA decay could be altered by culture conditions.

Fifth-passage HUVEC were cultured on collagen I-coated six-well plates in EGM-2 (see Experimental Procedures) containing 5% FBS. At 70% confluence, one set of cells (four wells) was maintained in EGM-2 plus 5% FBS (condition I, Fig. 1), whereas the other sets of cells were washed and transferred to EBM-2 containing 5% (condition II), 2.5% (condition III), or 1% (condition IV) FBS. EGM-2 and EBM-2 were identical except for the absence of supplemental growth factors in the latter. After 5 h, cells were lysed, and total RNA was isolated and northern blotted (see Experimental Procedures). APP-specific signals were normalized to those of actin and plotted for each condition (Fig. 1). To assess growth rates, separate sets of cells were trypsinized and counted at the start and at the end of the 5-h incubations (Table 1). Removal of all supplemental growth factors (condition I versus II) caused a significant decrease of ~30% in steady-state APP mRNA levels. Cells cultured in conditions I and II had identical growth rates (Table 1). Thus, changes in APP mRNA levels were not a result of alterations in the rates of cell proliferation. APP mRNA continued to decline as serum content of the medium was decreased further to 2.5 and 1% (Fig. 1, conditions III and IV). However, growth rates of cells cultured under conditions III and IV were inhibited considerably (Table 1). Cells remained differentiated and morphologically indistinguishable under all culture conditions.

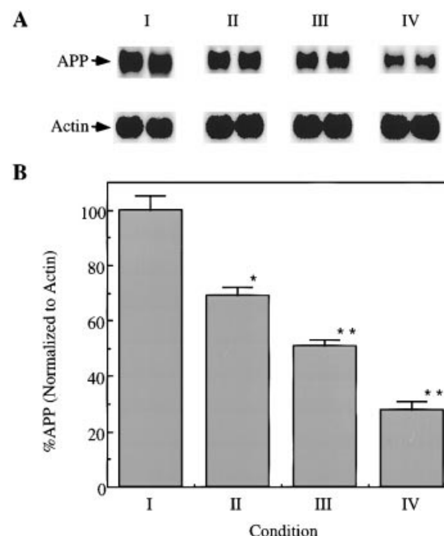

FIG. 1. Culture conditions modulate steady-state levels of endogenous APP mRNA in HUVEC. Fifth-passage HUVEC were cultured on collagen-coated plates to 70% confluence under condition I (Table 1). At this point, one set of cells was maintained in condition I. Other sets of cells were switched to either condition II, III, or IV (Table 1) and cultured for 5 h, after which cells were lysed and total RNA isolated for northern analysis. **A:** Northern blots were hybridized sequentially with cDNA probes for APP and actin. Each condition in this figure is represented by duplicate samples. **B:** Signals were quantified by phosphorimaging, and APP-specific signals were normalized to those for actin and plotted for the different culture conditions. Data points represent the means ± SD of five separate determinations (*p < 0.01; **p < 0.001).

Effects of medium growth factor and serum content on APP mRNA half-life

The medium-dependent alterations in APP mRNA steady-state levels could be the result of either changes in gene transcription (Goldgaber et al., 1989), mRNA turnover (Zaidi and Malter, 1994), or both. We therefore measured APP mRNA decay after blocking transcription with actinomycin D. The experiment described in Fig. 1 was repeated, and actinomycin D (5 μg/ml) was added following 5-h incubations in different culture conditions (I, II, III, or IV; see Table 1). Cells were lysed either immediately (0 time) or at 2, 4.5, and 7 h after actinomycin D addition. Total RNA was isolated from each sample and northern blotted (Fig. 2A). APP mRNA showed no perceptible decay ($t_{1/2}$ > 12 h) in growth factor-supplemented medium with 5% FBS (EGM-2, condition I). However, when cells were cultured for 5 h in a growth factor-lacking medium (EBM-2) supplemented with 5% FBS (condition II), a 35–50% decline in APP mRNA steady-state level (compare 0 times, condition I versus II) was accompanied by a greater than threefold decrease in APP mRNA half-life ($t_{1/2}$ = 4 h; Fig. 2B). The decline in APP mRNA steady state was therefore largely the result of accelerated decay. Varying the FBS content of EGM-2 from 2 to 10% did not alter APP mRNA steady-state level or half-life (>12 h; data not shown), thereby linking accelerated decay to the absence of supplemental growth factors. As the amount of serum in EBM-2 was lowered to 2.5 and 1%, APP mRNA steady-state levels continued to decrease (0 time, conditions III and IV, Fig. 2A). However, these de-

