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The Amino-Terminal Region of Amyloid Precursor Protein Is Responsible for Neurite Outgrowth in Rat Neocortical Explant Culture

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We have previously shown that secreted forms of β-amyloid precursor protein (APP^{s}s) promote neurite outgrowth in embryonic rat neocortical explant culture. To determine the region of APP^s responsible for its biological activity, we produced both amino- and carboxyl-terminal regions of APP^s using a yeast expression system. The purified fragment corresponding to the amino-terminal region (NAPP) enhanced neurite outgrowth of neocortical explants, but the carboxyl-terminal region fragment did not. The neurite-promoting activity of full length APP^s and NAPP was blocked by the antibody, 22C11, specific for the amino-terminal region, and the 16-mer peptide of epitope for 22C11 also enhanced neurite outgrowth. However, the 17-mer peptide which contains RERMS sequence did not enhance the neurite outgrowth, but promoted the survival of neocortical neurons in dissociated culture. These findings suggested that the amino-terminal region is responsible for the neurite-promoting activity of APP^{s}s.

Alzheimer's disease is characterized by the deposition of amyloid, both intracellularly, in the form of neurofibrillary tangles, and extracellularly, in the form of amyloid plaques and congophilic angiopathy. The major component of extracellular amyloid is amyloid β protein (Aβ) derived from its precursor, β-amyloid precursor protein (APP) (1-3). At least eight isoforms of APP have been identified as a result of alternative splicing of exon 7, 8, and 15 (4). The major pathway of APP metabolism involves an enzymatic cleavage within the Aβ sequence (5, 6) which releases secreted forms of APP (APP^{s}s) that contain most of the extracellular domain from the cell surface, resulting in high levels in the brain and cerebrospinal fluid (7, 8), which suggests that APP^{s}s play a significant role in the physiology of the central nervous system (CNS).

In fact, several biological functions and the regions of APP^s responsible for them have been reported. The amino-terminal region has been found to activate mitogen-activated protein kinase (MAPK) in PC12 pheochromocytoma cells (9). A fragment that includes the RERMS sequence adjacent to the Kunitz-type serine protease inhibitor (KPI) domain was found to regulate fibroblast growth (10) and promote neurite extension in a neuronal cell line (11), and mediation of modulation of intracellular calcium levels by K^+ channels appears to require the carboxyl region of APP^s (12).

We recently reported that recombinant secreted forms of APP695 and APP770 (APP695^s and APP770^s) produced by either transfected COS-1 cells or methylotrophic promote neurite outgrowth in embryonic rat neocortical explants (13). The neurite-outgrowth-promoting activity of both APP^{s}s was significant, indicating that the KPI domain is not responsible for this activity. In the present studies, the active domain of APP^s responsible for promoting neurite outgrowth in neocortical explant was identified as the amino-terminal region of APP^s. We found the epitope of 22C11, monoclonal antibody against the amino-terminal region of APP^s, also enhanced neurite outgrowth.

MATERIALS AND METHODS

Antibodies and peptides. Monoclonal antibodies against APP^s, 22C11 and Alz90, were purchased from Boehringer Mannheim. The

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Abbreviations: Aβ, amyloid β protein; APP, β-amyloid precursor protein; APP^s, secreted forms of β-amyloid precursor protein; BSA, bovine serum albumin; CAPP, carboxyl-terminal region of the secreted form of β-amyloid precursor protein; CBB, Coomassie Brilliant Blue; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; KPI, Kunitz-type serine protease inhibitor; NAPP, amino-terminal region of the secreted form of β-amyloid precursor protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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16-mer epitope peptide APP_{66-81} for 22C11 (KEGILQYCQEVYPELQ) (22), the 16-mer peptide of its reverse-sequence, and the 17-mer peptide containing RERMS sequence (AKERLEAKHRERMSQVM) (20) were purchased from Wako Co. Ltd. Japan. The purity of each peptide was >98%.

