Regulation of Amyloid Protein Precursor (APP) Binding to Collagen and Mapping of the Binding Sites on APP and Collagen Type I*

(Received for publication, September 7, 1995, and in revised form, November 2, 1995)

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The specific binding of the amyloid precursor protein (APP) to extracellular matrix molecules suggests that APP regulates cell interactions and has a function as a cell adhesion molecule and/or substrate adhesion molecule. On the molecular level APP has binding sites for collagen, laminin, and glycosaminoglycans which is a characteristic feature of cell adhesion molecules. We have examined the interactions between the APP and collagen types I and IV and identified the corresponding binding sites on APP and collagen type I.

We show that APP bound most efficiently to collagen type I in a concentration-dependent and specific manner in the native and heat-denatured states, suggesting an involvement of a contiguous binding site on collagen. This binding site was identified on the cyanogen bromide fragment αI(1)CB6 of collagen type I, which also binds heparin. APP did not bind to collagen type I-heparin complexes, which suggests that there are overlapping binding sites for heparin and APP on collagen. We localized the site of APP that mediates collagen binding within residues 448–465 of APP exon 12, which are encoded by the ubiquitously expressed APP exon 12, whereas the high affinity heparin binding site of APP is located in exon 9. Since a peptide encompassing this region binds to collagen type I and inhibits APP-collagen type I binding in nanomolar concentrations, this region may comprise the major part of the collagen type I binding site of APP. Moreover, our data also indicate that the collagen binding site is involved in APP-APP interaction that can be modulated by Zn(II) and heparin. Taken together, the data suggest that the regulation of APP binding to collagen type I by heparin occurs through the competitive binding of heparin and APP to collagen.

The amyloid precursor protein (APP) belongs to a gene family in which three genes are known. In addition to the APP gene, the genes for the amyloid precursor-related proteins APLP1 and APLP2 map to the human chromosomes 19 (APLP1) (1) and 11 (APLP2) (2, 3). APP is encoded by the APP gene on the long arm of human chromosome 21 and has attracted attention due to its involvement in the deposition of amyloid β44 protein in the brain of patients with familial and sporadic Alzheimer’s disease and individuals with trisomy 21 (4, 5).

The role of APP in the pathogenesis of Alzheimer’s disease has been underscored by the discovery of mutations within the βA4 or sequences flanking the βA4 (6–8). All of the identified susceptibility genes linked to Alzheimer’s disease appear to influence the βA4 amyloid formation (9–11).

From the high degree of evolutionary conservation of the endo- and ectodomain of APP and its widespread tissue expression, APP has been expected to be implicated in a variety of cellular processes and events. Secreted isoforms of APP (APPS) containing a region homologous to the Kunitz protease inhibitor consensus sequence have a role in regulation of extracellular protease activity (12) and are endocytosed by the low density lipoprotein receptor-related protein (13). A possible in vivo function is provided by the discovery that APP is a very potent inhibitor of factor XIa, and APP-factor XIa complexes might be involved in the regulation of the coagulation cascade (14, 15).

Another possible function of the ectodomain of secreted or membrane-associated forms of APP have also been shown to be involved in neuronal-cell or cell-matrix interactions and cell growth regulation (16–19). Here, the ability of APP to stimulate cell adhesion and growth does not depend on the Kunitz protease inhibitor domain but may derive from its high affinities for heparin, heparan sulfate proteoglycans, laminin, and collagen type IV (19–26).

APP has been shown to bind Zn(II) and Cu(II) at two distinct sites (27, 28) and was recently found to belong to a family of zinc-modulated, heparin-binding proteins (29). Zn(II) binding was shown to strengthen the binding of APP to heparin, thus demonstrating an interaction of residues involved in ligand binding which are located in different domains (21, 27). Binding of metallic cations like Zn(II) and Cu(II) may control APP conformation and stability (27, 28, 30) and thus may promote the binding of APP to extracellular matrix elements like heparan sulfate proteoglycans (30).