TABLE 1. *Culture conditions and growth of HUVEC*

	Supplemental growth factors[a]	% FBS	Cell count ($\times 10^{-4}$) (70% confluence)	
			0 h	5 h
Condition I (EGM)	Yes	5	7.12 ± 0.12	8.6 ± 0.05
Condition II (EBM)	No	5	7.06 ± 0.16	8.7 ± 0.12
Condition III (EBM)	No	2.5	6.91 ± 0.16	7.5 ± 0.21
Condition IV (EBM)	No	1	7.09 ± 0.12	7.3 ± 0.18

[a] EGF, bFGF, IGF-1, and VEGF.

creases were due mostly to transcriptional down-regulation, because APP mRNA remained very stable ($t_{1/2}$ > 12 h) under these conditions. Culture conditions can therefore induce dramatic changes in the amount of APP mRNA via both transcriptional and posttranscriptional events.

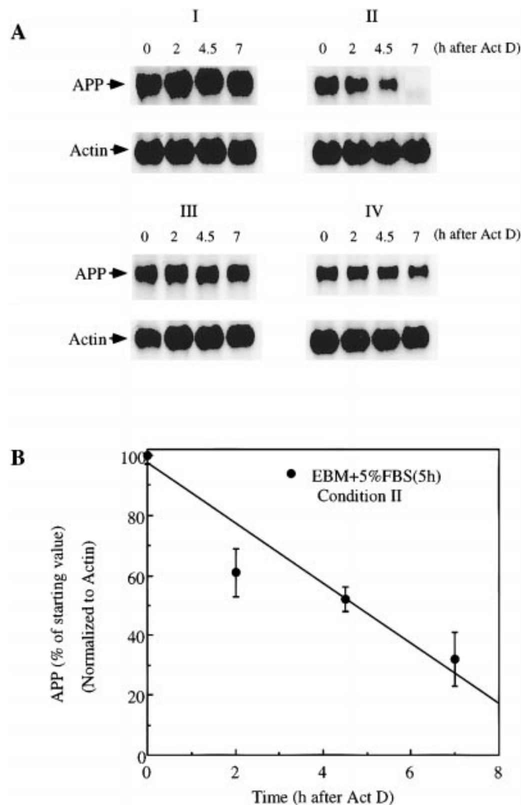

FIG. 2. Decay rates of endogenous APP mRNA in HUVEC are altered by growth factors and serum. Cells were cultured as indicated in Fig. 1. Following the 5-h incubation, transcription was blocked with actinomycin D (Act D; 5 μg/ml), and cells were lysed either immediately or 2, 4.5, or 7 h after actinomycin D addition. **A:** Total RNA was isolated and analyzed as indicated in Fig. 1. **B:** APP signals were normalized to those of actin and plotted versus time for condition II. Each time point is the mean ± SD of three determinations.

APP 3′-UTR-mediated decay of transfected mRNA: influence of growth factors

Using a rabbit reticulocyte lysate cell-free translation system, previous work from our laboratory had established a 29-nucleotide sequence in the 3′-UTR as necessary and sufficient for regulated APP mRNA decay (Rajagopalan et al., 1998). We therefore wanted to determine whether changes in APP mRNA half-life in HUVEC (Fig. 2, condition I versus II) were mediated by the same 29-nucleotide sequence. To do this, we constructed plasmids (see Experimental Procedures and Fig. 3) from which we transcribed chimeric Gl-APP mRNAs containing the entire 5′-UTR and coding region of globin fused to the 3′-UTR of APP. Gl-APPwt mRNA contained an intact 29-nucleotide element, whereas Gl-APPmut mRNA had a scrambled 29-nucleotide element. All mRNAs were capped at their 5′ ends and contained a 90-nucleotide poly(A) tail at the 3′ end. Chimeric RNAs were used to distinguish transfected mRNAs from endogenous APP mRNA and to permit identification of essential *cis*-elements that controlled decay.

