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Proteins that bind to the RERMS region of β amyloid precursor protein

Monika Pawlik[¶,1], **Deborah A.C. Otero**[#], **Minkyu Park**[§], **Wolfgang H. Fischer**[§], **Efrat Levy**[¶,&], and **Tsunao Saitoh**[*,+]

¶ *Nathan Kline Institute, 140 Old Orangeburg Road, Orangeburg, NY 10962, USA*

 # University of California, San Diego, Center for Molecular Genetics, 9500 Gilman Drive, La Jolla, CA 92037, USA

§ Peptide Biology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

** University of California, San Diego, Department of Neurosciences, 9500 Gilman Drive, La Jolla, Ca 92093-0624, USA*

& New York University School of Medicine, Departments of Psychiatry and Pharmacology, New York, NY 10016, USA

Abstract

The main objective of this study was to investigate the biological function of β amyloid precursor protein (APP), in particular its nerve growth factor-like activity. We hypothesize that the extracellular domain containing the sequence RERMS, amino acids 328-332 of APP_{695}, represents the active site for this function. Binding assays using peptide fragments of this domain have demonstrated specific and saturable binding to the cell surface with affinity in the low nanomolar range. This induced our quest for an APP-specific receptor. We chose different peptide fragments of the RERMS domain as ligands and displacing agents on affinity columns to purify APP binding molecules. Amino acid microsequencing yielded partial sequences of serum albumin, actin, two novel proteins of 41 and 63 kDa and human Collapsin Response Mediator Protein-2 (hCRMP-2). Because both APP and hCRMP-2 promote neuronal outgrowth and use a common signaling pathway, APP could be acting through a semaphorin receptor as well.

Keywords

β Amyloid Precursor Protein (APP); Neurotropic Activity; Receptor Candidates; Lipid Rafts; Semaphorin Family

A major constituent of amyloid plaques and cerebrovascular deposits in brains of patients with Alzheimer's disease (AD) is the amyloid β (Aβ) protein. It derives from the processing of APP, a type I membrane protein. Four of the APP isoforms encoding 695, 717, 751 and 770 amino acids, contain the Aβ protein. Several processing pathways for APP have been described. Cleavage at position 597 of APP_{695} by β-secretase results in the release of amino-terminal soluble APP, sAPPβ. The carboxyl-terminal fragment may undergo an additional cleavage by γ secretase leading to the generation of Aβ. Alternatively, processing by an α-secretase between

To whom correspondence should be addressed[1] Email address: MPawlik@nki.rfmh.org, fax: 845 398 5422
[+]In memory of

positions 612 and 613 of APP_{695} (16 and 17 of Aβ) precludes the release of intact Aβ and results in the release of sAPPα (for review: [1]).

Alteration of APP processing and overproduction of Aβ is often suggested as a primary event in the pathogenesis of AD ([2], [1]). However, it is also possible that the disrupted molecular function of APP and its connected signal transduction pathways contribute to or initiate the pathological process. A variety of biological functions were attributed to the extracellular, N-terminal region of APP. Activities specific for the longer isoforms, APP_{751} and APP_{770}, which contain a Kunitz type serine protease inhibitor (KPI) domain, include regulation of neurite extension, blood coagulation and wound-healing (for review: [2]). The APP_{695} isoform, predominantly expressed in the brain and utilized in this study, lacks the KPI domain, and therefore cannot be assumed to function as a protease inhibitor. Growth regulation and modulation of neuronal excitability, synaptic plasticity, and cell survival were suggested to be KPI independent functions of sAPP (for review: [3]). Neuron excitability has been proven sAPPα specific.

The growth-regulating activity of APP in fibroblast was localized to the N-terminal part of sAPP containing the five amino acids sequence RERMS ([4], [5]). The same sequence induced neurite outgrowth in B103 cells, a neuronal cell line from rat central nervous system ([6]). RERMS-containing APP peptides also promoted neuronal survival in primary rat cortical cells ([7]). One of the peptides, a 17 mer containing RERMS (see table 1), was tested for its ability to protect against neuronal loss in a rabbit spinal cord model for ischemia. This peptide increased significantly the ischemia duration required to produce paraplegia ([8]). Infusion of the same peptide into the intraventricular compartment of elderly rats, which had been trained in a Morris water maze, resulted in increased memory retention to more than double along with an 18% increase of synaptic density in the frontoparietal cortex ([9]).

