Protection against \( \beta \)-Amyloid-induced Apoptosis by Peptides Interacting with \( \beta \)-Amyloid*

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\( \beta \)-Amyloid peptide produces apoptosis in neurons at micromolar concentrations, but the mechanism by which \( \beta \)-amyloid exerts its toxic effect is unknown. The normal biological function of \( \beta \)-amyloid is also unknown. We used phage display, co-precipitation, and mass spectrometry to examine the protein-protein interactions of \( \beta \)-amyloid in normal rabbit brain in order to identify the biochemical receptors for \( \beta \)-amyloid. \( \beta \)-Amyloid was found to bind primarily to proteins involved in low density lipoprotein and cholesterol transport and metabolism, including sortilin, endoplasmic reticulum-Golgi intermediate compartment 2 (ERGIC2), ERGIC-53, steroid 5α-reductase, and apolipoprotein B. \( \beta \)-Amyloid also bound to the C-reactive protein precursor, a protein involved in inflammation, and to 14-3-3, a protein that regulates glycogen synthase kinase-3β, the kinase involved in tau phosphorylation. Of eight synthetic peptides identified as targets of \( \beta \)-amyloid, three were found to be effective blockers of the toxic effect of \( \beta \)-amyloid on cultured neuronal cells. These peptides bound to the hydrophobic region (residues 17–21) or to the nearby protein kinase C pseudo-phosphorylation site (residues 26–30) of \( \beta \)-amyloid, suggesting that these may be the most critical regions for \( \beta \)-amyloid effector action and for aggregation. Peptides or other small molecules that bind to this region may protect against \( \beta \)-amyloid toxic effect by competitively blocking its ability to bind \( \beta \)-amyloid effector proteins such as sortilin and 14-3-3.

\( \beta \)-Amyloid (A\( \beta \))\(^2\) is a toxic peptide produced by cleavage of amyloid precursor protein (APP) by \( \beta \)- and \( \gamma \)-secretases. Application of A\( \beta \) to cultured cells at micromolar concentrations causes apoptosis (1), and lower concentrations cause up-regulation of apoptosis markers caspase-3 (2) and annexin-(3). Biochemical effects of A\( \beta \) include activation of calcium channels (4, 5), inactivation of potassium channels (6), production of free radicals (7), excitotoxicity through activation of N-methyl-D-aspartate receptors (8, 9), inhibition of protein kinase C (10, 11), and glutamate accumulation leading to increased Ca\(^{2+} \) levels (12). Intracerebroventricular injection of A\( \beta \) produces impairment of spatial memory and non-spatial long term memory (13, 14), reduction of protein kinase C activity (15), induction of apoptosis (13), and activation of astrocytes and microglia to release excessive amounts of inflammatory cytokines (16). Transgenic animals expressing human A\( \beta \) exhibit many of the pathologies of Alzheimer disease (AD), including cognitive deficits (17), age-related formation of amyloid plaques, activation of astrocytes and microglial cells, vascular amyloid pathology, degeneration of cholinergic nerve terminals, and reduced lifespan (18). However, transgenic mice expressing normal human APP do not exhibit the neurofibrillary tangles and significant neuronal loss characteristic of AD (18).

Formation of A\( \beta \) from APP is dependent on the intracellular transport system. APP is transported from the ER and Golgi to the cell surface membrane (19), where it may be cleaved by \( \alpha \)-secretase. Protein kinase C-activated \( \alpha \)-secretases also reside in the trans-Golgi network (20), which is a major site for \( \beta \)-secretase activity. Uncleaved APP is then internalized into endocytic compartments, where it is cleaved by \( \beta \)- and \( \gamma \)-secretase to produce A\( \beta \) (21, 22). Both neurons and many non-neuronal cells also contain membrane \( \beta \)- and \( \gamma \)-secretases (23), which produce A\( \beta \) that is secreted into the extracellular space. In neurons, A\( \beta \) can also be produced in the endoplasmic reticulum (24, 25). However, A\( \beta \) does not remain in these two compartments. A\( \beta \) has been found in a number of other compartments, including cytosol (26–29), lysosomes (30, 31), mitochondria (32, 33), and even in cell nuclei (34). This widespread distribution may result in part from uptake of extracellular A\( \beta \) into neurons (28, 35) and astrocytes (36). Because A\( \beta \) lacks an ER signal sequence, it may also be identified by the ER as a misfolded protein and translocated across the ER or Golgi membrane (37) by sec61 (38).

The normal functions of APP and A\( \beta \) are unknown. APP is an integral membrane protein with high affinity for copper (39, 40). It has been suggested that APP is involved in neurodevelopment (41) and is essential for neuronal growth (42, 43). Mutant mice in which APP has been knocked out develop reactive gliosis, weight loss, cognitive deficits, and reduced levels of presynaptic marker proteins (44), indicating generalized central nervous system pathology. Down-regulation of APP inhibits neurite outgrowth (45), and anti-APP antibodies block memory formation in chicks (46). After being transported along nerve fibers, APP participates in synaptogenesis (47) and...
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cell adhesion (48). Thus, both APP and Aβ may play an important role in normal synaptic plasticity and neuronal growth.