We report here the regulation of APP binding to collagen type I by heparin and the mapping of the binding sites for APP to collagen type I and vice versa. Our binding studies reveal that APP binding to collagen type I is mediated by residues 448–465. Since synthetic peptides representing this region show self-aggregational properties, it is suggested that APP-binding may exist. APP was identified in the human platelet to be present in membrane associated and soluble forms (31), and collagen is particularly important for initiation of platelet activation leading to successful formation of a hemostatic plug (32). For this reason our findings suggest that APP as an adhesive glycoprotein may participate in collagen-induced platelet aggregation and as APPs may take
part in the regulation of the coagulation cascade at sites of vascular injury.

EXPERIMENTAL PROCEDURES

Materials—Collagen type I from bovine and human tissues (Sigma C-3511, C-7774) and collagen type IV (human placenta, Sigma C-7521) were used.

Different collagen types were dissolved in 10 mM NaOAc, pH 5.5, and with diluted with 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, to appropriate concentrations just before use.

Protein concentrations were determined by amino acid analysis.

Collagen type I was dissolved at 10 mg/ml in 70% formic acid, and solid CNBr (Merck) was added to a final concentration of 20 mg/ml. Digestion was allowed to proceed for 18 h at 4 °C.

Peptides were synthesized according to published methods (33) and purified on C-18 columns with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Sequences were confirmed by amino acid sequence analysis (477A, Applied Biosystems).

Construction of Expression Vectors—The plasmid pFd770IgA is based on the procaryotic expression vector pFd770IgA (28). For in vitro mutagenesis according to Kunkel (34), M13mp18 replicative form DNA was cut with Sall-HindIII and religated in the presence of dilugonucleotides TCCGACCTGACTGTGCA and AGGTGCGAATGTCAGG to create a Sall restriction site. A Sad-Spel fragment of pFd770IgA was cloned into M13mp18-Spel, and the dilugonucleotide CCCCACATCTTCTCGAACATCTATTATTTTGTGTGACAA was annealed to the single-stranded DNA. The mutated sequence was exchanged with the corresponding fragment of pFd770IgA (28) to construct the plasmid pFd770IgA.

Purification of APP—APP695 was isolated from rat brain as described previously (21).

A full-length (Fd-APP770) and truncated recombinant forms of human APP (Fd-APP23, TP-APP770) were prepared and purified in the form of a prokaryotic expressed Fd fusion protein by methods essentially as described by Weidemann et al. (35). TP-APP770 represents the secreted form of APP (in-secretase cleaved) (9).

After preparative SDS-PAGE and electrophoresis, the soluble protein was separated from salts and SDS by Excellulose GF-5 columns (Pierce).

Protein concentrations were determined by amino acid analysis according to the manufacturer's protocol after hydrolysis with 6 N HCl, 0.1% phenol for 24 h at 110°C (420A Amino Acid Analysis System, Applied Biosystems).

Binding Assay of Type I Collagen—The binding of proteins to collagen was determined by either dot blot or a solid-phase assay. The dot blot assay was performed as described previously for APP-heparin binding (21). The inhibitory effect of heparin was studied in the dot blot assay after preincubation of 3.5 pmol of 125I-rn-APP with 125 μg/ml heparin or 10 μg/ml heparin for 4 h at room temperature and separating the protein from salts and free heparin by Excellulose GF-5 columns (Pierce) and preincubation of dots coated with collagen in the presence of 40 μg/ml heparin in 1× PBS for 1 h.

After adsorption of collagen to microtiter wells, nonspecific binding sites were blocked by incubation for longer than 1 h with 1% BSA in PBS. 125I-labeled proteins were diluted with PBS and added to the wells (1×10^3 to 2×10^6 cpm/well), together with the reagents to be tested. After a 3-h incubation at room temperature, the wells were emptied. The plate was divided into separate wells, and the radioactivity of bound ligand was measured by liquid scintillation counting.

The value obtained with wells coated with BSA was subtracted, as this represents nonspecific binding. Results were expressed as the mean of duplicate determinations, which usually did not differ by more than 10%.

