Amino Acid Sequence RERMS Represents the Active Domain of Amyloid β/A4 Protein Precursor that Promotes Fibroblast Growth

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Abstract. The growth of A-1 fibroblasts depends on exogenous amyloid β/A4 protein precursor (APP), providing a simple bioassay to study the function of APP. Our preliminary study, testing the activity of a series of fragments derived from the secreted form of APP-695 (sAPP-695) on this bioassay, has shown that at least one of the active sites of sAPP-695 was localized within a 40-mer sequence (APP296-335, Kang sequence; Roch, J.-M., I. P. Shapiro, M. P. Sundsmo, D. A. C. Otero, L. M. Refolo, N. K. Robakis, and T. Saitoh. 1992. J. Biol. Chem. 267:2214-2221). In the present study, to further characterize the growth-promoting activity of sAPP-695 on fibroblasts, we applied a battery of synthetic peptides on this bioassay and found that: (a) the sequence of five amino acids, RERMS (APP328-332), was uniquely required for the growth-promoting activity of sAPP-695; (b) the activity was sequence-specific because the reverse-sequence peptide of the active domain had no activity; and (c) the four-amino-acid peptide RMSQ (APP330-333), which partially overlaps the COOH-terminal side of the active sequence RERMS, could antagonize the activity of sAPP-695. Furthermore, a recombinant protein which lacks this active domain (APP20-591 without 306-335) did not promote fibroblast cell growth, suggesting that this domain is the only site of sAPP-695 involved in the growth stimulation. The availability of these biologically active, short peptides and their antagonists should prove to be an essential step for the elucidation of APP involvement in regulation of cellular homeostasis.

The amyloid β/A4 protein is the major component of cerebrovascular amyloid deposits (Glennar and Wong, 1984) and the core of neuritic plaques (Masters et al., 1985; Selkoe et al., 1986), the latter of which is believed to be the hallmark of the pathology found in brain tissue of patients afflicted with Alzheimer's disease (AD). The protein is derived from a membrane-spanning protein, amyloid β/A4 protein precursor (APP) (Robakis et al., 1987; Kang et al., 1987; Goldgaber et al., 1987; Tanzi et al., 1987), of which at least five different forms of primary translation products are now known. Three forms (APP-563, -751, and -770) contain a domain showing a strong homology with protease inhibitors of the Kunitz type (KPI) whereas the other two forms (APP-695 and -714) do not (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; De Sauvage and Oltersdorf, 1989; Golde et al., 1990). Subsequent studies have shown the existence of secreted forms of APP (sAPP), either in the medium of cultured cells, such as PC12 and fibroblasts (Schubert et al., 1989a; Uéda et al., 1989; Weidemann et al., 1989), or in the cerebrospinal fluid (Palmer et al., 1989; Weidemann et al., 1989; Kitaguchi et al., 1990). Since the identification of KPI-containing forms of sAPP as protease nexin II (Oltersdorf et al., 1989; Van Nostrand et al., 1989), many reports have appeared describing biological functions for these forms of sAPP. These include roles in the regulation of neurite extension (Oltersdorf et al., 1989; Van Nostrand et al., 1989), the blood coagulation process (Cole et al., 1990; Smith et al., 1990; Van Nostrand et al., 1990), and the wound-healing process (Cunningham and Van Nostrand, 1991). Little is known, however, about the physiological function of sAPP-695 which lacks the KPI domain, in spite of the evidence indicating that APP-695 is the major form of APP mRNA in the brain (Neve et al., 1988; Ponte et al., 1988; Tanaka et al., 1988; König et al., 1991). Curiously, however, at the protein level, it has been reported that APP-695 represents only a minor fraction of total brain APP (Van Nostrand et al., 1991). Nevertheless, the fact remains that APP-695 is found almost exclusively in brain tissue and its activity could underlie a brain-specific mechanism.