The Aβ peptide is not only produced in AD but has also been found in cerebrospinal fluid and blood plasma of normal patients (49). The production and secretion of Aβ is regulated by neuronal activity (50). Kameneza et al. (50) found that APP reversibly depresses synaptic transmission by a mechanism mediated by activation of APP cleavage by N-methyl-D-aspartate receptors. This suggests that Aβ is normally produced by neurons and has one or more functions in normal cells. To identify the biochemical pathways in which Aβ participates, we performed phage display screenings and co-precipitation experiments to identify the binding partners of Aβ in normal rabbit brain. We also examined the ability of peptides based on these binding partners to block the toxic effects of Aβ. Aβ was found to interact with apolipoprotein B and the C-reactive protein precursor, the transporter proteins sortilin, ER-Golgi intermediate compartment 2 (ERGIC2), and ERGIC-53, and the regulatory proteins 14-3-3ε and 14-3-3γ. Peptides based on these binding regions were effective blockers of Aβ toxicity.

EXPERIMENTAL PROCEDURES

Materials—5′-Nucleotidase was purchased from Biomol International (Plymouth Meeting, PA). Phosphodiesterases 3A and 3B were obtained from EMD Biosciences (San Diego, CA). Antibodies against 14-3-3β, δ, γ, and -η were obtained from Upstate (Charlottesville, VA). Anti-ERGIC2 was obtained from Aviva Systems Biology (San Diego, CA). Kidins antibody was obtained from Orbigen (San Diego, CA). Sortilin antibody was obtained from Abcam Inc. (Cambridge, MA). Other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All β-amyloid and β-amyloid derivatives were purchased from AnaSpec (San Jose, CA). UltraLink protein A and protein G-agarose, sequencing grade trypsin, and Pro-Ject protein transduction reagent were purchased from Pierce. Caspase-3 substrate (Ac-DEVD-amidomethylcoumarin) was purchased from American Peptide Co. (Sunnyvale, CA). Rabbit and mouse antisera were obtained from Pel-Freez (Rogers, AR). Culture media were obtained from Invitrogen. Other chemicals were obtained from Sigma.

Cell Culture—Rat hippocampal H19-7/IGF-IR cells (ATCC, Manassas, VA) were plated onto poly-l-lysine-coated plates and grown at 35°C in DMEM/10% fetal calf serum for several days until ~50% coverage was obtained. The cells were then induced to differentiate into a neuronal phenotype by replacing the medium with 5 ml of N2 medium containing 10 ng/ml basic fibroblast growth factor at 39°C and grown in T-75 flasks at 37°C.

β-Amyloid Oligomerization—Human Aβ (AnaSpec 20276, 1 mg) was dissolved in 0.5 ml of deionized water by the addition of a minimum volume of 1% ammonium hydroxide and was incubated for 3 days at 37°C. The solution was then reduced in volume to 0.886 ml by lyophilization and incubated for an additional 48 h at 37°C. The oligomerized Aβ was then stored at ~80°C.

Characterization of Oligomerized Aβ—A 10-μl sample of Aβ was injected onto a Macrosphere GPC100 7-μm size-exclusion high performance liquid chromatography column (250 × 4.6-mm inner diameter) and eluted with 20 mM ammonium carbonate. Absorbance was monitored at 210 nm. The oligomerized Aβ used in each experiment was tested to ensure that a similar degree of oligomerization had occurred.

Synthetic Peptides—Aβ-binding peptides were synthesized by Genscript (Piscataway, NJ). All peptides were dissolved in phosphate-buffered saline at a concentration of 1 mM, except for peptide #3 (VSVGMLWC), which was dissolved at 0.1 mM. Peptides were then sterilized by filtration before use. One vial of Pro-Ject protein transduction reagent (Pierce) was then dissolved in 250 μl of CHCl₃. Four μl of Pro-Ject solution were transferred to 1.5-ml polypropylene centrifuge tubes and evaporated with nitrogen. Peptide was diluted in phosphate-buffered saline to 9.1 μg/ml, and 0.22 μg of peptide was added. The vial was incubated for 5 min, then vortexed, and the volume was brought to 0.5 ml with serum-free DMEM.

Cells were grown in 12-well plates containing 1 ml of N2 culture medium. When the cells reached 75–80% confluence, they were washed with serum-free DMEM, and peptide/Pro-Ject mixture dissolved in 0.5 ml of serum-free DMEM was added. After 4 h of incubation at 37°C, 0.5 ml of DMEM containing 20% fetal bovine serum was added. After 18 h, oligomerized Aβ was added. Cells were monitored daily. Under these conditions some cell death is visible within 48–72 h. The number of visibly apoptotic cells continued to increase for several more days. Therefore, we stopped the experiment after 7 days (6 days after adding Aβ). The medium was removed, and the cells were washed twice with 1× phosphate-buffered saline. A 100-μl aliquot of phosphate-buffered saline was added, and the cells were removed by gentle scraping. The cells were homogenized by sonication and stored at ~80°C.

