Alzheimer’s disease (AD) may be caused by toxic aggregates formed from amyloid-β (Aβ) peptides. By using Thioflavin T, a dye that specifically binds to β-sheet structures, we found that highly toxic forms of Aβ-aggregates were formed at the initial stage of fibrillogenesis, which is consistent with recent reports on Aβ oligomers. Formation of such aggregates depends on factors that affect both nucleation and elongation. As reported previously, addition of Aβ42 systematically accelerated the nucleation of Aβ40, most likely because of the extra hydrophobic residues at the C terminus of Aβ42. At Aβ42-increased specific ratio (Aβ40:Aβ42 = 10:1), on the other hand, not only accelerated nucleation but also induced elongation were observed, suggesting a pathogenesis of early-onset AD. Because a larger proportion of Aβ40 than Aβ42 was still required for this phenomenon, we assumed that elongation does not depend only on hydrophobic interactions. Without any change in the C-terminal hydrophobic nature, elongation was effectively induced by mixing wild type Aβ40 with Italian variant Aβ40 (E22K) or Dutch variant (E22Q). We suggest that Aβ peptides in specific compositions that balance hydrophilic and hydrophobic interactions promote the formation of toxic β-aggregates. These results may introduce a new therapeutic approach through the disruption of this balance.

Fibrillar amyloid-β peptide (Aβ) in β-sheet conformation is one of the main components of senile plaques, a pathological hallmark of Alzheimer’s disease (AD) (1, 2). Aβ is cleaved from β-amyloid precursor protein (APP) by secretases whose enzymatic components are suggested to include presenilins and β-site APP cleaving enzyme (3, 4). Most mutations of both APP and presenilins are associated with early-onset familial Alzheimer’s disease (5–10). Interestingly, such mutations are also associated with increased production of Aβ42, which is a longer isoform of various Aβ species (5–10). Aβ42 likely aggregates more rapidly than other Aβ peptides because it contains additional hydrophobic amino acids at its carboxyl terminus (11–15). The overexpression of structurally normal APP that results from an extra gene in trisomy 21 (Down’s syndrome) almost invariably leads to the premature occurrence of classic AD neuropathology during middle adult years (14). Brains from younger Down’s syndrome subjects often display “diffuse plaques” composed solely of Aβ42, whereas older Down’s syndrome subjects display “cored plaques” composed of Aβ40, a shorter form, at the plaque center (15). Together, these findings provide strong evidence for the role of Aβ42 in AD and AD-like pathology. Here, we report the importance of Aβ compositions for promoting toxicity-associated β-aggregation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Peptides—**Thioflavin T (ThT) was obtained from Sigma. N,N-diethylmethylamide, sodium lauryl sulfate (SDS), 28% ammonia solution, Tris (hydroxymethyl) aminomethane, and HEPES were purchased from Wako Pure Chemical (Osaka, Japan). Glycerol was obtained from Junsei (Tokyo, Japan). Wild type β-amyloid1–40 of four lots (510818, 511024, 520311, 520130) and wild type Aβ1–42 of three lots (510523, 520116, 520323) were obtained from Peptide Institute (Osaka, Japan). Italian variant Aβ1–40 of a lot (P0401071T) was purchased from American Peptide Company (Sunnyvale, CA). Italian variant Aβ1–40, Dutch variant Aβ1–40, Italian variant Aβ1–42, and four other non-pathogenic variants Aβ1–40 (E22R, E22shortK, E22D and E22longE) were synthesized and purified at Research Resources Center of RIKEN Brain Science Institute. “shortK” is an amino acid residue that has a similar structure to Lys with only three methyl groups prior to the terminal amine. “longE” is a residue that has a similar structure to Glu with an additional methyl group, three in total. All peptide samples were high pressure liquid chromatography-purified to be more than 90% pure and were in trifluoroacetic salt form.

**Aβ Sample Preparation—**A stock solution of Aβ was prepared by dissolving powdered Aβ peptide in 100% HFIP to a final concentration of 1 mg/ml. After shaking for two hours at 4 °C, the Aβ stock solution was aliquoted into siliconized tubes and stored at −80 °C. Just prior to each experiment, aliquots were spin-vacuumed using an Integrated SpeedVac system (Savant). For experiments shown in figures except Fig. 1, aliquots of Aβ40 were redissolved in 50% HFIP/14% NH3 solution and then spin-vacuumed. They were then dissolved in a HEPES-buffered solution.