 Determination of the Collagen Binding Domain of APP—Purified rat brain APP695 and fusion proteins Fd-APP770 and Fd-APP23 were incubated at 37°C overnight with endoprotease Lys-C (5 μg) (Boehringer Mannheim). After inactivating the protease for 1 min at 110°C, fragments were isolated in PBS overnight at room temperature with collagen type I bound to microtiter wells. After being washed three times with cold PBS, bound peptides were extracted with formic acid. High performance liquid chromatography revealed one major fragment (16 kDa) in the eluate. The NH2-terminal sequence was AAQIR-SQVMTHRLVYERMNQSLSLLYNVPAVA, corresponding to residues 448–480 of APP695. Similar digestion of 125I-rn-APP generated the same sized fragment as visualized by SDS-PAGE and autoradiography.

Synthetic peptides representing candidates for the collagen binding region on APP and control peptides were dissolved in 0.1× PBS and tested as competing ligands for 125I-APP-collagen type I (human placenta) binding. Collagen type I was dot-blotted in duplicate onto nitrocellulose (0.05–10 μg) and nonspecific binding sites were blocked with 1% BSA in 1× PBS for 1 h at room temperature. The dot blot was cut into strips and incubated with 125I-APP together with and without competing peptides for 3 h at room temperature in 0.1× PBS. After incubation the dot blot was washed one time with blocking buffer, and the dots were excised, placed in scintillator, and assayed by counting.

Variations of this binding assay were occasionally used and are indicated in the text and figure legends.

Determination of the APP Binding Domain of Collagen—In order to separate chains (1) and (2) of collagen type I, 2–3 mg of protein was dissolved in hypotonic buffer (14 mM NaCl, 0.27 mM KCl, 0.81 mM Na2HPO4, 0.15 mM KH2PO4) and subjected to liquid phase-high performance liquid chromatography (Aquapore RP-300, buffer A: 0.1% trifluoroacetic acid, buffer B: 0.05% trifluoroacetic acid in 70% CH3CN, gradient from 0% buffer B to 100% buffer B in 60 min).

CNBr cleavage of isolated chains was performed as described (36). The identity of the (1)IC6 fragment has been confirmed by NH2-terminal sequencing.

APP Binding to the Synthetic Peptide CBP (Residues 448–480) A synthetic peptide corresponding to residues 448–480 of APP695 was adsorbed to microtiter wells (0.5–5 μg/well) and incubated with 1% BSA in PBS for 1 h, 125I-labeled hs-APP was added (1×10^5 to 2×10^6 cpm/well) together with the competitors to be tested in PBS. After a 3-h incubation at room temperature the wells were emptied, dried, cut, and subjected to liquid scintillation counting. The mean of duplicate measurements is given which was corrected for nonspecific binding (BSA alone).

Adsorption of Protein to Microtiter Wells—Microtiter wells (Falcon, microtest III, 96 flatbottom wells, flexible) were coated with either 0.05–10 μg of collagen/well or 1% BSA in 200 μl of PBS by incubation at room temperature overnight. After a brief rinse, nonspecific binding sites in the wells were blocked with either 100 μl of 1% BSA in PBS. The wells were then rinsed with PBS and prepared for the appropriate assay.

To determine the amount of collagen adsorbed to microtiter wells, 5 μg of each collagen type were allowed to adsorb in microtiter wells overnight followed by a brief rinse. Then protein was extracted from microtiter wells with three successive rinses of 100 μl of formic acid and subjected to amino acid analysis after hydrolysis. The amount of protein adsorbed to the microtiter wells was quantified and calculated to be 50% of the amount of that was loaded.

Ligand Blot—Proteins were denatured for 5 min in SDS-sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose filters. Nonspecific binding sites were blocked with 1% BSA in 1× PBS for 1 h at room temperature. Immunoblotting was done using 125I or 35S-heparin (2×10^6 cpm/ml) or 35S-heparin (2×10^6 cpm/ml) in hypotonic buffer (14 mM NaCl, 0.27 mM KCl, 0.81 mM Na2HPO4, 2×H2O, 0.15 mM KH2PO4) for 3 h at room temperature. Filters were then washed three times for 5 min with binding buffer and exposed to Kodak XAR-5 films at –70°C.

Ligand blot of APP—105-kDa rat brain full-length APP was iodinated by the chloramine-T method (IODO-BEADS, Pierce) and separated from free iodine by heparin-Sepharose chromatography.