As an initial attempt to study physiological functions of APP, we established a fibroblast cell line, A-1, transfected...
with a plasmid expressing an antisense APP RNA (Saitoh et al., 1989). This cell line produces very low levels of APP mRNA and its translation products, resulting in a slow growth rate. The growth of A-1 cells can be restored to the level of normal (parent) fibroblasts (AG2804) by the addition of exogenous APP, either APP-695 or APP-751/770, into the culture medium (Saitoh et al., 1989; Bhasin et al., 1991). Although the biochemical basis for the retarded growth rate of A-1 cells is still unclear, the dependence of the growth of cells on exogenous APP provided us with a rather simple bioassay for the functional mapping of APP (Roch et al., 1992). Testing the activity of a series of sAPP-695 fragments obtained from our bacterial expression system on this bioassay, we have already shown that at least one of the sites of sAPP-695 responsible for the growth stimulation is located within a 40-mer sequence (APP296-335, Kang sequence) (Roch et al., 1992).

In the present study, to further characterize the growth-promoting activity of sAPP-695 on fibroblasts, we addressed the following questions: (a) Which amino acid sequence within the 40-mer domain is essential for the activity? (b) Is the activity sequence-specific? (c) Does the 40-mer domain contain the only site of sAPP-695 capable of growth stimulation?

We found that the sequence of five amino acids, RERMS (APP328-332), was uniquely required for the growth-promoting activity of sAPP-695 on fibroblasts. We also found that the four-amino-acid peptide RMSQ (APP330-333), which partially overlaps the COOH-terminal side of the active sequence RERMS, could antagonize the activity of sAPP-695. These findings firmly establish the growth-stimulating activity of sAPP-695 which lacks the KPI domain. The availability of these agonists and antagonists should be useful in further delineating the biological activity of sAPP-695 in physiological and pathological conditions.

Materials and Methods

Cell Culture

A-1 and AG2804 fibroblasts were maintained as described previously (Saitoh et al., 1989). In brief, AG2804 cells were cultured in DME containing 10% FCS in 75-cm² flasks, at 37°C in 8% CO₂, with one passage per week. A-1 cells were maintained in the same way with the exception that the growth of A-1 cells is still unclear, the dependence of the growth...
Results

In the previous report (Roch et al., 1992), we showed that both KB75 (APP20-591) and a 40-mer peptide (APP296-335) were active on the A-1 cell growth assay. To find out the amino acid sequence within the 40-mer peptide which is essential for its activity, we synthesized a battery of peptides and tested their activity on the A-1 cell growth assay. The amino acid sequences of the synthetic peptides used are given in Table I. First, three peptides which covered the NH₂-terminal (14-mer), mid- (13-mer), and COOH-terminal (17-mer) portions of the 40-mer were tested. Only the 17-mer was active (Fig. 1 A). Second, two peptides which covered the NH₂-terminal (8-mer) and COOH-terminal (11-mer) portions of the 17-mer were tested. Only the 11-mer was active (Fig. 1 B). Third, six peptides which covered the NH₂-terminal (N4 and N5), mid- (M5 and M6), and COOH-terminal (C6 and C7) portions of the 11-mer were tested. The M5 and M6 peptides were active, although the concentrations required for significant effects were 10-fold higher than those of other active peptides described above (Fig. 1 C). Neither N4/N5 nor C6/C7 were active. The negative results for C7 (ERMSQVM) and C6 (RMSQVM) suggested that the NH₂-terminal R and E residues of M5 (RERMS) were indispensable for the activity. To see whether the COOH-terminal S and M residues are dispensable or not, we tested M4 (RERM) and M3 (RER) and found no activity (Fig. 1 D), suggesting that both of the COOH-terminal residues were required for the activity. Thus, we have narrowed down the site responsible for the growth stimulation of fibroblasts to the five-amino-acid sequence RERMS (APP328-332, Kang sequence). To test the sequence specificity of the activity, we tested the reverse-sequence 17-mer and found no activity (Fig. 1 B). As an additional parameter of cell growth, incorporation of [³H]-thymidine into the cells was determined. Three days exposure of cells to either KB75 or 17-mer (both at 10 pM) caused a significant increase in the incorporation (Fig. 2) reflecting an increase in the cell number per well, whereas neither 14-mer nor 13-mer caused higher levels of [³H]-thymidine incorporation at the same concentrations, consistent with the results of cell numbers.