Construction of the expression vectors for recombinant APP^s. *Escherichia coli* JM109 was used as a host for the construction of each plasmid. Plasmids pHIL-D2, pHIL-S1, and pPIC9 were purchased from Invitrogen. The expression vector for APP695^s was described previously (13). In brief, the DNA fragment encoding from Met^1 to Lys^{612} of human APP695 was inserted downstream of the alcohol oxidase promoter in pHIL-D2, yielding pAPE1. For expression of the amino-terminal region of APP^s (NAPP), the DNA fragment encoding from Arg^{16} to Val^{290} of human APP770 was obtained by PCR with primers 5′-CGGCTCGAGCGCTGGAGG-3′ (5′ oligonucleotide) and 5′-GTTGAATTCACACCTCTCGAACC-3′ (3′ oligonucleotide) and subcloned into pHIL-S1 as a *Xho*I-*Eco*RI fragment, yielding plasmid pAPE3, an in-frame fusion of the *PHO1* secretion signal to Arg^{16}, and an insertion of the stop codon next to Val^{290}. For expression of the carboxyl-terminal of APP^s (CAPP), the DNA fragment encoding from Glu^{380} to Thr^{663} of human APP770 was obtained by PCR with primers 5′-CAAGTCTCGAGAGACACCTGG-3′ (5′ oligonucleotide) and 5′-CAGAGAATTCCTACGTCTTGAT-3′ (3′ oligonucleotide) and subcloned into pPIC9 as a *Xho*I-*Eco*RI fragment, yielding pAPE5, an in-frame fusion of the α-factor prepro region to Glu^{380}, and an insertion of the stop codon next to Thr^{663}. *Pichia pastoris* GS115 (*his4*) was transformed with each plasmid according to a method described previously (14). Briefly, after digestion of pAPE1 with restriction enzyme *Nco*I or pAPE3 and pAPE5 with restriction enzyme *Bgl*II, the fragments obtained containing a gene cassette for expression of a whole or part of APP^s and the *his4* gene were introduced into *P. pastoris*. Colonies of His^+ Mut^- phenotype, expected to be recombined at the *AOX1* sequence on the yeast chromosome, were then selected.

Expression and purification of recombinant proteins. Expression of APP695^s, NAPP, and CAPP in the recombinant yeast was induced by the method described previously (15). APP695^s containing from Leu^{18} to Lys^{612} of APP695, NAPP containing from Arg^{16} to Val^{290} of APP770, and CAPP containing from Glu^{380} to Thr^{663} following 6 amino acids derived from the α-factor prepro region, EAEAYI, were secreted into the culture medium. APP695^s was purified according to the method described previously (13). To purify NAPP and CAPP, the yeast cells were separated by centrifugation and 200 to 400 ml of the medium was concentrated to 15 ml by pressure filtration using a YM-10 Amicon ultrafilter. The concentrated sample was then loaded onto an anion exchange column, DE52 (1 × 5 cm; Whatman) and equilibrated with 20 mM phosphate buffer (pH 7.0). After washing with 25 ml of the buffer, bound protein was eluted with 4 ml of 0.5 M NaCl in 20 mM phosphate buffer pH 7.0. The eluted protein was desalted with a HiTrap Desalting column (Pharmacia), mixed with one volume of 4.0 M $(NH_4)_2SO_4$, and then loaded onto an alkyl-Superose column (0.5 × 5 cm; Pharmacia) at a flow rate of 0.5 ml/min. After washing with 50 ml of 2.0 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4 buffer (pH7.0), the bound protein was eluted with a linear gradient of 2.0-0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min over 40 min. To obtain NAPP, fractions corresponding to the first peak eluted with 1.4 M $(NH_4)_2SO_4$ were pooled. To obtain CAPP, fractions corresponding to the second peak eluted with 0.8 M $(NH_4)_2SO_4$ were pooled. After dialysis against phosphate-buffered saline (PBS), the purified proteins were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by staining with Coomassie Brilliant Blue (CBB) or Western blotting with antibodies 22C11 (1:1000) and Alz90 (1:50). Binding of primary antibodies was detected with anti-mouse immunoglobulin, peroxidase-linked $F(ab')_2$ fragment (1:1000; Amersham), and visualized by the ECL technique (Amersham).

Neurite outgrowth assay in rat neocortical explants. The effect of purified proteins and peptides on neurite outgrowth in rat neocortical explants was examined by the method described previously (13, 16). In brief, neocortical tissues from 17-day rat embryos were cleaned of meninges and chopped into pieces of various sizes (50-200 μm in diameter). The explants were then suspended in serum-free Dulbecco's modified Eagle's medium (DMEM) and plated in a polyethylenimine-coated 24-well plate (Coaster) at 100-150 explants per well. Each protein and peptide was added to the culture medium 2 h after seeding. In some experiments, proteins were pre-incubated with an antibody in DMEM at 37°C for 30 min before being added to the culture medium. After 2 days of cultivation, the explants were fixed with 2% glutaraldehyde and stained with CBB to visualize neurite outgrowth. The extent of neurite outgrowth was determined by examination under a microscope, as described elsewhere (17).