RESULTS

Characterization of Binding of APP to Collagen—During our initial overlay studies using 125I-rn-APP to identify the cell surface receptor of APP that had earlier been proposed to exist (37), we observed an APP-collagen interaction and decided to study this in more detail.

First, we tested which different collagen types interact with APP. A dot blot assay revealed that 125I-labeled rn-APP binding to fibriibrar collagen type I and basement membrane collagen type IV (38) was saturable and specific. APP bound most efficiently to collagen type I (data not shown).

To investigate the kinetics of APP binding to collagen types I and IV and to gelatin in more detail, we used two distinct assays. Radiolabeled rn-APP was incubated in microtiter wells with immobilized collagen types I and IV at room temperature.
in microtiter wells or BSA as a control. APP-gelatin interaction was tested by incubating the protein with gelatin-Sepharose beads.

The binding efficiency was observed to depend on salt concentration and showed substrate specificity (binding to collagens, but not to other extracellular matrix components, such as fibronectin or vitronectin). At the specific activity used, 10% of the 125I-labeled rr-APP bound to collagen type I in a dot blot assay. Binding was saturable and analyzed by using the methodology of Scatchard (39). Assuming the presence of only one binding site on the collagen types I and IV, K_d values in the range of 0.5 × 10^{-8} M for collagen type I (Fig. 1), 4.5 × 10^{-8} M for collagen type IV, and 1.5 × 10^{-8} M for gelatin (data not shown) were calculated for the binding of APP to native and heat-denatured collagen (gelatin).

Determination of the Collagen Binding Domain of APP—To map the putative and single collagen binding site, we used proteolytic fragments of purified fusion protein APP (Fd-APP770) obtained by endoproteinase Lys-C digestion. A mixture of fragments was incubated with collagen type I that had been immobilized to microtiter plates. Proteolytic fragments of APP that specifically bound to collagen type I were extracted from microtiter wells with formic acid. Amino acid sequence analysis of the only resulting fragment revealed the sequence to commence at residue 448 of APP_{695} (Fig. 2). Other peaks were found to be caused by collagen α1 and α2 chains of collagen type I eluting between 20 and 25 min, Fig. 2) that was initially immobilized to the wells and was co-extracted with formic acid.

To confirm that we identified the collagen binding site of APP, three peptides of residues 448–465, 448–480, and 471–480 (Table I) were still able to inhibit binding of APP to collagen type I although with a reduced capacity by a factor of 10. A possible explanation for this is given by the secondary structure prediction for CBP/hs-APP, CBP/mm-APLP1, and CBP/hs-APLP2 according to Chou and Fasman (Table III; Ref. 41) that changes from β-structure for the NH_{2} terminus of CBP/APP to α-structure in the same site of APLPs (Table III).

Furthermore, direct binding of peptides CBP and CBP1 to collagen type I was proven by surface plasmon resonance (BIAcore, Pharmacia Biotech Inc.). Collagen type I was immobilized to the dextran surface of the sensor chip (21), but the kinetics of the binding reaction could not be determined, because two independent binding events, CBP to collagen and CBP to CBP, were observed to superimpose. This led us to the conclusion that both peptides showed a strong tendency for self-aggregation and suggested that CBP could represent a binding site for APP.

If APP-APP binding could occur through the CBP sequence, APP should bind specifically to the synthetic peptide CBP. The binding was analyzed in a solid-phase assay. 125I-rr-APP was added to microtiter plates coated with the CBP in the absence or presence of Zn(II) and heparin. Binding of APP was found to be saturable and dependent of the amount of CBP coated to the microtiter plate (Fig. 4a). A dose-dependent inhibition of the binding of 125I-rr-APP to CBP by TP-APP_{770} was observed in nanomolar concentrations (Fig. 4b).

The inhibition of binding was also found to be concentration-
dependent for Zn(II) and Cu(II) but not for other divalent ions such as Fe(II), Co(II), and Ni(II) (Fig. 4). Zinc(II) and copper(II) reduced binding to 10% (Zn(II)) and 20% (Cu(II)) at 50 $\mu$M, 15% (Zn(II)) and 35% (Cu(II)) at 25 $\mu$M, 30% (Zn(II)) and 50% (Cu(II)) at 1 $\mu$M concentrations. These values fall within the physiological Zn(II) and Cu(II) concentrations (42, 43).

