Distinct Binding Sites in the Structure of $\alpha_2$-Macroglobulin Mediate the Interaction with $\beta$-Amyloid Peptide and Growth Factors*

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$\alpha_2$-Macroglobulin ($\alpha_2$M) and its receptor, low density lipoprotein receptor-related protein (LRP), function together to facilitate the cellular uptake and degradation of $\beta$-amyloid peptide (A$\beta$). In this study, we demonstrate that A$\beta$ binds selectively to $\alpha_2$M that has been induced to undergo conformational change by reaction with methyalamine. Denatured $\alpha_2$M subunits, which were immobilized on polyvinylidene difluoride membranes, bound A$\beta$, suggesting that $\alpha_2$M tertiary and quaternary structure are not necessary. To determine whether a specific sequence in $\alpha_2$M is responsible for A$\beta$ binding, we prepared and analyzed defined $\alpha_2$M fragments and glutathione S-transferase-$\alpha_2$M peptide fusion proteins. A single sequence, centered at amino acids (aa) 1314–1365, was identified as the only major A$\beta$-binding site. Importantly, A$\beta$ did not bind to the previously characterized growth factor-binding site (aa 718–734). Although the A$\beta$ binding sequence is adjacent to the binding site for LRP, the results of experiments with mutated fusion proteins indicate that the two sites are distinct. Furthermore, a saturating concentration of A$\beta$ did not inhibit LRP-mediated clearance of $\alpha_2$M-MA in mice. Using various methods, we determined that the $K_D$ for the interaction of A$\beta$ with its binding site in the individual A$\beta$2M subunit is 0.7–2.4 $\mu$M. The capacity of A$\beta$ to bind A$\beta$ and deliver it to LRP may be greater than that predicted by the $K_D$, because each A$\beta$2M subunit may bind A$\beta$ and the bound A$\beta$ may multimerize. These studies suggest a model in which $\alpha_2$M has three protein interaction sites with distinct specificities, mediating the interaction with A$\beta$, growth factors, and LRP.

Accumulation of $\beta$-amyloid peptide (A$\beta_{1–40}$ and A$\beta_{1–42}$) in the brain plays a central role in the development and progression of Alzheimer’s disease (AD) (1). Mutations in $\beta$-amyloid precursor protein (APP), which result in increased production of A$\beta$, are associated with autosomal dominant forms of familial AD in humans (2–4). Mutated forms of human APP may also induce changes consistent with AD when expressed as transgenics in mice (5–8). Furthermore, immunization with A$\beta_{1–42}$ prevents progression of AD in animal model systems and may reverse symptoms by promoting resorption of A$\beta$-containing plaques (9, 10). These results suggest that A$\beta$ accumulation in the brain is a dynamic and reversible process. Proteins other than antibodies with the capacity to bind A$\beta$ and promote its catabolism may influence disease progression.

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a 718-kDa homotetrameric glycoprotein, which is well characterized as an extracellular proteinase inhibitor (11) and as a carrier of specific growth factors, including transforming growth factor-$\beta$ (TGF-$\beta$) and nerve growth factor-$\beta$ (NGF-$\beta$) (12, 13). At least two separate polymorphisms in the A$\beta$2M gene may be associated with increased risk of late-onset AD. The first involves a region within intron 17, at the 5′ splice acceptor site for exon 18 (14). This exon is important because it encodes part of the bait region, where proteinases initiate reaction with $\alpha_2$M by cleaving susceptible peptide bonds (15, 16), and a segment of the growth factor binding sequence (17–19). In the second A$\beta$2M gene polymorphism, Val-1000 is replaced by Ile (20). The linkage of A$\beta$2M gene polymorphisms to late-onset AD remains incompletely understood, because the original observations have been confirmed in only a limited number of populations (21–25) and because there is no molecular explanation regarding how A$\beta$2M gene mutations may affect $\alpha_2$M structure, function, and expression.