These results indicate a biological function for the RERMS region of APP. Specific binding of the 17 mer peptide to the surface of B103 cells has been demonstrated with a predicted K_D of 20+/− 5 nM. The binding is saturable, reversible and has a B_{Max} value of 80 +/− 8 fmol / 10^6 cells. We proposed that the peptide binds to membrane receptors, which mediate the neurotropic activity of APP ([5], [6]). This study aimed to identify the proteins that bind to the RERMS domain of APP and might be receptor candidates. Using three different approaches, we identified five binding proteins including two novel proteins of 41 and 63 kDa.

MATERIALS AND METHODS

Pig cortex was kindly provided by Dr. Gamagami, General Surgery Office of the Veterans Hospital, San Diego. Human cortical tissue from patients with moderate to severe AD was kindly provided by Lawrence Hansen, the Alzheimer's disease Research Center at the University of California, San Diego. All peptides were prepared as described in Roch et al., 1992 [10]. ^{125}I-labeling was performed using Bolton-Hunter reagent as described in Ninomiya et al., 1994 [5]. All purification procedures except for the extractions were done at 4°C.

Protocol 1

Pig brain was homogenized in Buffer A (10 mM Tris HCl pH 8.0 containing 0.32 M sucrose, 3 mM EGTA, 0.5 mM $MgSO_4$, 1 mM EDTA and 5 mM Benzamidine) and briefly centrifuged at 1,000g. Supernatants were centrifuged for 50 minutes at 14,000g. The pellets were homogenized and extracted at room temperature with 1% Triton X-100 in Buffer B (10 mM Tris HCl pH 8.0 containing 3 mM EGTA, 0.5 mM Mg SO_4, 1 mM EDTA) and centrifuged for 60 minutes at 100,000g. Extracts were loaded onto a column of CN-activated 4B Sepharose (Pharmacia, St. Louis, MO) containing 13 μM sAPPα. sAPPα was expressed as a His-tagged fusion protein and purified by affinity chromatography using a Nickel column as described in

Li et al., 1997 [11] except for omission of Imidazole in equilibrating the column. Molecular weight and purity of sAPPα were verified by electrophoresis on a 4–12% Tris Glycine Acrylamide Gel (Novex/Invitrogen, Carlsbad, CA) and subsequent Western blotting with anti-APP monoclonal antibodies 22C11 (Boehringer Mannheim, Indianapolis, IN) and 6E10 (kindly provided by Dr. K.S. Kim and Dr. H.M. Wisniewski, now available from Signet, Dedham, MA). The blots were labeled with ^{125}I-protein A and exposed to autoradiographic film. While both antibodies recognize the full-length APP, 22C11 recognizes sAPPα and sAPPβ and 6E10 recognizes only sAPPα. sAPPα was quantified by densitometry (LKB UltraScan XL, Pharmacia, St. Louis, MO). It was measured with Coomassie blue stain against bovine serum albumin as standard.

Nonspecific binding was eluted with 0.8 mM scrambled 17 mer, prior to specific elution with 0.8 mM 17 mer (Table 1). The resulting eluates were monitored by spectrophotometer at OD_{280}. Protein-containing fractions were concentrated by ultrafiltration on Centriprep 10 columns (Pierce, Rockford, Ill), separated on a 10% Tris Glycine Acrylamide Gel and blotted onto 0.2 μm PVDF membrane (Biorad Laboratories, Hercules, CA). Proteins exclusively eluted with 17 mer peptide were sequenced without any further treatment by Edman degradation as described by Fischer et al. 1991 [12].