**Fluorescence Spectroscopy (ThT Assay)—**The degree of β-aggregation was determined using the fluorescent dye, ThT, which specifically binds to fibrous structures (16). First, Aβ stock solution (see above) was diluted with 5 or 10 mM HEPES-NaOH and 0.9% NaCl. ThT was added to each test sample to a final concentration of 10 μM. Each sample was prepared in 96-well Black Cliniplates (Labsystems) and shaken for 10 s prior to each measurement. Measurements were carried out every 20 min.

The relative degree of β-aggregation was assessed in terms of fluorescence intensity, which was measured at 37 °C using a Fluorescan Ascent FL spectrophotometer (Labsystems, Finland). Measurements were performed at an excitation wavelength of 445 nm and an emission of 485 nm, wavelengths that result in the optimum detection of bound ThT. To account for background fluorescence, the fluorescence intensity measured from each control solution without Aβ was subtracted from that of each solution containing Aβ. By making three or six wells of each condition, their standard deviations were first calculated. They were then divided by the square root of n.

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Received for publication, December 16, 2002, and in revised form, April 23, 2003. Published, JBC Papers in Press, April 25, 2003, DOI 10.1074/jbc.M212785200

Printed in U.S.A.

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Cell Cultures—Human embryonic kidney (HEK) 293 cells were used to test the toxic effects of Aβ-aggregation as assessed in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see below). They were grown in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (HyClone) and incubated in a humidified chamber (85% humidity) containing 5% CO2 at 37 °C. On the morning of Aβ treatment, the cell culture medium was replaced with serum-free Dulbecco’s modified Eagle’s medium, and the cells were plated onto a 96-well, coated plate (Corning) at a final cell count of 20,000 cells/well. The cell viability was subsequently assessed using the MTT assay (see below).

MTT Assay—The toxic effects of spontaneous β-aggregation of Aβ on cell cultures were assessed. Solutions containing Aβ were prepared as described for the ThT assay, and then the samples were transferred immediately into wells containing the HEK293 cell (see “Cell Cultures”). After certain periods, MTT was added to each well, and the plate was kept in a CO2 incubator for an additional 2 h. The cells were then lysed by the addition of a lysis solution (50% dimethylformamide, 20% SDS, pH 4.7) and were incubated overnight. The degree of MTT reduction in each sample was subsequently assessed by measuring absorption at 570 nm at 37 °C. Twenty thousand cells/well. The cell viability was subsequently assessed using the MTT assay (see below).

RESULTS

We first assessed the baseline kinetics of β-aggregation. The time course of β-aggregation was determined by incubating Aβ40 with ThT, a compound that fluoresces when specifically bound to β-sheet structures (16) (Fig. 1). Fluorometric measurements were carried out every 20 min for ~72 h at 37 °C. As expected (11-13), the β-aggregation kinetics was sigmoidal over the first 5 h of incubation (Fig. 1A). With increasing incubation, however, β-aggregation decreased slowly (Fig. 1B), decaying to an approximate asymptotic level of less than half the peak aggregation.