β-Galactosidase—We tested for senescence using the hydrolysis by β-galactosidase at pH 6 of X-gal, a commonly used β-galactosidase substrate. Under these conditions, β-galactosidase is easily detectable in senescent cells but undetectable in quiescent, immortal, or tumor cells (51). To measure β-galactosidase, cell homogenate (20 μl) was incubated in 100 μl of 0.5 M Tris-HCl, pH 6.8, containing 0.1 mg/ml X-gal. After 24 h at 37°C, the samples were diluted to 1 ml, and absorbance at 610 nm was measured.

pBad—Bad (Bcl-x₁/Bcl-2-associated death promoter) is a member of the Bcl-2 family and regulates the survival signal (52). Unphosphorylated Bad dimerizes with Bcl-2 and Bcl-x₁, which neutralizes their anti-apoptotic activity. Activation of the phosphoinositol 3-kinase pathway ultimately leads to activation of Akt, which phosphorylates Bad on serine 136. Activation of mitogen-activate kinase pathways results in phosphorylation of Bad on serine 112. Phosphorylated Bad is sequestered from its proapoptotic role by binding with 14-3-3-3 protein (53). Thus, a decrease in Bad phosphorylation indicates apoptosis (54–60).

pBad was measured by densitometry of Western blots stained with phospho-Bad antibody. Image quantitation and molecular weight estimation were done on 16-bit images using the Unix-based image analysis program Imaq. The background value for each band was calculated by fuzzy k-means clustering analysis of the appropriate region of the image (61–64). Densitometry results are expressed as units of relative staining.
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Caspase-3 Assay—Members of the caspase family of cysteine aspartyl proteases are related to the Caenorhabditis elegans CED-3 death protein. Caspases-8, -9, and -10 are activated by receptor clustering and are known as “initiator caspases” (65). Caspases-3, -6, and -8 are activated by changes in mitochondrial permeability that are associated with apoptosis and are known as “effector caspases.” Effector caspase-3 proteolyses a number of substrates, including DNA fragmentation factor/inhibitor of caspase-activated deoxyribonuclease (DFF45/ICAD), poly(ADP-ribose) polymerase, gelsolin, and nuclear lamins (66). Proteolysis of DFF45/ICAD liberates the DNase subunit of DFF to cause chromatin degradation (67). Proteolysis of poly-(ADP-ribose) polymerase has been used by many researchers as a marker for apoptosis. We measured caspase-3 activity fluorometrically using the commercially available substrate Ac-Asp-Glu-Val-Asp-NH2-methylcoumarin, which contains the poly(ADP-ribose) cleavage site (68). Hydrolysis of this peptide yields a species that fluoresces at 440–460 nm.

To measure caspase-3, samples (20 μl) were incubated with 20 μl of caspase buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% CHAPS, and 15% sucrose) containing 3% dithiothreitol and 0.54 μg of Ac-DEVD-amidomethylcoumarin peptide in 1.5-ml polypropylene centrifuge tubes. The samples were incubated in a total volume of 40 μl for 1 h at 37 °C and diluted to 1 ml, and fluorescence was measured (ex = 354 nm, em = 442 nm) in a Spex Fluorolog 2 spectrofluorometer.

Phosphodiesterase Assay—Phosphodiesterase was measured by the method of Alvarez and Daniels (69) with slight modifications. Fines were removed from acidic alumina (Brockmann grade 1) by resuspending several times in water. Bio-Rad Poly-Prep columns were packed with a slurry containing 1.3 g of alumina to a height of 3 cm. Columns were precycled with 0.1 M ammonium acetate to remove any cAMP, then equilibrated with water. In a 0.5-ml polypropylene centrifuge tube, a volume equivalent to 0.15 ml of rat brain homogenate was diluted to 100 μl with water and 10 μl of buffer (1 mM Tris-HCl, pH 7.5, plus 2 mM MgSO4), and 0.5 μl of [3H]cAMP were added. After 10 min of incubation at 30 °C, the samples were boiled and cooled, and 0.1 μl of 5'-nucleotidase was added (Biomol, 500 kilounits/ml). The samples were incubated for 30 min at 30 °C and applied to the column. The adenosine was eluted with 4 ml of 5 mM HCl, mixed with scintillation fluid, and counted in a scintillation counter.

2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase) Assay—CNPase was measured using the coupled enzyme method of Weissbarth et al. (70). Samples (20 μl) were incubated for 20 min at 30 °C with 4 mM cyclic NADP, 2 mM d-glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, and 0.025% Triton X-100 in 0.2 M MES buffer, pH 6.0, in a total volume of 100 μl. The reaction was stopped by adding 2 ml of 50 mM NaCO3, pH 10.5, and the fluorescence of the resulting NADPH was measured (ex = 360, em = 460 nm).