When we tested the effect of Zn(II) (27, 30, 44) on binding of $^{125}$I-hs-APP to collagen by the synthetic peptides CBP (diamonds), CBP1 (open squares), and CBP2 (filled squares), The values represent residual binding determined in dependence of the concentration of the synthetic peptides as the competing ligands in a dot blot assay.

**Fig. 3.** Dose-dependent inhibition of the binding of $^{125}$I-hs-APP to collagen by the synthetic peptides CBP (diamonds), CBP1 (open squares), and CBP2 (filled squares). The values represent residual binding determined in dependence of the concentration of the competing ligands in a dot blot assay.

**Table I**

| Peptide | Sequence | Position |
|---------|----------|----------|
| CBP     | AAQIRSQVMTHLRLVYERMNQSLSLLYMVPAA^COOH | 448–480 |
| CBP1    | AAQIRSQVMTHLRLVYERMNQ | 448–465 |
| CBP2    | SLLYMVPAAEEIQDEVDELLQ^COOH | 471–493 |

**Table II**

Homology of the collagen type I binding site (CBP) of the human, mouse, and rat CBP sequence (CBP/APP) to human and mouse APLP2 (CBP/mm-APLP2) and mouse APLP1 (CBP/mm-APLP1).

| Protein | Box residues that differ from APP-CBP |
|---------|-------------------------------------|
| hs-APP  | 446 AAQIRSQVMTHLRLVYERMNQSLSLLYMVPAA |
| mm-APLP2| 446 AAQIRSQVMTHLRLVYERMNQSLSLLYMVPAA |
| hs-APLP2| 446 AAQIRSQVMTHLRLVYERMNQSLSLLYMVPAA |
| mm-APLP1| 446 AAQIRSQVMTHLRLVYERMNQSLSLLYMVPAA |

**Table III**

Homology of the collagen type I binding site (CBP) of the human, mouse, and rat CBP sequence (CBP/APP) to human and mouse APLP2 (CBP/hs-APLP2) and mouse APLP1 (CBP/mm-APLP1).

| Residues that differ from the CBP/APP sequence are boxed. The amino acid sequence of CBP/hs-APLP2 was taken from Ref. 69 and is completely homologous to mouse APLP2 (70). |

**Table IV**

Comparison and secondary structure prediction of the amino acid sequences of CBP/APP, CBP/APLP2, and CBP/APLP1.

Amino acid residues identical in APLP2 and APLP1 are indicated by a dash. The secondary structure was predicted according to Chou and Fasman (41) using the Proxe program (Lasergene computer program package by DNASTAR, version 1.20).

**Fig. 4.** Determination of the APP Binding Domain of Collagen—To further examine this, we decided to investigate whether the high affinity heparin binding site of type I collagen that has been localized in the COOH-terminal CB fragment of the molecule (46) is the critical site responsible for the binding of heparin and chondroitin sulfate to collagen type I (Table IV), because chondroitin sulfate does not bind to APP (30). Radioiodinated APP was saturated with heparin and separated from unbound, free heparin by desalting. It was found to bind to collagen type I, but collagen preincubated in a dot blot assay with heparin did not bind any longer to APP. In summary, these results show that heparin bound to collagen, but not bound to APP, inhibits the interaction between APP and collagen in a concentration-dependent manner (Table IV, Fig. 5).

Determination of the APP Binding Domain of Collagen—To further examine this, we decided to investigate whether the high affinity heparin binding site of type I collagen that has been localized in the COOH-terminal CB fragment of the molecule (46) is the critical site responsible for the binding of APP to collagen type I. We examined the abilities of collagen type I to bind heparin by affinity chromatography and performed binding studies with $^{35}$S-heparin and $^{125}$I-mm-APP. Since the binding of collagen to heparin-Sepharose is necessary but not sufficient to fulfill the criteria of heparin binding, CB fragments of collagen type I eluting at 600 m NaCl from heparin-Sepharose were separated by SDS-PAGE, transferred to nitrocellulose filters, and incubated with $^{35}$S-heparin. Heparin binding was found in the covalently linked, partially digested dimers or trimers of the $\alpha$-Chains, the $\alpha$II(1)CB8 and the $\alpha$II(1)CB6 fragments (Fig. 6, A and B).