Protocol 2

Human brain tissue was homogenized in Buffer A and briefly centrifuged at 1,000g. Supernatants were centrifuged for one hour at 100,000g. The protein contents of the supernatants were estimated by measurement at OD_{280}. A so prepared crude membrane fraction of 20 mg protein was sonicated in Buffer B and extracted with 3% of the anionic detergent cholate in Buffer B. After centrifugation at 100,000g the supernatant was diluted with Buffer B to 0.8% cholate and loaded on a SulfoLink column (Pierce, Rockford, Ill). The specific ligand on the column was 1 mM 22 mer peptide (Table 1). After incubation for one hour at 4°C bound proteins were eluted with 10 μM 16 mer (Table 1). The eluates were concentrated, separated on 4–12% Tris Glycine Acrylamide gel (Novex/ Invitrogen, Carlsbad, CA) and blotted for sequence analysis as described above.

Protocol 3

A crude membrane fraction of human brain tissue was prepared as described in protocol 2 and extracted with 0.64% non-ionic detergent Igepal CA-630 (Sigma-Aldrich, St. Louis, MO) in Buffer B. After centrifugation for two hours at 28,000g the supernatant was loaded on a 60 ml DE52 column (Whatman Inc., Florham Park, NJ) in 20 mM Tris HCl pH 8.0 with 3 mM EGTA, 0.5 mM Mg SO_4, 1 mM EDTA and 0.05% Igepal (critical micelle concentration = 0.005%) and eluted by NaCl gradient.

Aliquots were checked for binding by three methods:

1. Crosslinking followed by autoradiography: Aliquots of 10 μl containing between 10 and 40 μg protein were incubated with 35 nM ^{125}I-labeled 16 mer peptide (Table 1), 0.5 mM crosslinking reagent disuccinimidyl suberate (DSS) in phosphate buffered saline pH 7.4 with 0.15% gelatin, 0.2 mM Mg SO_4 and 0.55% DMSO for 30 minutes at room temperature. After electrophoresis in 4–12% Tris Glycine Acrylamide and dehydration with 40% Methanol and 5% Glycerol, the gel was exposed to autoradiography film.
2. Centrifugation followed by scintillation counting: Aliquots of 50 μg total protein were incubated with 35 nM ^{125}I-labeled 16 mer peptide (Table 1), 0.5 mM DSS in phosphate buffered saline pH 7.4 with 0.15% gelatin, 0.2 mM $MgCl_2$ and 0.55%

DMSO for 30 minutes at room temperature, centrifuged for one hour at 100,000g and measured by liquid scintillation counting.

3. Binding of labeled peptides to a gel followed by phospoimaging ("geloverlay"): An aliquot of a peak fraction containing 0.1 μg protein was separated on 4–12% Tris Glycine Acrylamide. The gel was incubated for one hour with 31 nM ^{125}I-labeled 16 mer peptide (Table 1) in Buffer B containing 0.15% gelatin, washed twice with phosphate buffered saline pH 7.4, once with water, dehydrated and the bands measured in Phosphoimager Trinitron (Molecular Dynamics, Sunnyvale, CA).

Fractions showing binding activity according to the various protocols were dialyzed and loaded onto a SulfoLink column (Pierce, Rockford, Ill) with 2.4 mM 21 mer peptide (Table 1). The column was incubated with 3.4 mM 16 mer (Table 1) for 17 hours. The eluates were concentrated, separated on 4–12% Tris Glycine Acrylamide and blotted for sequence analysis. Flow through fractions as well as fractions from the NaCl elution of the affinity column were processed in parallel to control for nonspecific bands.

RESULTS

Three different protocols (Table 2) were used in order to identify brain proteins, which bind to the RERMS sequence within the N-terminal domain of sAPP. In protocol 1 membrane proteins derived from Triton extraction were selected for specific binding to sAPPα and to 17 mer peptide by affinity chromatography. In protocol 2 a crude membrane fraction was extracted by cholate, because cholate was found in preliminary experiments to result in fractions with high affinity for the 16 mer and 17 mer peptides. This extract was submitted to affinity chromatography to select for proteins that bind to the 22 mer peptide and with even higher affinity to the 16 mer. In protocol 3 a crude membrane fraction was extracted with Igepal, another non-ionic detergent, and then fractionated according to ionic strength by exchange chromatography. Specific binding for the 16 mer was checked by three different methods using autoradiography, liquid scintillation counting and phosphoimaging. The most promising fractions were combined and once more selected for specific binding to the 21 mer and 16 mer peptides. Thus, in all three protocols the fractions were screened two- and threefold respectively for specific binding before they were submitted to sequence analysis.