Because there are several competing hypotheses concerning the factor(s) associated with AD pathogenesis, it was of interest to analyze the toxicity, biochemical constituents, and structure of the aggregates during β-aggregation. At selected intervals during the first two days of incubation, Aβ/ThT samples were collected, transferred to HEK293 cell cultures grown in serum-free medium, and the toxicity of the Aβ/ThT solution was examined using the MTT assay, which assesses cell viability by measuring mitochondrial activity (17) (Fig. 1C). Even after only a short period of incubation, the β-aggregates that formed were highly toxic. With longer incubation, however, the β-aggregates were less toxic. We next determined the time course of the formation of different Aβ intermediates during the course of β-aggregation. The Aβ/ThT solutions were collected at selected times during aggregation and were analyzed by Western blot. Aliquots were applied to SDS-polyacrylamide gels and immunoblotted with an anti-Aβ antibody (Fig. 1D). Samples collected immediately after being incubated separated out as a thick band the size of the Aβ monomer and a thinner, less distinct band the size of the Aβ dimer, indicating that some dimerization had occurred almost immediately upon solubilization. After 2 h of incubation, the Aβ monomer band was less distinct, and an immunoreactive smear appeared, suggesting the formation of Aβ polymers. As incubation progressed, the amount of monomeric Aβ decreased. After extended incubation, we noticed increased staining at the bottom of the loading wells that presumably represented large aggregates that could not be resolved by the gel. The macromolecular structures of these various intermediates were examined using electron microscopy (Fig. 1, E-H). No distinguishable fiber-like structures were observed in samples collected soon after incubation began or in samples collected after 1 h of incubation. After 2 h of incubation, however, small oligomeric aggregates and long fibrils were observed (Fig. 1, E and F). The fibrils appeared to be comprised of two smaller filaments that were twisted around each other (Fig. 1F), but no further appreciable association between fibrils was observed. In contrast, after 4 h of incubation, the time point at which the greatest amount of β-aggregates formed (Fig. 1C), long fibers appeared to be gathered closely together in a net-like structure (Fig. 1G). After 27 h of incubation, Aβ fibers, aligned side by side and formed numerous fiber bundles (Fig. 1H). Interestingly, samples from this time period in the toxicity experiments were only moderately toxic; the most toxic Aβ-aggregates were those from the initial growth stage of fibrillogenesis. These findings prompted us to search for factors that cause an acute increase in the initial stage of β-aggregation.

Because individuals with familial Alzheimer’s disease have larger proportions of Aβ42 compared with normal individuals (5-10), we examined the hypothesis that elevated levels of Aβ42 may play a role in the initial stage of β-aggregation. Thus, we measured the time course of β-aggregation formed by various ratios of Aβ40 and Aβ42 (Fig. 2A). Consistent with previous reports (11-13), incubation of Aβ42 alone showed signs of β-aggregation almost immediately after incubation began. After this initial increase, however, aggregation remained at a fairly constant, modest level without further significant increase. Pretreatment of Aβ42 with either HFIP or HFIP/NH3 did not significantly alter the time course of β-aggregation. On the other hand, although the lag time until the start of β-aggregation of HFIP/NH3-treated Aβ40 was much longer, the formation of β-aggregates increased to higher levels compared with that for Aβ42. Because longer lag time seemed more appropriate to examine the initial steps of aggregation in detail, HFIP/NH3-treated Aβ40 was used in the rest of the experiments. When the proportion of Aβ42 was increased to the Aβ40:Aβ42 ratio of 10:1, β-aggregation kinetics systematically shifted to the left. Thus, Aβ42 accelerated the seeding or nucleation of Aβ40. When the proportion of Aβ40 to Aβ42 mimicked the proportion reported in some familial Alzheimer’s disease mutants (i.e. Aβ40:Aβ42 ratio of 10:1) (10, 18), β-aggregation was further accelerated and also increased to a much higher level. Moreover, these Aβ42-induced aggregates were highly toxic when assessed with the MTT assay (Fig. 2C). At the end of the incubation period, the level of Aβ42-associated aggregation was lower than that of Aβ40 (Fig. 2B). Aggregates formed by Aβ42 alone were less toxic than aggregates formed by Aβ40 alone. The most toxic aggregates were in samples with the highest levels of aggregation (i.e. Aβ40:Aβ42 combined at a ratio of 10:1). Thus, it was of interest to examine the macromolecular structure of these aggregates (Fig. 2, D-F).