In initial studies, we transfected Gl-APPwt mRNA into HUVEC at 70% confluence (see Experimental Procedures), cultured for 5 h in either EGM-2 plus 5% FBS (condition I) or EBM-2 plus 5% FBS (condition II) as described in Fig. 1. At the indicated time points after

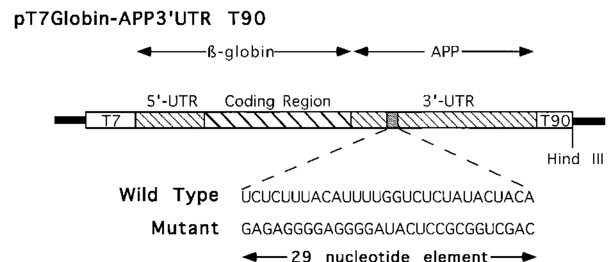

FIG. 3. T7Globin-APP3′UTR T90 in vitro transcription vectors. Chimeric cDNA was constructed containing the 5′-UTR and coding sequence of globin followed by the entire 3′-UTR of APP with either a wild-type (Gl-APPwt) or mutated (Gl-APPmut) 29-nucleotide element (see Experimental Procedures). The chimeric constructs were cloned into transcription vectors, downstream from a T7 RNA polymerase site and upstream from a 90-nucleotide oligo(dT) tract. Linearization of the transcription vectors at the unique *Hin*dIII site permitted the synthesis of mRNAs containing 90-nucleotide poly(A) tails.

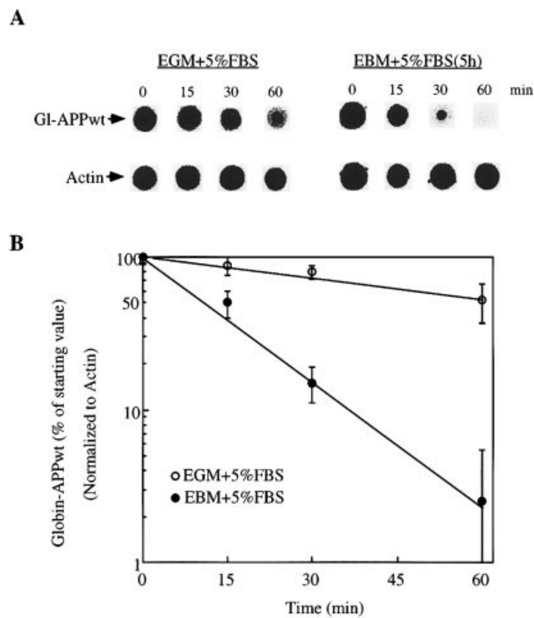

FIG. 4. Growth factors stabilize transfected Gl-APPwt mRNA. HUVEC were cultured for 5 h in either condition I or condition II, as described in Fig. 1. Capped, polyadenylated, in vitro transcribed Gl-APPwt mRNAs were delivered into cells via the particle-mediated gene transfer technology. At the indicated time points following transfection, cells were trypsinized, washed twice in HEPES buffer, and then lysed. **A:** Total RNA was isolated from each sample and dot blotted. Blots were then sequentially hybridized with cDNA probes for globin and actin. **B:** Gl-APP-specific signals were normalized to those of actin and plotted versus time. Each time point is the mean ± SD of six determinations.

transfection (Fig. 4), cells were trypsinized and washed twice in HEPES-buffered saline before lysis and isolation of total RNA. This procedure eliminated any mRNA bound to the collagen matrix or to the plasma membrane. Transfected intracellular mRNA was then detected by dot blot hybridization with radiolabeled globin cDNA probes (Fig. 4A). Nontransfected HUVEC showed no detectable endogenous globin mRNA (data not shown). Gl-APP-specific signals were normalized to those of endogenous actin to control for variations in cells harvested, and plotted versus time (Fig. 4B). Each time point is the mean ± SD of four to eight separate transfections.