Isolation of a 220 kDa Protein

Protocol 1, the Triton based isolation procedure, utilized pig brain and thus allowed the shortest possible postmortem time. The binding experiments revealed proteins of approximately 36 kDa and 40 kDa and a doublet at 220 kDa to be highly specific to 17 mer. The protein of approximately 45 kDa was strongly visible with the 17 mer elution but in traces apparent in the elution with scrambled 17 mer as well (Fig 1). The ~ 36 kDa protein was identified as mitochondrial Malate Dehydrogenase which is a frequent contaminant in membrane preparations. Sequence analyses of two of the other proteins were unsuccessful most likely because of N-terminal blockage. The stronger upper band of the doublet at 220kDa contained estimated 1 μg of protein not enough for sequence analysis at such a high molecular weight.

Cholate Extraction of a Novel APP Binding 41 kDa Molecule

Protocol 2, based on the ionic extraction of human brain proteins, yielded two strong bands using only one hundredth the amount of material needed in protocol 1. Sequencing of the 67 kDa band showed serum albumin. Sequence analysis of the 41 kDa band revealed the N-terminal sequence Pro-X-Gln-Tyr-Pro-Ala-Leu-X-Ser-X-Gln-Glu-Leu-Asn. This sequence could not be matched to any known protein. Using 50 nM ^{125}I-16 mer peptide as ligand (Table 1), we have observed that the APP binding fraction of the cholate extracts floats after 8 hours

centrifugation at 485,000g in the upper third of a 5–20% sucrose gradient. This suggests that the 41 kDa protein is a raft protein.

Identification of Actin, CRMP-2 and a Novel 63 kDa Molecule

Protocol 3, the non-ionic Igepal extraction from human brain, provided three proteins. The specific elution of the affinity column resulted in a main peak and a trailing shoulder peak containing roughly 1/10 as much protein as the main peak. Those fractions had been sequenced separately. Both peaks contained protein of apparent molecular weight of 63 kDa. The protein from the main peak was digested with trypsin and the fragments were separated by reverse-phase HPLC before sequencing. The resulting sequence of Gly-Thr-Val-Val-X-Tyr-Gly-Glu-Pro-Ile-Thr-Ala-Ser-Leu-Gly matched human Collapsin Response Mediator Protein-2 (hCRMP-2). N-terminal sequencing of the other band at 63 kDa from the “shoulder” provided the sequence: Pro-Ile-Leu-Ala-Glu-Val-Thr-Val-Asn-Gly-X-Asp with no homology to any known protein. In addition, the main peak contained a protein of 40 kDa, which according to the seven N-terminally sequenced amino acids is Actin.

DISCUSSION

We used three different peptide fragments containing the RERMS domain within sAPP as ligands and displacing agents on affinity columns in order to identify brain proteins that bind to this sequence. This approach yielded five proteins, and the high binding specificity makes it likely that all of them are involved in the biological function of the RERMS region. Three of them may provide leads towards the purification of an APP specific receptor. While sequence analysis revealed some bands corresponding to known proteins, namely serum albumin, actin, and CRMP-2, two turned out to be novel proteins. In addition, a band of 220 kDa was identified following isolation protocol 1. Its binding specificity to APP was assured by using three steps of selection: binding to sAPPα and to the 17 mer RERMS fragment and its lack of binding to a 17 mer scrambled peptide. While we were not able to sequence this protein, its size suggests that the band represents an APP-receptor complex.

Serum albumin has been previously reported as APP binding protein and the binding site was suggested to be within the Aβ region ([13]). Our results reveal binding of serum albumin to the N-terminal RERMS region of APP.

The novel protein of 41 kDa is not a typical receptor candidate because it was extracted from the membrane fraction with an ionic detergent. Its identification from a much smaller amount of material than three of the other proteins, suggests high affinity to the ligands. It could be a soluble factor interacting with the membrane or it could derive from a lipid raft. Lipid rafts, formerly often referred to as caveolae-like membranes, are biochemically defined by their relative insolubility at low temperature in non-ionic detergents. They represent functionally distinct membrane microdomains, which are thought to be involved in signal transduction and proteolytic processing. It was suggested that a portion of APP might be present in caveolae-like membranes ([14]) and furthermore that it may bind to a raft-associated specific sAPP receptor ([15]).