Western Blots—Protein was measured using the Bradford dye binding technique (71). Samples were boiled in SDS sample buffer and loaded on a 4–20% polyacrylamide gel. Identical amounts of protein were applied to each gel. The samples were subjected to SDS-polyacrylamide gel electrophoresis, nitrocellulose blotting, and antibody staining using commercial antibody conjugates and alkaline phosphatase-conjugated secondary antibody as described previously (72).

Co-precipitation—One rabbit brain was homogenized by sonication in 3 volumes of 10 mM Tris-HCl, pH 7.4, containing 50 mM NaF and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 20 min at 100,000 × g. The supernatant was then re-centrifuged to produce the cytosolic fraction, and the original pellet was sonicated in the original volume of 10% N-lauryl sarcosine or 10% CHAPS and centrifuged for 20 min at 100,000 × g. The detergent extracts and cytosol were divided into 4-ml aliquots and incubated in 17 × 100-mm polypropylene tubes (Falcon 2059) with 10 μg of biotin-LC-β-amyloid-(1–42) (Anaspec) for 1 h at room temperature. Avidin-agarose (50 μl) was added, and the incubation was continued on an orbital shaker (800 rpm) for an additional 60 min. The mixture was cooled on ice, transferred to a Bio-Rad Poly-Prep column, and rapidly washed with 2 ml of ice-cold 10 mM Tris-HCl plus 100 mM NaCl using pressure. The co-precipitated proteins were eluted with 2 ml of 4 M NaCl and 2 ml of 0.2 M glycine-HCl, pH 2.2. The eluted fractions were concentrated and desalted in Centricon-3 ultrafiltration units, then mixed with SDS sample buffer for Western blotting. The non-eluted proteins were eluted from the agarose beads by boiling with SDS sample buffer. Samples were applied to a 4–20% SDS-polyacrylamide gel and stained with Coomassie Blue.

Phage Display Screening—Phage display screening was performed using a Ph.D. 7 peptide display system (New England Biolabs). Aβ-(1–42), Aβ-(1–20), Aβ-(25–35), or Aβ-(12–28) (37.5 μg) were coated onto an Immulon 4 HBX flat-bottom microtiter plate (Thermo Labsystems, Waltham, MA) and panned with 4 μl of phage display peptide library according to the manufacturer’s instructions. Bound proteins were eluted with 40 μl of 0.2 M glycine, pH 2.2, followed by 20 μl of Tris-HCl, pH 9, and 20 μl of Tris base. Four rounds of panning were performed on the plate. Because phage display screening using low M, peptides can be inefficient, we also performed a solution panning using mouse anti-Aβ-(18–30) antibody (Calbiochem 171587), which was determined to give the broadest reactivity for all forms of Aβ of the four antibodies we tested. Phage were purified using Ultralink protein A-agarose alternating with Ultralink Protein G-agarose to avoid purifying phage that had affinity for protein A or protein G. Three rounds of solution panning were performed.

Identifying Aβ Targets by Mass Spectrometry—One rabbit brain was sonicated in 3 volumes of buffer (10 mM Tris-HCl pH 7.4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.3 mM...
leupeptin, 0.125 mM pepstatin, and 5% CHAPS) and centrifuged at 100,000 × g. The supernatant was aliquoted into four 17 × 100-mm Falcon 2059 tubes and incubated for 1 h at room temperature in the presence of 10 μM CuCl₂ or 1 μM EDTA with 100 μg of fresh biotin-LC-β-amyloid-(1–42) or biotin-LC-β-amyloid-(1–42) that had been preincubated at 37 °C for 4 days. After incubation, 0.5 ml of avidin-agarose was added, and incubation was continued with shaking for 30 min. The sample was cooled to 4 °C, transferred to a disposable column, and rapidly washed with 4 ml of ice-cold homogenization buffer. The proteins with affinity for Aβ were then sequentially eluted with 2 ml of 0.5 M NaCl, 1.5 M NaCl, 4 M NaCl, 1 M Tris-HCl, pH 9, or 0.2 M glycine HCl, pH 2.2. All buffers contained 0.1% CHAPS plus 1 mM phenylmethylsulfonyl fluoride. All NaCl solutions also contained 10 mM Tris-HCl, pH 7.4. The eluates were concentrated and desalted in Centricron-3 ultrafiltration units. The proteins were then separated by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Mass Spectrometry—After excision, Coomassie-stained protein bands were destained by washing with acetonitrile/acetic acid/water (1:1:1), reduced, alkylated, and digested with 120 ng of trypsin using the in-gel method (73, 74). Digestion with trypsin was carried out overnight at 37 °C. Peptides were twice extracted from the gel into 50% acetonitrile, 5% formic acid. The extracts were pooled, the volume was reduced by vacuum centrifugation, and the final volume was brought up to 6 μl with 25 mM ammonium bicarbonate. The peptides from the tryptic digests were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Liquid chromatography was performed using an LC Packings UltiMate NanoLC at 250 nl/min using a PepMap C18 reverse-phase 100-Å pore column. Peptides were separated using a linear gradient from 95% A plus 5% B to 5% A plus 95% B (A = H₂O plus 0.1% formic acid; B = 80% acetonitrile plus 0.1% formic acid) over 38 min followed by 95% B for 17 min. The LC effluent was electrosprayed directly into the sampling orifice of an LTQ mass spectrometer (Thermo Finnigan) using a nanospray interface. The LTQ was operated to collect MS/MS spectra in a data-dependent manner with up to five of the most intense ions that exceeded a pre-set threshold being subjected to fragmentation and analysis. The MS/MS data generated were analyzed, and matches to protein sequences in the NCBI non-redundant nr data base (human/trypsin subset) were determined using SEQUEST (75) program.