$\alpha_2$M is expressed by microglia, which accumulate near amyloid plaques (26). Thus, locally synthesized $\alpha_2$M may affect AD progression by regulating the activity of various proteinases or by binding important growth factors. The previously demonstrated ability of $\alpha_2$M to bind and neutralize the activity of TGF-$\beta$ (12, 13, 27–29) may be detrimental in AD, because TGF-$\beta$ stimulates A$\beta$ clearance by microglial cells and reduces A$\beta$ accumulation in the brain parenchyma of mice that overexpress human APP (30). Furthermore, TGF-$\beta$ has been reported to antagonize the cytotoxic activity of A$\beta$ (29, 31, 32).

Another mechanism whereby $\alpha_2$M may regulate AD progression involves its ability to bind A$\beta$, forming a complex that is internalized by the $\alpha_2$M receptor, low density lipoprotein receptor-related protein (LRP) and then degraded (33–35). Du et al. (36) originally reported that A$\beta_{1–40}$ and A$\beta_{1–42}$ bind to native $\alpha_2$M and to $\alpha_2$M that has been transformed into the LRP-recognized or “activated” conformation by reaction with methyalamine ($\alpha_2$M-MA). Narita et al. (33) subsequently reported selective binding of A$\beta_{1–40}$ and A$\beta_{1–42}$ to the activated conformation of $\alpha_2$M. $\alpha_2$M-MA apparently binds A$\beta_{1–40}$ and A$\beta_{1–42}$ with equivalent affinity (33). Hughes et al. (37) executed a yeast two-hybrid screening using A$\beta_{1–42}$ as bait and...
identified a 250-amino acid peptide from the C terminus of a2M as a strong and specific interactor. The same group also reported experiments confirming the interaction of Aβ with intact a2M; however, they did not demonstrate that the sequence identified by yeast-two hybrid screen is responsible for the binding of Aβ to intact a2M.

The growth factor binding site in a2M is contained within a 16-amino acid peptide located —500 amino acids N-terminal to the Aβ-binding site identified by yeast-two hybrid screen (19). The growth factor binding sequence is composed mainly of hydrophobic amino acids with two potentially important acidic residues. TGF-β, platelet-derived growth factor-BB (PDGF-BB), and NGF-β all interact with the growth factor-binding site in a2M (18, 19), despite the fact that these proteins demonstrate limited sequence identity. Based on this promiscuous behavior, we hypothesized that the growth factor-binding site in a2M may also function as an Aβ-binding site.

To test our hypothesis, we undertook a comprehensive molecular analysis to identify sequences in a2M with Aβ binding activity. Our results demonstrate that a single sequence, located near the C terminus of the a2M subunit, constitutes the only significant Aβ-binding site. Importantly, this sequence is entirely distinct from the growth factor-binding site. The LRP recognition sequence is also located near the C terminus of the a2M subunit (38–42); however, our evidence indicates that the LRP recognition site and the Aβ binding sequence are distinct. Thus, in addition to the bait region, the a2M subunit has at least three distinct “protein interaction sites” with distinct binding specificities. These sites mediate interactions with growth factors, Aβ and LRP.