CRMP-2, which has been independently identified as Ulip2, CRMP-62, TOAD-64, and DRP-2, belongs to a new protein family of at least five isoforms. Members of this protein family are involved in axonal outgrowth in response to Collapsin/ Sema3A, a member of the semaphorin protein family (for review: [16], [17]). Sema3A binding to its receptor neuropilin-1 leads ultimately to growth cone collapse, which is crucial for the formation of a normal pattern of innervation in developing neurons. CRMP-2 has also been reported by Yoshida et al. 1998 [18] to be associated with neurofibrillary tangles (NFT), another pathological hallmark of AD. It was identified through immunoaffinity purification specific for one fraction of paired helical

filaments, the main structural components of NFTs. Yoshida et al. 1998 [18] hypothesized that incorporation of CRMP-2 into the tangles may deplete neurons of necessary CRMP, lead to abnormal neuritic and/or axonal outgrowth and thereby accelerate neurodegeneration. The high affinity binding of CRMP-2 to APP demonstrated here suggests a functional connection of the two as cause for the trapping of CRMP-2 within the tangles. Because the growth cone collapse is for the most part carried out by a localized rearrangement of the actin cytoskeleton, it is interesting that we identified a molecule, which is probably actin, as binding partner to the neurotrophic region of APP. Actin has already been reported to colocalize with APP in neuroglioma cells by Sabo et al. 2001 [19] who used Rac1, a small GTP-binding protein of the Rho family, as their marker for the mobile lamellipodia, which contain APP and actin as well as Fe65. Rac1 is the same signaling molecule most likely involved in semaphorin-induced rearrangement of the actin cytoskeleton ([16], [17]).

Previously, various other strategies resulted in the proposal of a number of receptors or binding molecules for different regions within APP. Two of them, RAGE and LRP, had been deduced through structural similarities between their known ligands and APP. RAGE, a receptor for advanced glycation end products ([20]) mediates neurotrophic as well as neurotoxic responses. However, binding of synthetic Aβ to RAGE induces cellular oxidative stress, while neurotrophic responses are attributed to different ligands. LRP, the low-density lipoprotein receptor-related protein, binds the KPI domain of APP and can internalize the KPI-containing forms of APP ([21]). Several other molecules bind to the non-secreted cytoplasmic domain of APP (for review and a more comprehensive list see [3]). We have previously investigated signal transduction systems associated with the neurotrophic activity of sAPP located in the RERMS region. Our results suggested an involvement of phosphoinositide breakdown ([6]). Other studies (for review: [22]) suggested that excitoprotective as well as neurotrophic activity of sAPP is transduced by cyclic GMP (which agrees with unpublished results from binding experiments in our lab), followed by activation of potassium channels and reduction of calcium ion concentrations. These effects have been localized to amino acid positions 444-592 of secreted APP_{695}. The phosphatidylinositol-3-kinase (PI_3K)-Akt kinase pathway and a PI_3K-independent pathway for p42/ p44 mitogen-activated protein (MAP) kinases have been proposed to mediate neurotrophic and excitoprotective activity of sAPPα, but were not specifically localized ([23]). Only p42 MAP kinase activity had been previously localized to the cysteine-rich amino-terminal end of sAPP ([24]). Phospholipase D_2 , an enzyme involved in signal transduction of cell responses, can be stimulated by APP and inhibited by CRMP-2 in neuronal cells ([25], [26]). Phospholipase D_2 catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid and choline in response to various signals, including growth factors. Phospholipase D_2 appears to be associated with the plasma membrane, may be present in caveolae, and may co-localize with the actin cytoskeleton (for review: [27]), all of which makes it a promising link in a signaling system for the proposed receptor for APP.