The approximate diameter of long fiber bundles formed by
Fig. 1. **Time course of β-aggregation and toxicity of wild type Aβ40.** Aβ40 (5 μM) was pretreated with HFIP, a solvent that converts Aβ into a structural conformation normally found in cell membranes and was incubated with 10 μM ThT in 5 mM HEPES (pH 7.4) and 0.9% NaCl. A, early stage of β-aggregation, showing sigmoidal kinetics. B, entire time course, showing decay of β-aggregation as incubation progressed. C, toxicity of β-aggregates to cultured cells. At each time indicated, an Aβ/ThT sample was collected from the incubation well, and the well was rinsed with 50 μl of buffer. The rinsed solution was mixed with the Aβ/ThT sample. Five microliters of this mixture (i.e. 0.1 of the total volume of culture medium) was added to HEK293 cell cultures; the final concentration of Aβ was ~330 nM. The cells were incubated with this mixture for a total of 4 h; MTT was added to the cell cultures at the 2-h point to assess the degree of MTT reduction (i.e. cell viability). Fluorescence values (bar graphs) and % MTT values (line graph) are means ± S.E. throughout this report. D, Western blot showing the time course of β-aggregation. The formation of intermediate β-aggregates was also assessed by SDS-PAGE and immunoblotting using monoclonal antibody 4G8. E–H, electron photomicrographs showing macromolecular structures formed during the time course of β-aggregation. Five micromolar Aβ40 solution samples were incubated for 2 h (E), 2 h (F), 4 h (G), and 27 h (H). After incubation, 3 μl of Aβ40 solution was applied to Formvar-coated copper grids and negatively stained with neutralized tungstophosphoric acid. Magnification is 80,000; scale bars are 100 nm.
Aβ40 varied from 8 to 16 nm. On the other hand, closely associated fibers of aggregates formed by Aβ42 were shorter and had a maximum diameter of ~8 nm; thus, we provisionally called these fibers “protofibrils” (Fig. 2E). In samples containing Aβ40 and Aβ42 at a 10:1 ratio, aggregates formed fiber bundles that appeared shorter than extended Aβ40 fibers (compare Fig. 2F and D). It is possible that, via the rapid formation of protofibrillar nuclei or seeds, Aβ42 enhanced β-aggregation as well as increased the toxicity of the Aβ40:Aβ42 mixture. However, our results thus far suggest that Aβ42 alone does not...
cause large elongation. In the next set of experiments, we sought to determine whether certain other factors affect the elongation step of β-aggregation formation. As previously suggested, the hydrophobic nature of the Aβ42 C terminus is critical for accelerating the seeding process (11–13). Thus, if the C terminus is related to seeding, we hypothesized that the N terminus of Aβ may be related to elongation.

To test this hypothesis, we assessed the β-aggregation kinetics of several Aβ40 peptides with pathogenic mutations in the N-terminal domain, Italian variant Aβ40 (E22K) and Dutch variant (E22Q). Previously, such substitutions were suggested to affect the intersheet stacking between β-sheets (19–21). We compared the β-aggregation time courses of wild type Aβ40 (E22K), Italian variant (E22K), Dutch variant (E22Q), and their mixtures (Fig. 3A) (20, 21). None of the variants by themselves showed significantly higher levels of β-aggregation compared with that of the wild type. Indeed, Italian variant (E22K) showed undetectable β-aggregation. However, only when the Italian or Dutch variants were mixed with wild type Aβ40 in a 1:1 ratio did β-aggregation increase significantly during the early stage of elongation. Although the Dutch variant alone formed β-aggregates faster than wild type Aβ40, the mixing of the two induced and enhanced elongation. Although β-aggregation by the Italian variant and wild type Aβ40 mixture was faster and more robust compared with that of the wild type alone, β-aggregation by the Italian variant alone was undetectable. Clearly, the interactions between hydrophilic residues in the N-terminal domain play an important role in elongation under our experimental conditions.

As before, we assessed the impact of Aβ42 on β-aggregation, this time in ratios reported for wild type Aβ40 and Italian variant Aβ40 in vitro (22) (Fig. 3B). When wild type or variant Aβ42 was added to the wild type Aβ40/Italian Aβ40 sample, an even larger elongation was observed compared with that when Aβ42 was absent (Fig. 3C). This combination was very toxic as well (Fig. 3D). The most probable reason to explain the difference between the time courses of aggregation of a mixture containing wild type Aβ40 and Italian Aβ40 in Fig. 3, A and B would be the difference between two lots of Italian Aβ40 peptides, one lot from American Peptides (Fig. 3A) and the other from RIKEN BSI (Fig. 3, B–D and Fig. 4B). Nonetheless, one is tempted to draw conclusions about phenotypes from these in vitro results. The differences in nucleation and elongation of β-aggregation resulting from different Aβ combinations do not completely explain differences in the pathological features of these mutations that show altered Aβ40/Aβ42 ratios and Aβ sequence mutations. However, because conditions that mimic these pathogenic mutations both induce and accelerate β-ag-