One possible mechanism for the action of these APP peptides is the interaction of APP with specific cell-surface molecule(s). The sequence specificity of the activity, as described above, seemed to support this idea. If this is the case, it is expected that the inactive peptides which partially overlap the active sequence RERMS might have an antagonistic action against the active peptides. According to this idea, we tested the activity of either 17-mer or KB75 (at 10 pM) in the presence of excess concentration (10 nM) of inactive peptides which partially overlaps RERMS, and we found that both C7 and C6, but not N4, antagonized the activity of the peptides (Fig. 3). This antagonistic action was not an artifact caused by DMSO because C4 (RMSQ), a water-soluble derivative of C6, had a similar antagonistic action (Fig. 3). C4 by itself had no growth-stimulating activity (data not shown).

The fact that the same peptide (C4) could antagonize the activities of either 17-mer or KB75 to a similar extent was indirect evidence that this domain (17-mer) represented the only active site of KB75. To see more directly whether this domain is the only active site of sAPP-695 or whether there is an additional active site, we engineered a construct which encodes APP20-591 without 306-335 (pKB75Δ) (Fig. 4 A and B). Recombinant protein KB75Δ was obtained through bacterial expression and protein purification procedures, which were exactly the same as employed for the preparation of KB75 (Roch et al., 1992). Immunoblotting with anti-GID antibody detected the expression of KB75Δ in the partially purified bacterial lysate, with a predicted difference in the molecular weight from that of KB75 (Fig. 4 C). The peptide was further purified, through heparin-agarose column and

### Table 1. Amino Acid Sequences of the Peptides Used in the Present Study

| Peptide           | Sequence                                      |
|-------------------|------------------------------------------------|
| KB75              | APP20-591, Kang sequence                      |
| KB75Δ             | APP20-591 without 306-335                    |
| 40-mer            | TPDADVKEYLETPSDENEHAFQKAKERLEAKHRERSQVM (APP296-335) |
| 14-mer            | TPDADVKEYLETPGD                               |
| 13-mer            | GDENEHAFQKAK                                  |
| 17-mer            | AGERLEAKHRERSQVM                              |
| Reverse sequence 17-mer | MVGSRHERKAEKALREKA                        |
| 8-mer             | AKHRERSQVM                                   |
| 11-mer            | AKHRE                                        |
| N5                | HRERS                                        |
| M5                | RERMS                                        |
| M4                | RER                                          |
| M3                | RMSQ                                         |
| C7                | RMSQ                                         |
| C6                | RMSQ                                         |
| C4                | RMSQ                                         |

* KB75 and KB75Δ were expressed in our bacterial expression system. All the other peptides were chemically synthesized. See Materials and Methods for details.
Figure 1. Growth-promoting activity of APP peptides on A-1 cells. The growth assay on A-1 cells was performed as described under Materials and Methods, using increasing concentrations of APP peptides. The amino acid sequence of each peptide is given in Table I. In each panel, the lower dotted line and the shaded area represent the mean ± SEM of the percentage increase of A-1 cell numbers after 3 d culture in the regular media (DME/10% FCS) (basal increase). As shown, the basal increase in the number of A-1 cells was virtually zero. The upper dotted line and the shaded area represent the mean ± SEM of the percentage increase of AG2804 cell numbers after 3 d culture in the regular media, which was determined in parallel in each experiment. Each point represents the mean ± SEM of at least four determinations, each done in triplicate. * p < 0.05, significantly different from the basal increase (differences were assessed by ANOVA followed by the Dunnett's test).

Figure 2. Effects of APP peptides on [3H]thymidine incorporation into A-1 cells. After 3 d incubation in the presence or absence (control) of the indicated peptide, [3H]thymidine incorporation into the cell layer was determined as described under Materials and Methods. The concentration of each peptide was 10 pM. Each bar represents the mean ± SEM of three determinations, each done in triplicate. * p < 0.05, significantly different from the control value (differences were assessed by ANOVA followed by the Dunnett's test).