Survival assay in neocortical neuronal culture. The primary culture of neocortical neuron was prepared from 17-day rat embryos by the method described previously with slight modification (20, 25). In brief, neocortical tissues were cleaned of meninges, minced, and treated with trypsin (0.25%) and DNaseI (0.01%). After mechanical dissociation by pipetting, cells were resuspended in Minimal Essential Medium supplemented with insulin 10 μg/ml, transferrin 5.5 μg/ml, putrescine 100 μM, sodium selenite 6.7 ng/ml and penicillin (50 units/ml)-streptomycin (100 μg/ml), and plated onto poly-L-lysine-coated 24 well plates (Coaster) at the density of 1×10^5 cells/cm^2. After 2 h of incubation, each peptide (final 1μM) was added and cells were further incubated for 4 days. Number of surviving neurons was counted under a phase-contrast microscope in three fields per well for three independent wells.

RESULTS

Two protein fragments of APP^s, containing an amino-terminal region upstream of KPI domain and a carboxyl-terminal region downstream of KPI domain were expressed in a yeast expression system using *P. pastoris* as a host (see Fig. 1A). For secretion of amino-terminal and carboxyl-terminal regions, DNA fragments encoding a *PHO1* secretion signal and α-factor prepro region under the control of *P. pastoris* alcohol oxidase gene promoter were fused in frame to PCR products encoding each region (see Materials and Methods). As expected, NAPP containing from Arg^{16} to Val^{290} and CAPP containing from Glu^{380} to Thr^{663} following 6 amino acids derived from α-factor prepro region, EAEAYI, were secreted into the culture medium (data not shown). The secreted protein fragments were purified to a single band monitored by SDS-PAGE (Fig. 1B). Final yields of NAPP and CAPP were 6.1 mg and 0.7 mg per liter culture medium, respectively. The identity of the purified fragments was confirmed by Western blotting with antibodies 22C11 for the amino-terminal region and Alz90 for the carboxyl-terminal region of APP^s. Full length APP695^s was recognized by both antibodies, but only NAPP and CAPP were detected with 22C11 and Alz90, respectively (Fig. 1C).

To investigate the biological activity of the fragments, we examined the neurite-promoting activity of each fragment in cultured neocortical explants. As reported previously (13), APP695^s displayed a neurite-promoting effect on explants at a concentration of 100