Sequence identification was based on the cross-correlation normalized for peptide length (Xcorr‘) and δ correlation (ΔCn) scores. SEQUEST-derived peptide identifications and protein identifications were evaluated for statistical significance and filtered with the Peptide Prophet and Protein Prophet software tools (76). In each case the predicted Mᵢ and pI of the identification matched the Mᵢ and pI values on the gel within ±5%.

RESULTS

To identify the proteins that interact with Aβ, we performed phage display screenings using Aβ-(1–42), Aβ-(25–35), Aβ-(12–28), and Aβ-(1–20). To account for the possibility that the binding site of the peptides might be hindered by binding of the peptide to the polystyrene plate, we also performed a solution screening with all four peptides. The peptides were allowed to interact in solution with phage and then recovered with a specific Aβ antibody, and the phage was isolated using alternate pannings with protein A-agarose and protein G-agarose. A total of 160 clones were sequenced, yielding 61 different peptides. The peptides are shown in Table 1.

Although an unambiguously identified cannot be made from a seven-amino acid sequence, in most cases a mammalian-
A constrained BLAST search identified only a single protein or in some cases two candidate proteins as having a close match (Table 1). These ambiguities were resolved by co-precipitation studies (below). The peptides found by phage display contained sequences found in phosphodiesterase 3A and 3B and a number of proteins involved in cholesterol transport and metabolism (sortilin and steroid 5α-reductase type 2). The strongest binding (17 clones) was to a peptide found in the P2X2 purinergic receptor and the protein Suppressor of Ty 3 homolog (SUPT3H) (77).

To confirm the interactions between Aβ and the candidate proteins and to resolve the ambiguities in the phage display results, rabbit brain cytosol and brain N-lauroyl sarcosine extract or CHAPS extract were incubated with biotin-LC-β-amyloid-(1–42). Aβ-binding proteins were collected using avidin-agarose and analyzed by Western blotting. Because the protein interactions of some candidate proteins (such as 14-3-3) depend on Ca²⁺, the co-precipitation experiments were carried out in the presence and absence of added calcium. Because copper also binds to Aβ (78, 79), Cu²⁺ (10 μM) was also added to some samples. These co-precipitation experiments confirmed that Aβ interacts with the following proteins in rabbit brain extracts: C-reactive protein precursor, 14-3-3ε, 14-3-3γ, GDNF receptor, ERGIC-53, P2X2, ERGIC2, apolipoprotein B, and sortilin (Fig. 1; see Table 3). No co-precipitation could be found for phosphodiesterase 3A, phosphodiesterase 3B, CNPase, tubulin, SorLa, KIDINS-220, SUPT3H, or CD34. The interactions of Aβ with 14-3-3 and ERGIC-53 were calcium-dependent. The co-precipitation signal with C-reactive protein precursor was very weak, possibly because of the low levels of expression of C-reactive protein precursor in brain. Aβ co-precipitated with the P2X2 purinergic receptor but not with CD34 or SUPT3H, indicating that peptide YQDSAKT corresponded to P2X2. Similarly, co-precipitation experiments indicated that the peptide SVLDRQR corresponded to sortilin and not KIDINS-220 because Aβ co-precipitated only with sortilin (Fig. 1); however, the sortilin band was relatively weak, suggesting low levels of sortilin in the rabbit brain. No copper dependence was observed for any Aβ-binding protein.
The inability to detect tubulin by co-precipitation in either cytosol or N-lauroyl sarcosine extract despite the strong signal observed by mass spectrometry is not surprising since binding of Aβ may be specific to particular oligomeric or fibrillar forms of Aβ. Tubulin binding to Aβ (80) and carboxyl-terminal fragments of Aβ precursor protein (81) has been observed by previous researchers. Antibodies were not available for tumor differentially expressed protein or TMS membrane protein; thus, it was not possible to determine which of these two proteins bound Aβ. The proteins that were confirmed by co-precipitation to interact with Aβ are summarized in Table 2.