Figure 3. Excess C6, C7, or C4, but not N4, antagonized the activity of 17-mer or KB75. The growth assay on A-1 cells was performed as described under Materials and Methods. The active peptide included was 17-mer (A) or KB75 (B), both at 10 pM, in the absence (white bars) or presence (gray bars) of the second peptide as indicated. The concentrations of N4, C7, C6, and C4 were 10 nM. The amino acid sequence of each peptide is given in Table I. Each bar represents the percentage increase of the number of cells compared to the number of seeded cells (mean ± SEM of three determinations, each done in triplicate. * p < 0.05, significantly different from the values in the absence of the second peptide (differences were assessed by ANOVA followed by the Dunnett's test).
Figure 4. Deletion of the active domain of sAPP-695. (A) The structure of pKB75 encoding APP20-591 was described in the previous report (Roch et al., 1992). Using the APP-695 cDNA as a template, we carried out PCR to produce a DNA fragment coding for the APP-695 region extending from Arg-336 to Ile-591 (end of KB75). The PCR primers were designed so that this fragment would be flanked by two Xhol restriction sites, thus allowing it to be easily ligated into the unique Xhol site of pKB75. This ligation resulted in a DNA fragment encoding a new version of KB75 where the domain extending from Thr-306 to Met-335 is deleted, therefore resulting in a fusion of Glu-305 to Arg-336. To prevent translation from proceeding beyond the BglII site, which would result in the production of an aberrant peptide, a STOP codon was included in the 3' primer of the PCR. (B) This scheme shows the regions of APP-695 encoded by the plasmids pKB75 and pKB75&. None of them contains the signal sequence, or the O/A form, or the transmembrane or cytoplasmic domains. The KPI domain is shown only to indicate its insertion point in APP-751, but it is not present either in KB75 or KB75&. (C) Identification of APP-related proteins in bacteria. SNI fractions (25 μg of total protein in each lane) from bacteria carrying either pKB75 or pKB75& were prepared as described (Roch et al., 1992), separated by SDS PAGE, and analyzed by immunoblotting with anti-GID antibody, diluted 1:2,000 (a rabbit polyclonal antibody against the peptide 175-186 of APP; see Saitoh et al., 1989). As expected, the form of APP encoded by pKB75& was slightly smaller than that encoded by pKB75.

Figure 5. KB75& lacked growth-promoting activity on A-1 cells. The growth assay on A-1 cells was performed using increasing concentrations of KB75 or KB75&. See legend to Fig. 1 for details. Each point represents the mean ± SEM of at least four determinations, each done in triplicate. *, p < 0.05, significantly different from the basal increase (differences were assessed by ANOVA followed by the Dunnett's test).
In another set of experiments, the synthetic peptide corresponding to the deleted sequence in KB75~ (40-mer, with the profile of NaCl concentration. The elution profiles of KB75 and KB75~ were different. Consistent with our previous finding (Roch et al., 1992), KW/5 was eluted at about 0.8 M NaCl. In contrast, KB756 was eluted at 0.45 M, showing thus a reduced affinity for heparin. In another set of experiments, the synthetic peptide corresponding to the deleted sequence in KB756 (40-mer, open circles; 17-mer, closed circles), each 10 µg, was loaded to the same column and eluted in the same way. In this case, absorbance at 215 nm was monitored. As shown, both of the peptides were recovered in the flow-through fractions (PBS; 0.15 M NaCl), indicating the poor affinity of these peptides to heparin.

hemin-binding affinity. To see which was the case, we applied the synthetic peptides corresponding to the deleted sequence (i.e., 40-mer and 17-mer) to the same heparin affinity column but found no heparin-binding capacity for the peptides (Fig. 6). Thus, although the deleted sequence in KB756 might contribute to the heparin-binding capacity of sAPP-695 in an unknown way, the biological activity of the active domain included in the deleted sequence apparently is not due to heparin binding.