**Fig. 3.** Effect on time-course of β-aggregation and toxicity by various Aβ variants having mutations in the N-terminal domain. A, β-aggregation kinetics of wild type Aβ40 (●), Italian variant Aβ40 (E22K) (■), Dutch variant (E22Q) (▲). The final Aβ concentration for each of these samples was 5 μM. β-aggregation was also assessed for samples containing a 1:1 ratio of wild type Aβ40 and different Aβ variants as indicated: Italian (□), Dutch (●). B, impact of Aβ42 on aggregation. The following were assessed: wild type Aβ40 (●), wild type Aβ42 (■), wild type Aβ40 and Aβ42 (10:1) (▲), and wild type Aβ40 and Aβ42 (15:1) (▲). β-aggregation was also assessed for samples containing a 1:1 ratio of different wild type Aβ species and various Italian variant Aβ species as indicated: wild type Aβ40 and Italian Aβ40 (1:1) (○), wild type Aβ42 and Italian Aβ42 (1:1) (□), wild type Aβ40/Italian Aβ40 (1:1) and wild type Aβ42/Italian Aβ42 (1:1) at ratio of 10:1 (○), and wild type Aβ40/Italian Aβ40 (1:1) and wild type Aβ42/Italian Aβ42 (1:1) at a ratio of 15:1 (△). For comparison, the mixture of wild type Aβ species was also measured. C and D, toxicity of aggregates formed by various Aβ variants. After 12 h of incubation, cell viability was assessed as described under “Experimental Procedures.” Bar graphs in panel C represent the degree of β-aggregation; those in panel D represent the viability of the cells. Numbers on the horizontal axis represent the following: 1, wild type Aβ40; 2, wild type Aβ42; 3, wild type Aβ40/wild type Aβ42 (10:1); 4, Italian Aβ40; 5, Italian Aβ42; 6, Italian Aβ40/Italian Aβ42 (10:1); 7, Italian Aβ40:Italian Aβ42 (15:1); 8, wild type Aβ40:Italian Aβ40 (1:1); 9, wild type Aβ42:Italian Aβ42 (1:1); and 10, wild type Aβ40/Italian Aβ40 (1:1) and wild type Aβ42/Italian Aβ42 (1:1) at a ratio of 15:1.
aggregation, as well as elevate toxicity (Fig. 3, C and D), the acute increase in β-aggregation at the initial stage is consistent with pathogenesis of amyloid-related disorders.

Our results thus far clearly hint that particular proportions of Aβ40 and Aβ42 either enhance or retard nucleation and elongation. Our findings are also consistent with previous stud-
ies showing that the accelerating effect of Aβ42 resides in the hydrophobicity of its C terminus (11–13), and the hydrophilic side chains in the N terminus most probably have an effect on intersheet stacking (19). In this last set of experiments, because overall β-aggregation depends on the relationship of factors that affect nucleation and elongation, we took a closer look at the respective role(s) of such factors, as such the Aβ42 C terminus and Aβ40 N terminus, in β-aggregation. To determine whether disproportionately greater concentrations of Aβ42 affect nucleation, we first assessed the aggregation kinetics of samples containing broader ratios of Aβ40:Aβ42. As seen in Fig. 2, greater proportions of wild type Aβ42 accelerated the seeding of Aβ40 (Fig. 4A). In terms of the elongation process, however, the proportion of Aβ42 appeared to have an inverse effect. In samples containing larger proportions of Aβ42 than Aβ40, the overall level of β-aggregation was lower (Fig. 4A). On the other hand, although Italian variant Aβ40 induced the seeding of wild type Aβ40 less effectively compared with that by wild type Aβ42, it effectively induced the elongation of aggregates formed by wild type Aβ40 (Fig. 4B).