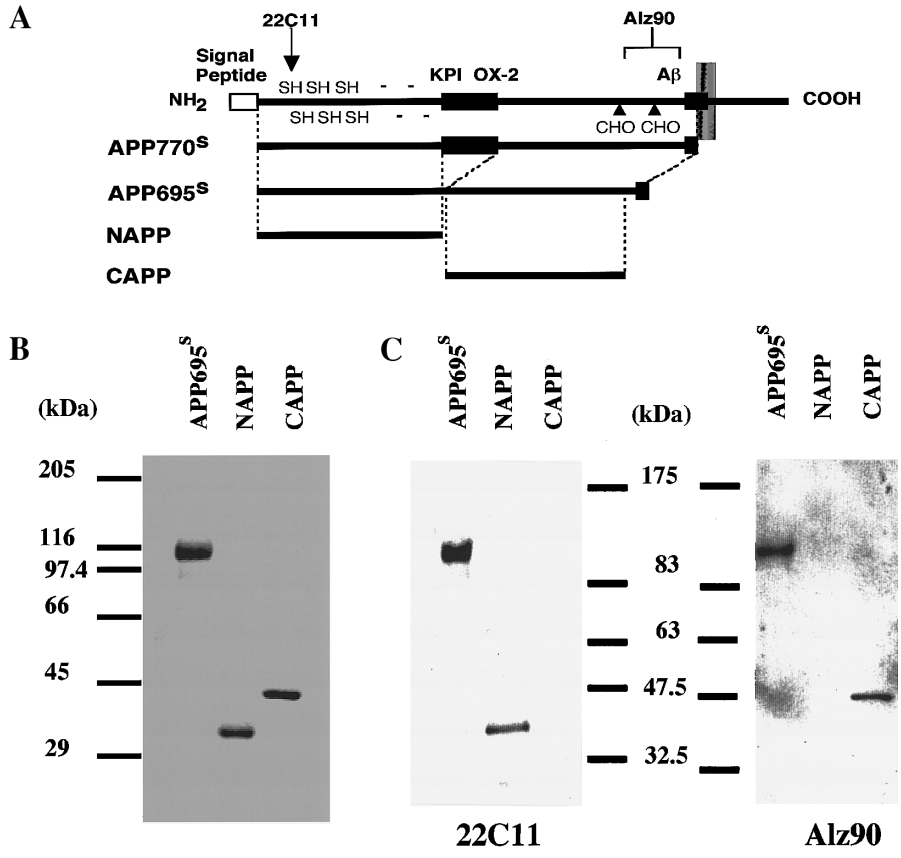

FIG. 1. Expression of fragments containing a portion of APP^s using a yeast expression system. **A:** Schematic drawing of the fragments containing a portion of APP^s (NAPP and CAPP) expressed by yeast. The fragments containing full length APP^s (APP770^s and APP695^s) are also shown. The approximate positions of the cysteine-rich region (SH), the acidic residue-rich region (−), the sites of *N*-glycosylation (CHO), and the binding sites for monoclonal antibodies 22C11 and Alz90 are indicated. Purified NAPP and CAPP (1 μg per lane for CBB stain and 200 ng per lane for Western blotting) were subjected to a 4-20% gradient gel and analyzed by staining with CBB (**B**) or transferred to nitrocellulose membrane for Western blotting with monoclonal antibody 22C11 or Alz90 (**C**).

ng/ml (1.5 nM), compared to the control (bovine serum albumin; BSA) (Fig. 2A and 2B). NAPP (100 ng/ml; 3.2 nM) also promoted neurite outgrowth, but CAPP (100 ng/ml; 3 nM) did not (Fig. 2A and 2B). The neurite-promoting activity of NAPP was dose-dependent, and the increase was significant at concentrations above 0.3 nM (Fig. 2C). The effective dose of NAPP is equivalent to that of APP695^s (0.44 nM) as reported (13). The ability of APP695^s to promote neurite outgrowth was significantly suppressed by the addition of antibody 22C11, which recognizes the amino terminal region of APP^s (Fig. 2D). When considered as a whole, these results clearly indicated that the amino-terminal region is sufficient to promote neurite outgrowth in neocortical explant culture.

Antibody 22C11 has been reported to increase G Protein coupling function of APP-transmenbrane form (23). This finding led us to speculate that the epitope of 22C11 included in NAPP might have an important role of APP^s function. Then, we examined the neurite-promoting activity of the epitope. As shown in Fig. 3A, the 16-mer peptide of the epitope of 22C11 (N16) enhanced neurite outgrowth as a dose-dependent manner and the effective dose of N16 was about 0.1 μM, but the peptide of the reverse-sequence of N16 (R16) did not. The result suggested that the cysteine-rich domain of NAPP including the epitope of 22C11 might be significant to the neurite-promoting activity of APP^s. It has also been reported that carboxyl-terminal domain containing RERMS sequence adjacent to the KPI domain stimulates neurite outgrowth of B103 neuronal cell line (11). However, the 17-mer peptide containing RERMS sequence (RERMS17) did not increase a number of explant containing neurites longer than their diameter (Fig. 3B). On the other hand, Yamamoto *et al.* has been described that the peptides containing RERMS sequence have an activity to support the neuronal survival in dissociated neocortical cultures (20).