In conclusion, we hypothesized that the RERMS region of APP transmits its neurotrophic effect through an APP specific receptor. We used three different peptide fragments of the RERMS domain as ligands and displacing agents on affinity columns in order to identify specifically binding molecules. This approach yielded three important leads towards the purification of an APP specific receptor: a putative APP receptor/receptor complex of 220 kDa; a novel APP binding molecule of 41 kDa that is either a soluble ligand for the receptor or a component of a lipid raft; and an APP binding protein of 63 kDa identified as hCRMP-2, a mediator for the semaphorin receptor complex involved in growth regulation. The high affinity between CRMP and sAPP suggests functional interaction of APP with the semaphorin receptor complex or very similar characteristics for the specific APP receptor to a semaphorin receptor.

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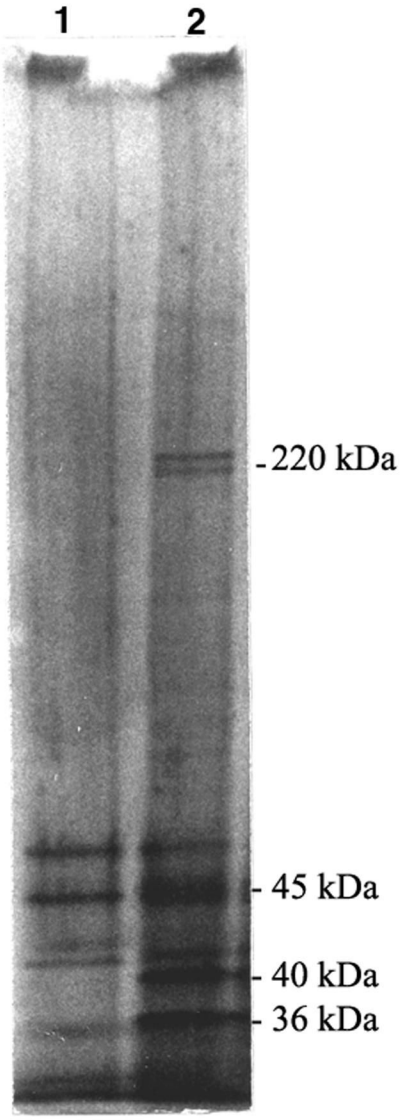

Fig 1.

Eluates of sAPPα column with scrambled (1) and normal (2) 17 mer on 4-20% Tris Glycine gel, stained with SilverStain Plus (Biorad Laboratories, Hercules, CA). The aliquots represent about 1/100 of the combined fraction.

Table 1

Peptides used for the purification of APP-binding proteins. N-terminal Cys was added to facilitate coupling to the column.

16 mer	APP317-332	Gln-Lys-Ala-Lys-Glu-Arg-Leu-Glu-Ala-Lys-His-Arg-Glu-Arg-Met-Ser
17 mer	APP319-335	Ala-Lys-Glu-Arg-Leu-Glu-Ala-Lys-His-Arg-Glu-Arg-Met-Ser-Gln-Val-Met
scrambled 17 mer	Same amino acids as in APP319-335 in random sequence	Lys-Ala-Ala-Arg-Met-Arg-Val-Met-Glu-Gln-Arg-Glu-Lys-His-Leu-Ser-Glu
22 mer	APP312 –332, with N-terminal Cys	Cys-Glu-His-Ala-His-Phe-Gln-Lys-Ala-Lys-Glu-Arg-Leu-Glu-Ala-Lys-His-Arg-Glu-Arg-Met-Ser
21mer	APP312 -332 without Phe316, with N-terminal Cys	Cys-Glu-His-Ala-His-Gln-Lys-Ala-Lys-Glu-Arg-Leu-Glu-Ala-Lys-His-Arg-Glu-Arg-Met-Ser

Table 2

Comparison of the three methods used for the purification of APP-binding proteins.

Protocol	Source	Main Purification Steps	Identified Proteins
1	70 g pig brain	1% Triton extraction, sAPPα column eluted with 17 mer for 1 hour	Proteins of 40, 52, 56 and 220 kDa
2	0.6 g human brain	3% Cholate extraction, 22mer Sulfolink column eluted with 10μM 16mer for 1 hour	Serum Albumin 41 kDa Novel Protein
3	120 g human brain	0.64% Igepal extraction, 21 mer Sulfolink column eluted with 3.4 mM 16 mer for 17 hours	hCRMP2 Actin 63 kDa Novel Protein