Discussion

We have mapped the growth-promoting activity of sAPP-695 to a domain of five amino acids RERMS (APP328-332) (Fig. 1). The concentration of peptide needed for a significant effect, however, was ~10-fold higher for RERMS than for either the 17-mer or 17-mer, suggesting that the amino acid sequence in the vicinity was necessary for the full activity. The reverse-sequence 17-mer had no activity (Fig. 1 C), further indicating the sequence specificity of the activity. We also found that the four-amino-acid peptide RMSQ (APP330-333), which partially overlaps the COOH-terminal side of the active sequence RERMS could antagonize the activity of either the 17-mer or KB75 (Fig. 3). The lack of activity of KB756 in the A-1 cell bioassay can be explained if the deleted sequence in KB756 (APP306-335) contains the only site capable of growth stimulation, or if a conformational modification results in a loss of activity. A change in conformation may be induced by the deletion of APP306-335 in KB756 because we observed a reduced affinity for heparin of KB756 as compared to KB75, in spite of the absence of heparin-binding activity in the 40-mer and 17-mer peptides. However, the activity of KB75 could be totally antagonized by the tetramer RMSQ, indicating that the RERMS domain is uniquely responsible for the growth promotion, and that no other site in KB75 could stimulate the growth of fibroblasts.

The RERMS motif and its NH2-terminal side amino acid sequence have some interesting characteristics. First, the sequence RERMS is unique to APP; we found no match in the peptide sequences so far registered in GenBank. Second, it is within one of the evolutionarily highly conserved regions of APP (Rosen et al., 1989). Exactly the same sequence as 40-mer is found in mammalian APPs (rat, mouse, and monkey). In the Drosophila "APP-like" molecule, there is a sequence REKVT (aa 423–427; Rosen et al., 1989); the polarity of the side chains of the corresponding residues is very similar to RERMS (K and R have basic, V and M have non-polar, and T and S have uncharged polar side chains, respectively). The uniqueness and the high evolutionary conservation both suggest the fundamental role of the sequence in the physiological function(s) of sAPP.

We could thus identify a unique region of APP which was active on our A-1 cell bioassay. The biochemical mechanism of the activity, however, is still unknown. In previous reports (Saitoh et al., 1989; Roch et al., 1992), we proposed two possible mechanisms. The first is the possible effect of APP on A-1 cell adhesion. Many reports have suggested the role of APP in cell adhesion (Shubert et al., 1989a; Breen et al., 1991; Chen and Yankner, 1991). APP binds to heparan-sulfate proteoglycan (Narindrasorasak et al., 1991), which is supposed to be involved in cell-extracellular matrix interaction. Our preliminary experiments indicated a significant reduction in the adhesiveness of A1 cells compared to that of the parent cells, AQ2804 (data not shown). Because we have not been able, so far, to increase the adhesiveness of A-1 cells using soluble or immobilized KB75, more work is needed for a better understanding of the involvement of APP in cell adhesion. It is possible that co- and post-translational processing, such as glycosylation, or transmembrane config-

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**Figure 6.** Elution profiles of KB75, KB75 and synthetic peptides from heparin-agarose column. SN111 fractions from bacteria carrying either pKB75 or pKB75 were prepared as described (Roch et al., 1992). 30 mg of total protein of each fraction were then mixed and loaded onto a heparin-agarose column, in PBS at 4°C (total amount of protein loaded, 60 mg). The flow-rate was 40 ml/h. Elution from the column was monitored by absorbance at 280 nm (not shown). After washing with PBS, a linear gradient of NaCl (0.15–1.0 M) was run through the column. Fractions of 1 ml were collected. Aliquots of 25 µl of each fraction were then analyzed by SDS PAGE and immunoblotting with the anti-GID antibody. Only the fractions containing APP-related proteins are shown on this figure and aligned with the profile of NaCl concentration. The elution profiles of KB75 and KB75~ were different. Consistent with our previous finding (Roch et al., 1992), KB75 was eluted at about 0.8 M NaCl. In contrast, KB756 was eluted at 0.45 M, showing thus a reduced affinity for heparin. In another set of experiments, the synthetic peptide corresponding to the deleted sequence in KB756 (40-mer, open circles; 17-mer, closed circles), each 10 µg, was loaded to the same column and eluted in the same way. In this case, absorbance at 215 nm was monitored. As shown, both of the peptides were recovered in the flow-through fractions (PBS; 0.15 M NaCl), indicating the poor affinity of these peptides to heparin.
uration, in contrast to secreted form, are required for the cell adhesive property of APP. The second possible mechanism we proposed is the effect(s) of sAPP on the intracellular signal transduction machineries through an interaction with cell-surface receptor molecule(s). The sequence specificity of the activity and the antagonistic actions of partially overlapping, inactive peptides are compatible with this notion, although direct evidence for the presence of a cell-surface recognition molecule for sAPP is still lacking.