To identify Aβ-interacting proteins by mass spectrometry, biotin-LC-β-amyloid-(1–42) was incubated with CHAPS extract from rabbit or mouse brain, collected with avidin-agarose, washed, and eluted with increasing salt and buffer concentrations. In one experiment, fresh and oligomerized biotin-LC-β-amyloid were also compared. Because Cu²⁺ has been shown to bind to Aβ (78, 79), 10 μM CuCl₂ was also added to some samples. Rabbit brain was used because the sequence of rabbit is identical to that of humans. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis, eluted from the gel, and identified by liquid chromatography/ion trap tandem mass spectrometry. The proteins are shown in Table 3.

The predominant Aβ-binding proteins found by mass spectrometry were 14-3-3 and CNPase. Similar patterns were observed for rabbit and mouse, although more mouse proteins than rabbit proteins were identified, presumably because the rabbit NR data base is relatively incomplete. The addition of than rabbit proteins were identified, presumably because the rabbit protein (81) has been observed by previous researchers. Antibodies were not available for tumor differentially expressed protein or TMS membrane protein; thus, it was not possible to determine which of these two proteins bound Aβ. The proteins that were confirmed by co-precipitation to interact with Aβ are summarized in Table 2.

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Protection against β-Amyloid—To test whether the Aβ-binding peptides could protect against the toxic effects of Aβ, we applied eight synthetic peptides mixed with Pro-Ject, a cationic lipid protein transduction reagent, to cultured H19-7/IGF-IR cells. These cells are hippocampal neurons from Rattus norvegicus that have been immortalized by retroviral transduction of temperature-sensitive tsA58 SV40 large T antigen (84). After 18 h, oligomerized Aβ-(1–42) was added to the cells. The cells were collected after 7 days in culture and analyzed for markers of apoptosis (caspase-3 and pBad) and senescence (β-galactosidase). The peptide sequences are shown in Table 3.

The oligomerized Aβ, analyzed by size-exclusion high-performance liquid chromatography, consisted predominantly of oligomeric species between 10 and about 60 kDa (Fig. 4).

Phosphorylated Bad was significantly decreased in H19-7/IGF-IR cells after Aβ-(1–42) treatment (Fig. 5, upper), indicating early stages of apoptosis. Peptides #3 (VSVGMLWC, representing steroid 5α-reductase) and #4 (SVLDRQRC, representing sortilin-related receptor) prevented the decrease, with #3 almost completely blocking the decrease. Similar peptides, #3, #4, and #5 (LGYSKPSG, representing ERGIC2) also prevented the increase in caspase activity produced by

### Table 2

| Protein                        | Subcellular fraction       | Method              | Eluent        |
|-------------------------------|----------------------------|---------------------|---------------|
| C-reactive protein precursor  | Cytosol                    | Protein A-Sepharose | NA            |
| 14-3-3x                       | Membrane (NLS extract)     | Protein A-Sepharose | NA            |
| 14-3-3y                       | Membrane (NLS extract)     | Protein A-Sepharose | NA            |
| GDNF receptor                 | Membrane (NLS extract)     | Protein A-Sepharose | NA            |
| ERGIC2                        | Cytosol                    | Biotin-Aβ           | Glycine       |
| ERGIC-53                      | Cytosol                    | Biotin-Aβ           | Glycine       |
| Sortilin                      | Cytosol                    | Biotin-Aβ           | Non-eluted    |
| P2X2                          | Biotin-Aβ                  | Biotin-Aβ           | Non-eluted    |
| Apolipoprotein B              | Membrane (CHAPS extract)   | Biotin-Aβ           | Non-eluted    |
| Steroid 5α-reductase          | Membrane (CHAPS extract)   | Biotin-Aβ           | Non-eluted    |

### Table 3

| Fraction | Score | Accession no. | Identity |
|----------|-------|---------------|----------|
| Rabbit   | 4 M NaCl, pH 9 | 858 | 56757656 | Spectrin |
|          | 60    | 71153780     | 14-3-3x  |
| Non-eluted | 74   | 3915087      | Tubulin β1 |
|          | 60    | 399268       | CNPase   |
|          | 60    | 17378880     | Myelin basic protein |
|          | 108   | 32363170     | Histone H4 |
|          | 640   | 60389477     | Actin    |
| Mouse    | 0.5 M NaCl | 320 | 2506796 | Pyruvate kinase |
|          | 70    | 71153780     | 14-3-3x  |
|          | 60    | 55976637     | 14-3-3x  |
|          | 80    | 66773956     | Peroxiredoxin |
|          | 208   | 1709259      | Neurofilament triplet L protein |
|          | 140   | 5138761      | CNPase   |
|          | 110   | 117487       | C-reactive protein precursor |
|          | 306   | 1709947      | Pyruvate carboxylase |
|          | 230   | 48429133     | Syntaxin-binding protein |
|          | 150   | 5138761      | CNPase   |
|          | 90    | 112696       | 14-3-3x  |
|          | 50    | 55976637     | 14-3-3x  |
|          | 70    | 14917049     | Tubulin β1 |
Protection against β-Amyloid