In cells cultured in condition I, transfected Gl-APPwt mRNA decayed with a half-life of 60 min (Fig. 4B). With cells cultured in the absence of all supplemental growth factors (condition II), decay of transfected Gl-APPwt mRNA was accelerated sixfold ($t_{1/2}$ = 10 min; Fig. 4B). The relative change in decay of transfected Gl-APPwt mRNA upon removal of growth factors was therefore similar to that observed with endogenous APP mRNA. However, the absolute decay rate of transfected Gl-APPwt mRNAs was considerably faster than that of endogenous APP mRNA after transcription was blocked with actinomycin D (compare Figs. 2B and 4B). We have observed previously a similar phenomenon in peripheral blood mononuclear cells with transfected versus endogenous GM-CSF (granulocyte-macrophage colony-stimulating factor) mRNAs (Rajagopalan and Malter, 1996).

APP mRNA decay in HUVEC is mediated by the 29-nucleotide 3′-UTR element

To examine whether the 29-nucleotide APP 3′-UTR element was necessary for rapid APP mRNA decay in the absence of supplemental growth factors, we transfected cells with chimeric Gl-APP mRNA in which the 29-nucleotide sequence had been mutated (Gl-APPmut; Fig. 3). Experiments were performed under identical cell culture conditions as described in Fig. 4. In the presence of growth factors (condition I), the decay of Gl-APPmut mRNA was identical ($t_{1/2}$ = 60 min; Fig. 5) to that of Gl-APPwt mRNA. Whereas removal of all supplemental growth factors accelerated the decay of transfected Gl-APPwt mRNA sixfold (Fig. 4), it produced only a modest decline in the half-life of transfected Gl-APPmut mRNA ($t_{1/2}$ = 40 min; Fig. 5). Thus, modification of the 29-nucleotide element was sufficient to confer near constitutive stability to Gl-APP mRNA. The 29-nucleotide element therefore functions to destabilize APP mRNA in the absence of the complete mixture of supplemental growth factors. Conversely, the functionality of the element can be masked by the inclusion of EGF, bFGF, IGF-1, and VEGF in the culture medium. We are currently examining the individual and synergistic contributions to APP mRNA decay of these as well as other growth factors, such as tumor necrosis factor-α, IL-1 (α and β), and TGF-β, that are also elevated in AD.

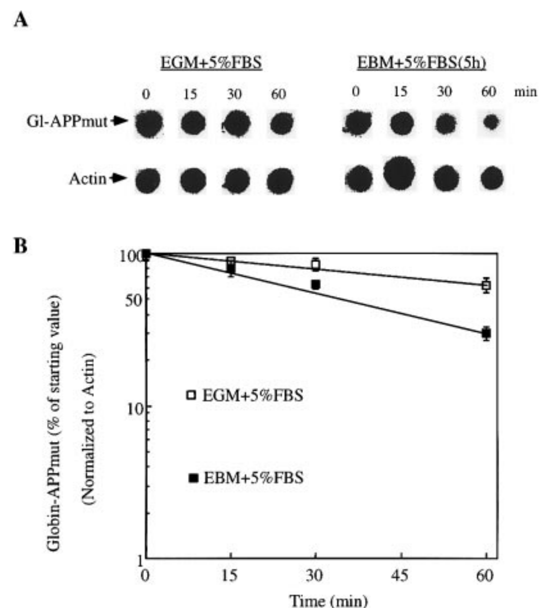

FIG. 5. Decay and growth factor-mediated stabilization of Gl-APP mRNA are sequence-specific. This experiment was performed as in Fig. 4, except that cells were transfected with Gl-APPmut mRNAs. Each time point is the mean ± SD of six determinations.

DISCUSSION

Growth factor-mediated stabilization of APP mRNA could play a crucial role in APP overexpression and accelerated βA4 deposition. This observation is particularly relevant in AD brain, where a growing list of inflammatory cytokines and growth factors, such as IL-1α (Griffin et al., 1989), bFGF (Stopa et al., 1990), TGF-β (van der Wal et al., 1993), IGF-1 (Connor et al., 1997), and VEGF (Kalaria et al., 1998) are chronically elevated. Epidemiological studies have shown that anti-inflammatory drugs can significantly delay the clinical expression of AD (McGeer et al., 1996). Further, after traumatic head injury, a strong risk factor for AD (Mayeux et al., 1993; Nicoll et al., 1995), proliferating microglia and reactive astrocytes synthesize and secrete high levels of tumor necrosis factor-α and TGF-β (Mattson et al., 1997). Thus, inflammatory mediators likely participate in the initiation and maintenance of the pathological cascade resulting in βA4 deposition.