APP is one of the heparin-binding proteins (Schubert et al., 1989b), the reason why we used heparin-agarose column to purify it from the bacterial lysate (Roch et al., 1992). In the course of purification of KB75 and KB756 expressed in bacteria, we noticed that the binding affinity of KB75 to heparin-agarose column was lower than that of KB75 (Fig. 6). The lack of the heparin-binding capacity of biologically active, synthetic peptides corresponding to the deleted sequence in KB75 (Fig. 6), however, excluded the active involvement of the binding to heparin or heparinlike molecules, which are presumably present in the assay media or on the cell surface, in the growth-promoting activity. Further evidence for the absence of contribution of heparin-binding capacity to the activity is that the inclusion of either heparin or heparan sulfate in the assay media (both at 100 μg/ml) failed to inhibit the effect of either 17-mer or KB75 (data not shown). A potential heparin-binding site on sAPP-695 predicted from the consensus sequence (Cardin and Weintraub, 1989) is not on the active domain but on APP98-104 (Narindrasorasak et al., 1991). Thus, although the sequence APP306-335 may contribute to the heparin-binding capacity of sAPP-695 in its native form of the molecule, the growth-promoting activity of the domain in itself had little to do with the heparin-binding capacity.

In summary, using our bioassay on A-1 cells, we showed that the site of sAPP-695 responsible for the growth stimulation is represented by the five-aminoo-acid sequence RERSM (APP328–332), and that this site is unique in the APP molecule. The biochemical basis for the activity is still unknown. Recent studies in our laboratory have revealed that the biological activity of this domain of sAPP is not confined to this particular cell line; the addition of synthetic peptides corresponding to this domain to the culture media caused significant morphological and biochemical changes in either neuroblastoma cells from rat brain or primary cortical neurons from newborn rat (manuscript in preparation). Thus, sAPP seems to be involved in the regulation of cellular homeostasis in various cell lines. In a previous study, we found that cultured fibroblasts from the skin of AD patients produced a smaller amount of APPs compared to fibroblasts from normal individuals (Uedaa et al., 1989). There have been several reports describing the reduction of the relative proportion of APP-695 to the KPI-containing forms of APP in postmortem brains from AD patients (Tanaka et al., 1988; Johnson et al., 1990; Neve et al., 1990). More recently, Van Nostrand et al. (1992) reported a significant reduction of sAPP in the cerebrospinal fluid of live AD patients. These findings raised a possibility that, in addition to the COOH-terminal β/44 portion of APP which is involved in amyloidogenesis, the secreted portion of APP is also involved in the pathogenesis of AD. We expect that the biologically active peptides and their antagonists described in the present study will be useful tools to study the possible involvement of the biological activity of sAPP-695 in various experimental model systems for the pathogenesis of AD.

This work was supported by grant AG 05131 from the National Institute on Aging. Dr. Ninomiya was supported by a Public Health Service Fogarty International Research Fellowship (No. 5FO5TW04602-02), and Dr. Roch was supported by a fellowship from the Swiss National Science Foundation (No. 823A-028366). Received for publication 30 September 1992 and in revised form 24 February 1993.

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