Figure 3. Effect of Aβ peptides on CGMP-inhibited 3′-5′-cAMP-phosphodiesterase (EC 3.1.4.17) and CNPase (EC 3.1.4.37) activity. A, total 3′-5′ phosphodiesterase activity in rabbit brain homogenate. Production of [3H]adenosine from 3′-5′-[3H]cAMP in the presence of 5′-nucleotidase was measured in total rabbit brain homogenate over a period of 10 min at 30 °C. B, effect of Aβ (1–42) treatment (Fig. 5, middle), with peptides #3 and #5 being equally potent at blocking caspase activation. Peptides #4 and #5 also reduced the increase in β-galactosidase produced by Aβ-(1–42) treatment (Fig. 5, lower), indicating that they were partially protective against Aβ-induced senescence. In contrast, peptides #1 (YQDSAKTC, P2X2 purinergic receptor) and #2 (NDRGLLAC, TMS membrane protein) had either no effect or a slight worsening effect. The three peptides with the strongest beneficial effect (#3, #4, and #5) corresponded to sortilin, steroid 5α-reductase, and ERGIC2, Aβ-interacting proteins that are involved in cholesterol, low density lipoprotein, or ER membrane transport. Steroid 5α-reductase is involved in the production of neurosteroids.

Discussion

Identification of the functional motifs of a protein along with identification of its binding partners can give valuable clues about its biological role and, in the case of Aβ, can provide insight on ways of blocking its toxicity. We have shown that Aβ peptide binds to 14-3-3, ERGIC-53, ERGIC2, sortilin, P2X2, apoB, steroid reductase, and the C-reactive protein precursor. The interactions with 14-3-3 and sortilin are of particular relevance to Alzheimer disease. Sortilin expression has been shown to correlate inversely with AD neuropathology (85). Levels of LR11/SorLA, which is also the receptor for apolipoprotein E, are decreased in sporadic AD (86). Its apparent role is to target proteins in the Golgi for transport to late endosomes.

The 14-3-3 protein, which has many similarities to the Parkinson disease-associated protein α-synuclein (87), is found in neurofibrillary tangles (88), binds to tau, and is involved in phosphorylation of tau by glycogen synthase kinase 3β (89, 90). Glycogen synthase kinase 3β, which is the principal enzyme involved in phosphorylating tau, is regulated by 14-3-3 (91). Increases in 14-3-3 have been reported in patients with Alzheimer disease (92). Phosphorylation of Ser-9 in glycogen synthase kinase 3β (GSK-3β) promotes binding of GSK-3β to 14-3-3. Although Aβ is produced in the trans-Golgi and ER, it has also been found in the cytosol (26–29), where 14-3-3 is usually

Table 4
Sequences of Aβ-binding peptides tested in cultured cells for their ability to protect against Aβ

| Peptide | Aβ region | Sequence | Probable identity (BLAST) |
|---------|-----------|----------|--------------------------|
| #1      | 12–28     | YQDSAKTC | P2X2 purinergic receptor  |
| #2      | 12–28     | NDRGLLAC | TMS membrane protein      |
| #3      | 12–28     | VSVGMLWC | Steroid 5α-reductase      |
| #4      | 25–35     | SVLDRQRC | Sortilin-related receptor |
| #5      | 1–42      | LGSYRPSG | ERGIC2                    |
| #6      | 1–42      | IQLHPRLC | Ceramide kinase           |
| #7      | 1–42      | OATGLLAC | TD metalloprotein/PDE 3B  |
| #8      | 25–35     | SARTFLPC | GDNF receptor type α2     |

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Aβ-(1–42) in cultured PC12 cells (99). Because of its low molecular weight, Aβ is difficult to detect in SDS gels, and interaction of Aβ with itself was not seen in our experiments. The fact that Aβ was not seen in our phage display may mean that the affinity of Aβ for itself is lower than its affinity for the other proteins detected. This is consistent with our experience and the experience of previous researchers with Aβ, which requires a minimum of 48 h before oligomers form, even in a 250 μM solution of pure Aβ (cf. Fig. 4). It is also possible that, because our Aβ was already aggregated, it becomes less likely to bind to additional Aβ molecules.

Therefore, it is likely that the hydrophobic region of Aβ and the nearby protein kinase C binding region are the most critical regions of Aβ for both its effector action and for aggregation of Aβ. Peptides or other small molecules that bind to this region should protect against the toxic effects of Aβ by competitively blocking its ability to bind Aβ aggregate proteins such as sortilin. This was confirmed by our neuroprotection studies. Peptides #3 and #4, based on steroid reductase and sortilin, respectively, were the most effective at protecting cultured neurons from the apoptotic effects of Aβ.

Further experimentation is necessary to determine whether these or similar peptides, when administered intracerebroventricularly or delivered across the blood-brain barrier, can protect against Aβ in vivo. However, delivery to the brain may not be necessary for Aβ-binding peptides to be effective. Experiments and clinical tests with Aβ antibodies have shown that chelation of Aβ in the circulation is useful in principle as a therapeutic strategy against AD.