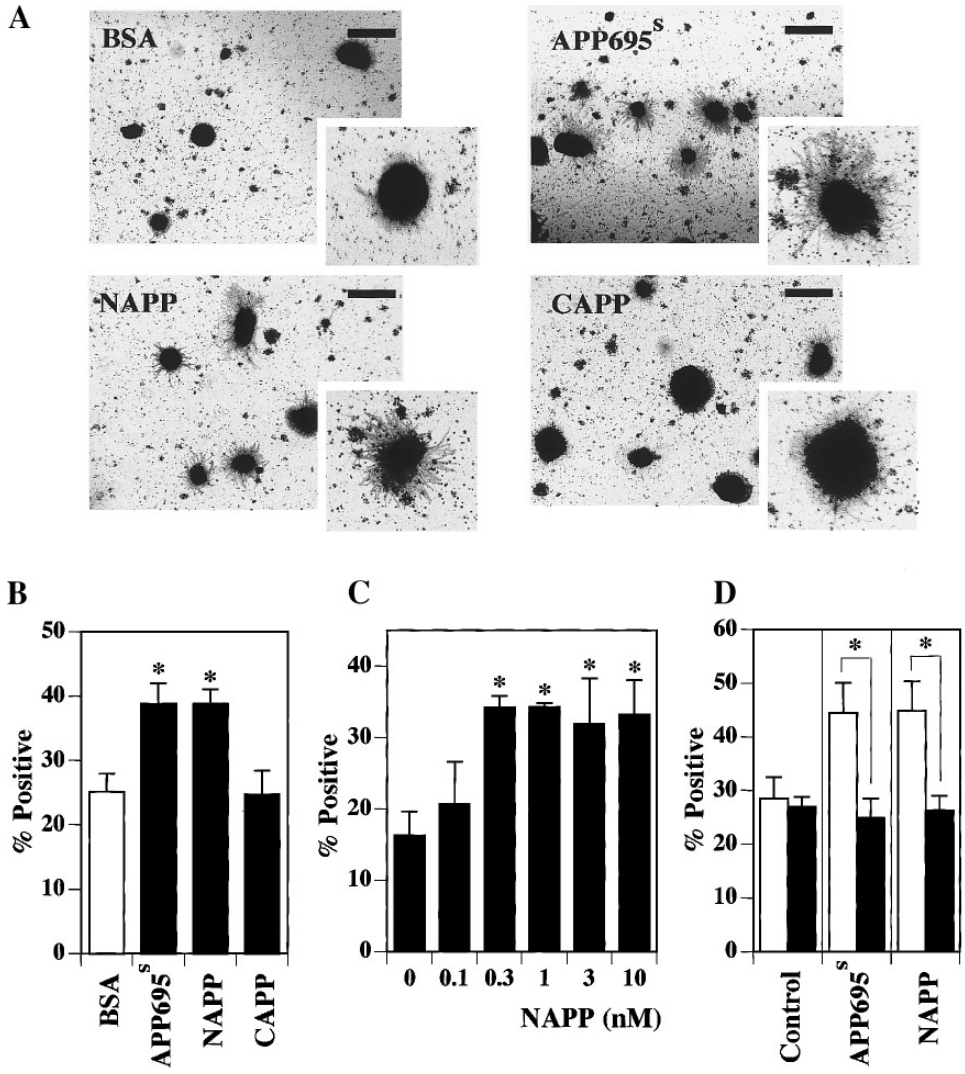

FIG. 2. Effects of NAPP and CAPP on neurite outgrowth in neocortical explants. **A:** CBB-stained explants with 100 ng of each protein per ml of medium. Bars represent 200 μm. A magnified photograph is included at the bottom right of each panel. **B-D:** Semi-quantitative analysis of neurite promoting activity. Results are expressed as percentages of explants containing neurites longer than their diameter. Means and SD for three wells are presented. Significance ($p < 0.05$) was assessed by Bonferroni method. Similar results were obtained from three (**B** and **C**) or two (**D**) independent experiments. In **B,** 100 ng of the protein indicated was added per ml of medium. *Significantly different from the addition of BSA. In **C,** the concentration of NAPP indicated was added. *Significantly different from the addition of 0 nM. In **D,** rAPP695^s (100 ng/ml) or NAPP (30 ng/ml) plus 1 μg/ml of normal mouse immunoglobulin G (IgG) or antibody 22C11 was added. No proteins were added to the negative control. *Significant difference.

Therefore we examined the effects of each peptide (N16, R16, or RERMS17) on the survival of rat neocortical neurons. Indeed, we have detected the survival-promoting activity in RERMS17 peptide (Fig. 4). However, neither N16 nor R16 supported the neuronal survival.

DISCUSSION

We previously reported that two isoforms of APP^s, APP695^s and APP770^s, produced by yeast had the same neurotrophic activity in neocortical explants, suggesting that the KPI domain is not responsible for this activity (13). Their molecular weights, calculated from SDS-PAGE, and elution profiles of a heparin column, were quite similar to those of APP^{s}s purified from the human brain (18). Thus, the expression system using yeast is useful in investigating the regions responsible for the biological activities of APP^s. To identify the region of APP^s responsible for neurite-promoting activity, we produced both the amino-terminal and carboxyl-terminal regions of APP^s (NAPP