As mentioned, the induction effect of wild Aβ42 is related to the hydrophobic nature of its C-terminal, resulting in β-sheet formation (11–13). On the other hand, the effect of Italian variant Aβ40 is due to the substitution of a negatively charged Glu at position 22 to a positively charged Lys. This likely induces β-aggregation through electrostatic interaction of side chains, thus promoting intersheet stacking (19) and elongation. Because this is an interaction between paired β-sheets having oppositely charged side chains, Italian variant Aβ40 does not aggregate by itself. This notion was further supported by experiments mixing four kinds of non-pathogenic mutants at position 22 with wild type Aβ40 (Fig. 4, D–G). Although the degree of induction effect was slightly different, two mutant Aβ40s having positively charged Arg and shortK (see “Experimental Procedures”) induced the elongation of wild type Aβ40 in a manner similar to the Italian variant (E22K) (see “Experimental Procedures”) and the wild type Aβ40 (Fig. 4, D–G). Therefore, the size of side chains at position 22 also tested two other mutants with negatively charged Asp and longE (see “Experimental Procedures”). Both mutants by themselves showed lower levels of β-aggregation than wild type Aβ40 (Fig. 4, F and G). Moreover, as the proportion of E22D and E22longE was increased, the β-aggregation was decreased in a dose-dependent manner, suggesting the importance of size of side chains in β-aggregation. In contrast, although all the mutants with positively charged residue at 22 (E22K, E22R, and E22shortK) by themselves also had lower β-aggregation level than wild type, when their small proportions were mixed with wild type, β-aggregation was significantly elevated. These results suggest the important role of electrostatic interactions of side chains in the β-aggregation process.

Substantial induction effects by both wild Aβ42 and Italian variant Aβ40 on the overall β-aggregation of wild Aβ40 were observed when the proportion of inducers was smaller than wild Aβ40. Because overall β-aggregation depends on both β-sheet formation (i.e. nucleation) and intersheet stacking (i.e. elongation), specific balance between these interactions determines the formation of fast and induced β-aggregation that leads to toxic activity. The importance of such balance is underscored by our finding that Italian variant Aβ42 suppressed the aggregation of wild type Aβ40 at all ratios tested (Fig. 4C). This is because the hydrophobic effect at the C terminus and electrostatic interactions between side chains are physically opposed. Only when they balance at the certain composition of Aβ peptides are toxic β-aggregates formed.

DISCUSSION

There is tremendous evidence that supports a single hypothesis for the pathogenesis of AD. This so-called “amyloid hypothesis” suggests that the process of Aβ deposition is intimately connected to the initiation of AD pathogenesis and that all other features of the disease are secondary to this initiation (23, 24). A recent, novel suggestion relating to this hypothesis is that oligomeric aggregates of Aβ peptides are the forms that generate neurotoxicity (25, 26). The aggregation kinetics presented in Fig. 1 show that initially formed aggregates are indeed more toxic than fiber bundles formed after a long incubation. However, one cannot conclude from these observations that deposited amyloids or bundles of fibers are innocuous but suggest that they are possibly less neurotoxic than initially formed Aβ-aggregates.

Mixing Aβ40 and Aβ42 at specific ratios induced and accelerated β-aggregation. These results suggest that particular ratios of Aβ species are very important for the toxic β-aggregation process and perhaps in AD pathogenesis. However, elevated ratios of Aβ42/40 have not been observed in the media collected from cells transfected with several intra-Aβ mutations, including the Dutch and Italian cases (22). The predominant increase of β-aggregation observed by mixing both wild type and Italian variant Aβ suggests that elongation is largely dependent on interactions between the N-terminal side chains of β-sheets, which are nucleated by hydrophobic interactions of C-terminal regions. Hydrophobic residues in the N-terminal domain may also induce nucleation or elongation by an entropically driven process, whereas other polar or charged side chains will interact more directly with each other. The latter type of interaction is hydrophilic and thus is generally known to be weaker in stabilizing proteins. Because these two types of interactions usually repel each other in a manner similar to oil and water, Aβ compositions that balance these interactions in a specific way determine whether the formation of toxic β-aggregates proceeds effectively. This is relevant to the possible mechanism of Aβ toxicity. One idea is that β-aggregates penetrate the membrane structure either extracellularly or intracellularly (27). β-sheets β-aggregates formed under conditions in which polar-nonpolar interactions are balanced in a specific way would make an alignment of charged residues that perhaps disrupt functions of the cell membrane. This is consistent with a recent finding that non-fibrillar, ThT-positive aggregates (i.e. aggregates of β-sheets) of a wide range of misfolded proteins exhibit similar toxicity to that shown in the present study (28).

Acknowledgments—We thank Y. Tatebayashi and T. Miyasaka for critical advice, A. Sekimata-Murakami for technical assistance, and N. Hirotsu for synthesizing peptides.

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