Research with a similar goal was carried out by Blanchard et al. (100, 101), who used a combinatorial library of hexapeptides containing random combinations of Ala, Ile, Val, Ser, Thr, and Gly, selected on the basis of their ability to complex with Aβ and to inhibit its fibrillogenesis. Additional peptides were engineered by adding prolines and amino- or carboxyl-terminal modifications. However, none of the peptides in either Blanchard et al. or Orner et al. (97) matched any proteins found in the present study. This would not be unexpected, considering that Blanchard et al. used rational peptide design rather than library screening to create their peptides. A similar approach was taken by Chalfour et al. (102), who used rational peptide design to create inhibitors of Aβ fibrillogenesis using D-amino acids.

Our work confirms the interactions between Aβ and tubulin, CNPase, and myelin basic protein, found by Verdier et al. (80), who studied synaptosomal proteins that co-precipitated with fibrillar Aβ. Differences between their results and ours may be
attributed to their focus on membrane-extracted proteins from the synapse. Because Aβ is created in the endoplasmic reticulum (Aβ-(1–42)), trans-Golgi network (Aβ-(1–40), and endocytic compartments (Aβ-(1–40) (24, 103), the results of Verdier et al. (80) may relate more specifically to the possible functions of Aβ in synaptic function or synaptic vesicle endocytosis.

Our evidence from mass spectrometry and phage display as well as that of Verdier et al. (80) indicate that some form of phosphodiesterase 3 or CNPase interacts with Aβ. However, in our purified system Aβ had only a minor effect on PDE activity, and co-precipitation experiments from brain cytosol or detergent extracts showed little evidence of a direct interaction. Therefore, it is possible that the interaction between Aβ and PDE is indirect. Phorbol ester-induced phosphorylation of PDE3A promotes binding to 14-3-3 (104). Phosphorylation of PDE3B by protein kinase A also promotes binding to 14-3-3 (105). Thus, the interaction of Aβ with phosphodiesterase and CNPase may be mediated by 14-3-3. Further investigation of the interactions between Aβ and 14-3-3 may shed additional light on the possible involvement of PDE and CNPase.

We have also confirmed that Aβ interacts with proteins involved in inflammation. C-reactive protein, a member of the pentraxin family, is an acute-phase protein normally found in plasma. However, C-reactive protein immunoreactivity is also detectable in temporal cortex of AD patients (106), more specifically in neurofibrillary tangles (107, 108). C-reactive protein mRNA is also detectable in pyramidal neurons, indicating that it is synthesized in the brain, and C-reactive protein is up-regulated in AD (109). Patients with the pathogenic apolipoprotein APOE4 allele have lower levels of C-reactive protein than normal patients (110).

Aβ also binds to the ERGIC marker protein ERGIC-53, which is involved in the calcium-dependent transport of glycoproteins such as APP from the ER to the Golgi intermediate compartment (111), where presenilin is located (112). Thus, ERGIC-53, like sortilin, may serve a transport function. Nicasrin, a glycoprotein component of the γ-secretase complex, interacts with ERGIC-53 (113). However, a role of ERGIC-53 in AD has not been established. ERGIC2 (originally named PTX1) is a similar transport protein originally found in prostate (114) and may also serve to transport APP or Aβ.

**Apolipoprotein B**—Apolipoprotein B, like ApoE, is a component of low density lipoproteins and is a suspected factor in atherosclerosis. Western blots, expressed as percent of control staining level. Middle, caspase-3 enzymatic activity. Lower, β-galactosidase activity. *, p < 0.05; **, p < 0.001.

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**FIGURE 5. Protection against apoptosis by Aβ-binding peptides.** The indicated concentrations of Aβ-(1–42) were applied to H19-7/IGF-IR neuronal cells. After 7 days the cells were collected, washed, and analyzed for pBad, caspase-3, and β-galactosidase. Upper, pBad levels measured by densitometry of Western blots, expressed as percent of control staining level. Middle, caspase-3 enzymatic activity. Lower, β-galactosidase activity. *, p < 0.05; **, p < 0.001.

**FIGURE 6. Binding regions of Aβ-interacting proteins.** The copper α-, β-, and γ-secretase cleavage sites are also shown.

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**Apolipoprotein B**—Apolipoprotein B, like ApoE, is a component of low density lipoproteins and is a suspected factor in athero-
osclerosis. ApoE4 is a genetic predisposing factor in AD. Serum apoB levels are increased in AD (115, 116), and although predominantly a serum protein, apoB is also found in hippocampus, where it is associated with hippocampal amyloid deposits and neurofibrillary tangles (117). Overexpression of apoB in mice increases APP expression in mice fed a high cholesterol diet (118).

P2X2—Purinergic receptors are up-regulated in AD. Recent studies have shown that caffeine and adenosine receptor antagonists prevent Aβ-induced cognitive deficits in mice (119) and reduce Aβ production (120). P2X2 has been shown to interact with Fe65 (121), an adaptor protein for APP that associates with tau in vivo (122).

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