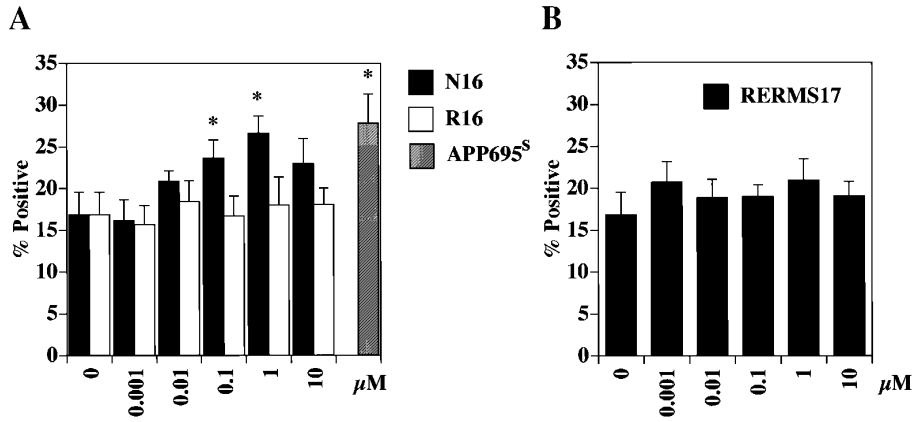

FIG. 3. Semi-quantitative analysis of neurite promoting activity of synthetic peptides containing epitope of 22C11 (**A**) and RERMS (**B**). Results are expressed as percentages of explants containing neurites longer than their diameter. Means and SD for three wells are presented. Significance ($p < 0.05$) was assessed by Bonferroni method. Similar results were obtained from ability of NAPP to promote neurite extension two independent experiments. In **A,** APP695^s (1 nM) was added to the positive control. *Significantly different from the addition of 0 μM.

and CAPP) using a yeast expression system. The fragments containing a portion of APP^s were secreted by a signal sequence derived from yeast and accumulated in the culture medium as a major and soluble protein. Therefore, the fragments were easily purified without any detergent.

NAPP, which corresponds to the Arg^{16} to Val^{290} region of human APP770, promoted neurite outgrowth, but CAPP, which contained from Glu^{380} to Thr^{663}, did not, and a monoclonal antibody, 22C11, recognizing the amino terminal region suppressed the activity of APP695^s. These findings indicated that the amino-terminal region, which contains the cysteine-rich domain and an area rich in acidic residues, should be sufficient for neurite-promoting activity. Extracellular cysteine-rich domains are common feature of the protein families, such as epidermal growth factor and tumor necrosis factor.

Recently, it has been reported that transmembrane form of APP forms a complex with the heteotrimeric G protein G_o in a receptor-like manner (24), and antibody 22C11 used as a ligand specifically activated G_o in APP695/G_o vesicles (23). Then, we speculated that the cysteine-rich domain recognized by 22C11 might be of importance to the function of APP and APP^s. The neurite-promoting activity of the peptide corresponding to 22C11 epitope (Fig. 3A) was good agreement with the speculation. However, the effective concentration of the peptide was about 0.1 μM, whereas the effective concentration of NAPP was about 0.32 nM (Fig. 2C), which was the same as the effective concentration of APP695^s. It has been also reported that both neurite-promoting activity in chick sympathetic and mouse hippocampal neurons and MAPK-stimulating activity in PC12 cells appear to require a cysteine disulfide bond in the amino terminal region of APP^s (19, 9). These findings led us to speculate that the conformation of cysteine-rich region in NAPP might be of importance in promoting the neurite outgrowth and the epitope peptide for 22C11 might not form appropriate conformation.

Furthermore, carboxyl-terminal domain containing RERMS sequence adjacent to the KPI domain has been reported to stimulate fibroblast growth, neurite outgrowth of B103 neuronal cell line, and the survival of primary cultured neurons (10, 11, 20). We also observed that the peptide, RERMS17, supported survival of neocortical neurons, but the peptide corresponding to 22C11 epitope did not (Fig. 4). However, neither the CAPP including RERMS sequence nor RERMS17 promoted neurite outgrowth in explant culture (Fig. 2 and Fig. 3B), suggesting that the fuctions of RERMS sequence is different from that of the peptide corresponding to 22C11 epitope. Indeed, Yamamoto *et al.* have discussed the possibility that the region containing the RERMS sequence is an active domain for the neuronal survival promoting activity of APP^s other than neurite extension (20). Our present findings supported this possibility. On the other hand, B5 antibody directed against the carboxyl-terminal region of APP^s suppressed the APP^s-induced stabilization of intracellular calcium levels in cultured hippocampal neurons (12, 21). When all of the above findings are considered together, it appears that APP^s play an important role in the brain as a multifunctional protein. The molecular mechanisms of the physiological actions of APP^s will have to be further elucidated to understand the roles of APP^s in greater detail.