In an overlay assay $^{125}$I-mm-APP binding to collagen type I was observed to partially digested dimers or trimers of the
α-chains and to the α1(I)CB6 fragment as the only CNBr fragment showing up at an apparent molecular mass of 25 kDa (Fig. 6C). To determine the identity of this fragment, it was purified from SDS gels and characterized by amino acid sequence analysis. Higher bands represent uncomplete digested and/or covalently cross-linked dimers of the α-chains. The results presented above show that the α1(I)CB6 fragment of collagen type I contains binding sites for both APP and the glycosaminoglycan heparin.

Since it has been indicated that the triple helical structure of collagen is important for the interaction with heparin (46), the α1 and α2 collagen chains and the CNBr fragment α1(I)CB6 were separated by SDS-PAGE, electroeluted, and assayed separately in a dot blot apparatus by incubation with 125I-rn-APP. Binding was strongest to the α1 chain of collagen type I, less binding was observed to the α1CB6 fragment, and no specific binding could be observed to the α2 chain of collagen type I (Fig. 7, A and B). This result is consistent with binding studies for which 125I-rn-APP and gelatin-Sepharose affinity chromatography indicated that APP recognizes heat-denatured collagen as well as triple-helical fibrillar collagen. Apparently, the APP binding site in the α1CB6 fragment has less tight specificity, probably due to the change of conformation of surrounding residues.

In conclusion, our observations reveal a mechanism of APP binding to collagen that is regulated by glycosaminoglycans that compete with APP for an overlapping binding site of the α1CB6 fragment of collagen. Although both components can interact with glycosaminoglycans (21, 47), our assay system allowed us to determine that inhibition of APP binding to colla-
Binding to denatured collagen is not observed, a finding that is consistent with the high affinity heparin binding site of APP695 (21) with residues 316–337. The synthetic peptide HP-1 representing the high affinity heparin binding site of APP695 with residues 316–337 does not inhibit the binding of 125I-APP to collagen type I in the presence of free heparin and collagen. The total amount of protein immobilized to nitrocellulose was determined by amino acid analysis. The inhibitory effect exerted through peptides CBP and CBP1 on APP-collagen binding must be taken as an indication.

Collagen binding of APP was mapped to the carbohydrate domain of APP corresponding to amino acid residues 448–465, not including the carbohydrate attachment site at asparagine in position 467 (Table I). The sequence of peptide CBP1 is in position 467 (Table I). The sequence of peptide CBP1 is identical to that of CBP and CBP1 on APP-collagen binding must be taken as an indication.

In this study we concentrated on collagen type I that showed the highest affinity of those that were tested. The affinity is in good agreement with the Kd values for the binding of 1 glycoproteins and NCAM to collagens (40, 49).

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outgrowth from peripheral neurons and also promote outgrowth from at least some central neurons (64).

An involvement of APP in these processes was earlier suggested by Breen et al. (23) who found cell binding to a collagen substrate (neuron-neuron and neuron-glial binding) inhibited by antibodies against the extracellular portion of APP. Neuronal regeneration is associated with the expression of extracellular matrix proteins (65, 66). A direct interaction of mature transmembrane APP with components of the extracellular matrix might reflect the importance of APP as a neuronal cell adhesion molecule. The interaction of APP with extracellular matrix molecules may turn out to have a role in the pathogenesis of Alzheimer’s disease. Proteoglycans appear to be increased in the vicinity of Aβ4 amyloid plaque deposition (67, 68). Collagen-like molecules such as the A12 form of acetylcholinesterase are also increased in the areas of amyloid. Alterations in the affinity of APP for extracellular matrix molecules by zinc may also be relevant to Alzheimer’s disease (21, 27).

The normal processing of APP in the central nervous system may therefore be affected by APP-matrix interactions and deserves closer scrutiny.

Acknowledgments—We are grateful to Andreas Schlicksupp and Santosh Pinto for technical assistance and thank Konrad Beyreuther for critically reading the manuscript.

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