An understanding of the cellular and molecular mechanisms connecting inflammatory cytokines to APP gene regulation, however, is limited. Studies with primary human vascular endothelial cells (HUVEC; Goldgaber et al., 1989) and neuronal cells (Ringheim et al., 1997; Yang et al., 1998) have shown that bFGF and IL-1 (α and β) increased APP mRNA and protein levels. This was partly attributable to an up-regulation of APP promoter activity. The APP gene promoter has multiple regulatory sequences that are conserved across species (Adroer et al., 1997). Of these, the AP-1 (transcription factor) consensus binding sequence was essential for growth factor-mediated up-regulation of APP production and secretion. More recently, enhanced translation was shown to be solely responsible for IL-1 (α and β)-mediated increases in astrocyte APP expression (Rogers et al., 1999). The regulatory sequence mapped to the 5′-UTR of APP mRNA and was homologous to translational control elements in the 5′-UTRs of ferritin mRNAs (Rogers et al., 1999).

The half-life of APP mRNA, however, varied greatly among the different cell-types, ranging from as little as 4.5 h in unstimulated HUVEC (Goldgaber et al., 1989) to >12 h in cell lines such as PC12 and SH-SY5Y (L. E. Rojagopalan and J. S. Malter, unpublished observations). We therefore reasoned that posttranscriptional changes in APP mRNA half-life could also contribute to expression. Here we have used a primary human vascular endothelial cell model to examine this important aspect of regulation. HUVEC were cultured to 70% confluence, in accordance with the manufacturer's protocol for optimal growth, in an EGM (see Experimental Procedures) containing EGF, bFGF, IGF-1, VEGF, and 5% FBS (condition I). Under these conditions, endogenous APP mRNA was very stable ($t_{1/2} > 12$ h). Upon shifting to a basal medium also containing 5% FBS (EBM, condition II) for 5 h, APP mRNA was rapidly destabilized ($t_{1/2} = 4.5$ h). Cell proliferation rates were indistinguishable between these two conditions, eliminating cell-cycle effects as a possible cause. Changing the serum content of the growth medium (EGM) from 2 to 10% had no effect on APP mRNA decay, thereby eliminating serum concentration as a contributing factor. These data suggest that the supplemented growth factors (EGF, bFGF, IGF-1, and VEGF) together mediate enhanced APP mRNA stability, presumably through cell-surface receptor signaling. Previously, we have shown that stabilized APP mRNAs accumulate and serve as coding templates for proportionally greater protein synthesis (Rajagopalan et al., 1998). This results from reuse of APP mRNAs by the protein synthetic machinery.

In this study, steady-state APP mRNA levels were also altered by cell (HUVEC) proliferation rates. In cells arrested by low FBS and EBM-2 media, we observed a significant stabilization of APP mRNA ($t_{1/2} > 12$ h). mRNA stabilization due to serum deprivation and cell-growth arrest has been reported for a number of other eukaryotic mRNAs, such as those encoding lysyl oxidase (Gacheru et al., 1997), insulin-like growth factor II (Scheper et al., 1996), and collagen (Kindy et al., 1988). However, in our studies, APP mRNA steady-state levels declined substantially despite stabilization, probably due to simultaneous down-regulation of the APP promoter (Goldgaber et al., 1989). Thus, growth factors and rates of cell proliferation can influence APP expression through transcriptional, as well as posttranscriptional, events.

Our earlier observations in a cell-free system identified a conserved 29-nucleotide destabilizing element ~200 nucleotides downstream from the APP mRNA stop codon (Zaidi and Malter, 1994; Rajagopalan et al., 1998). Mutation of this element resulted in substantial stabilization of APP mRNA and a two- to fourfold increase in APP synthesis (Rajagopalan et al., 1998). We were therefore interested in determining (a) whether this sequence functioned as a destabilizing element in intact, proliferating cells, and (b) whether it mediated APP mRNA decay in response to extracellular stimuli, such as growth factors. To accomplish this, we transfected cells with in vitro synthesized chimeric Gl-APP mRNAs containing either an intact (Gl-APPwt) or mutated (Gl-APPmut) 29-nucleotide element (Fig. 3). This enabled us to distinguish transfected mRNAs from full-length, endogenous APP mRNA and to examine mRNA decay rates in the absence of global transcription blockade. Although the use of transcriptional poisons, such as actinomycin D, has permitted measurements of relative mRNA decay rates, there is growing evidence that such drugs have profound effects on mRNA turnover. Sequences that normally destabilize c-*fos* (Shyu et al., 1989), c-*myc* (Wisdom and Lee, 1991), or erythropoietin (Goldberg et al., 1991) mRNAs are nonfunctional in the presence of actinomycin D. In addition, actinomycin D specifically and rapidly stabilized transfected GM-CSF mRNAs, suggesting direct inhibition of mRNA decay pathways (Rajagopalan and Malter, 1996).