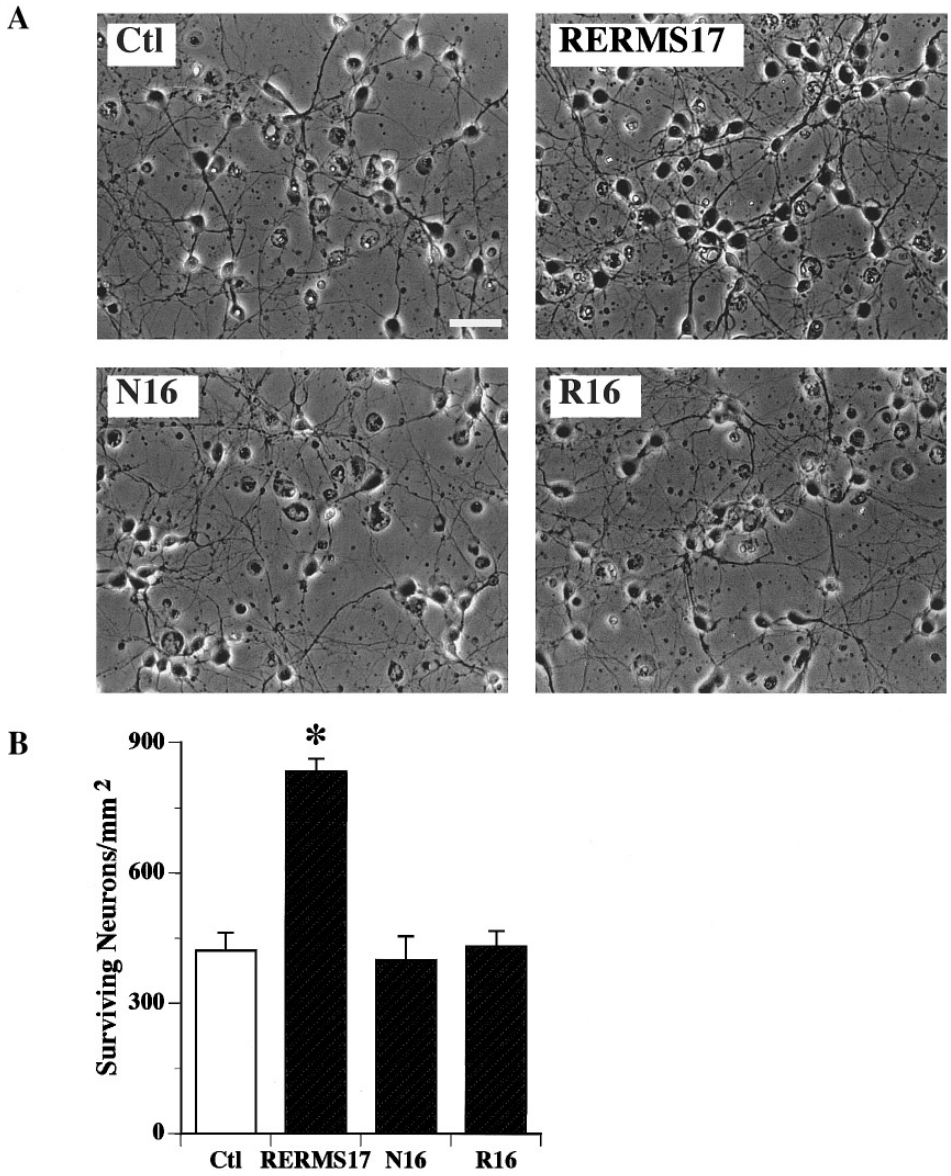

FIG. 4. Effect of peptides on survival of rat neocortical neurons. No proteins were added to the negative control (Ctl). **A:** Phase-contrast views of neocortical neurons with 1 μM of each peptide. A bar represents 50 μm. **B:** Number of cells survived. Means and SD for three wells are presented. *Significance ($p < 0.05$) from control was assessed by Bonferroni method. Similar results were obtained from two independent experiments.

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