mRNAs were delivered into HUVEC via particle-mediated gene transfer (Rajagopalan and Malter, 1996).

By this method, cells can be transfected rapidly with little damage (10–15% cell death) and transfected mRNAs are quickly mobilized onto polysomes for translation (Rajagopalan and Malter, 1996). When cells were cultured briefly in basal medium (EBM-2), transfected Gl-APPwt mRNA decayed very rapidly with a half-life of 10 min. Mutation of the 29-nucleotide sequence (Gl-APPmut) stabilized the mRNA ($t_{1/2}$ = 40 min), thereby establishing this sequence as a functional destabilizing element in primary human cells. Inclusion of EGF, bFGF, IGF-1, and VEGF in the culture medium stabilized Gl-APP mRNA in a sequence-specific manner. The half-life of Gl-APPwt mRNA increased sixfold from 10 to 60 min, whereas that of Gl-APPmut mRNA showed only a modest increase from 40 to 60 min. Decay rates of transfected mRNAs are generally very rapid and are likely a truer representation of actual decay rates (Rajagopalan and Malter, 1996). Decay measurements made on endogenous mRNAs after transcription is blocked with actinomycin D probably overestimate true half-lives. The turnover of labile cellular components, essential for rapid decay (Brewer and Ross, 1989; Altus and Nagamine, 1991), as well as a direct inhibitory effect on turnover, by actinomycin D (Rajagopalan and Malter, 1996), are probable reasons. However, the relative rates of decay are preserved under both situations. We therefore have shown for the first time that a *cis*-element can specifically target APP mRNA for rapid decay in cells. When present together in the culture medium, EGF, bFGF, IGF-1, and VEGF, growth factors that are elevated in AD brain, mask this instability element, thereby stabilizing APP mRNA.

Signal transduction pathways that link cell-surface events to mRNA decay are poorly understood. Recently, the c-*jun* amino-terminal kinase pathway was implicated in the regulation of IL-2 (Chen et al., 1998) and IL-3 mRNA (Ming et al., 1998) turnover, mediated by specific *cis* sequences in the 5′-UTR, coding region, and 3′-UTR of these mRNAs. Thus, defined *cis*-acting sequences can mediate mRNA turnover through distinct signaling pathways. We are currently examining the role of growth factor-mediated signaling in modulating APP mRNA decay. Further, as both the soluble form of βAPP and the insoluble βA4 can independently activate mitogen-activated protein kinase pathways (Greenberg et al., 1994; McDonald et al., 1998), it is interesting to postulate that synergy may exist with growth factors in regulating APP mRNA turnover.

The APP destabilizing sequence described here is rich in uridine and cytidine residues. A very similar 26-nucleotide mRNA-destabilizing sequence has been identified in the 3′-UTR of the growth-associated protein-43 (GAP-43) mRNA (Chung et al., 1997). GAP-43 is a phosphoprotein expressed at high concentrations in neuronal growth cones during development and axonal regeneration. In AD, as well as in Parkinson's disease and DS, the neuronal expression of GAP-43 mRNA and protein are considerably reduced, whereas astrocyte expression is up-regulated (de la Monte et al., 1995). Thus, posttranscriptional events regulating the expression of key neuronal, glial, and vascular endothelial proteins could have considerable significance on the onset of neurodegenerative cascades.

The posttranscriptional regulation of APP gene expression is therefore complex and likely has significant implications regarding the production of APP and βA4 and the development of AD. We have a valid and versatile system with which to examine the individual and synergistic contributions to APP gene regulation of key cytokines and growth factors that are elevated in AD brain. Our data suggest that dysregulation of APP mRNA decay may play a role in APP overproduction.

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