MATERIALS AND METHODS

Proteins and Reagents— a2M was purified from human plasma by the method of Imber and Pizzo (43). a2M-MA was prepared by dialyzing a2M against 200 mM methylamine-HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22 °C and then exhaustively against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4. Modification of a2M by methylation was confirmed by demonstrating the characteristic increase in a2M electro- phoretic mobility by non-denaturing PAGE (15). a2M-MA was radiiodinated using IODO-READs (Pierce) and stored at 4 °C for no more than 2 weeks. The specific activity was 0.5–1.0 Ci/mg. Receptor-associated protein (RAP), which blocks binding of A2M-MA to LRP (65), was expressed as a glutathione S-transferase (GST) fusion protein in bacteria and purified by chromatography on glutathione-Sepharose. Aβ1–40 was purchased from Bachem and radioiodinated using 125I-labeled Bolton-Hunter reagent (di-iodinated, PerkinElmer Life Sciences). Biotinylated Aβ1–40 was prepared by reacting Aβ1–40 with 4 μM sulfo-N-hydroxysuccinimide biotin (Pierce) for 2 h at 4 °C in sili- conized tubes. The reaction mixture was dialyzed extensively against water. Biotinylated Aβ was stored for up to 1 month at 4 °C or frozen at −80 °C and thawed once without affecting its ability to bind to a2M. GST-specific IgG, bovine serum albumin (BSA, greater than 99% pure), diithiothreitol (DTT), and iodoacetamide were from Sigma Chemical Co. 3-(Benzosulfonyl) isothiocyanate (BST) and horseradish peroxidase-conjugated avidin were from Pierce. Polyclonal Aβ-specific rabbit antibody was from Zymed Laboratories Inc.

Methods for Defined Fragmentation of a2M—When a2M is treated with papain under mildly acidic conditions, an 18-kDa fragment is released from the C terminus of each a2M subunit (aa 1314–1451) (38). The 18-kDa fragment includes the intact receptor-binding site and is thus referred to as the receptor binding fragment (RBF). The residual 600-kDa a2M remnant retains the major structural features of the parent molecule (45). To obtain the 18- and 600-kDa a2M fragments, 4.0 μg a2M-MA was treated with 2.4 μM papain in 50 mM sodium acetate, 1 mM cysteine, pH 5.0, for 20 h at 22 °C. The pH of the reaction mixture was increased to 7.4, and the products were purified by molecular exclusion chromatography on Ultrigel AcA-22.

Each a2M subunit has a single thiol ester bond formed by the side chains of Cys-949 and Gin-952 (11, 46, 47). When Aβ is heated in the presence of SDS, the thiol esters react internally, and, as a result, the a2M peptide backbone is cleaved (47, 48). The products include a 120- kDa N-terminal heat fragment and a 60-kDa C-terminal heat fragment. To produce a2M heat fragments, native a2M was incubated at 100 °C in 2 M sodium phosphate, 50 mM Tris-HCl, pH 7.4, for 2 h at 37 °C. In some experiments, increasing concentrations of the 18-kDa RBF (0.2–2.8 μM) were co-incubated with 125I-Aβ and a2M. Reaction mixtures were subjected to non-denaturing PAGE, using the buffer system described by Van Leuven et al. (49). 125I-Aβ binding to a2M was detected as radioactivity co-migrating with the a2M band. In control experiments, free 125I-Aβ did not migrate near a2M. To quantitate 125I-Aβ binding to a2M, gels were subjected to PhosphorImager analysis using ImageQuant software. Non-denaturing PAGE preserves non-covalent interactions; however, the amount of binding detected may be influenced by dissociation of protein complexes during electrophoresis (13).

Determination of Apparent Equilibrium Dissociation Constants—Because Aβ binding to a2M is reversible and probably subject to rapid dissociation when methods such as non-denaturing PAGE or chromatography are used, we utilized the BST3 rapid cross-linking method to determine the apparent KD for the binding of Aβ to a2M. This method has been used previously to determine KD values for the interaction of a2M with multiple growth factors and cytokines (12, 13, 50).

Increasing concentrations of a2M-MA were incubated with 25 nM 125I-Aβ for 2 h at 37 °C. freshly dissolved BST3 (5 mM) or vehicle (H2O)
was then added for 5 min. Cross-linking reactions were quickly terminated by rapid acidification, followed by transfer to buffered SDS. Under pseudo-first order conditions, a constant fraction of the non-covalent $^{125}$I-Aβ-αM-MA complex is covalently stabilized by the BS3 (13). To quantitate the amount of covalently stabilized complex, BS3-treated and vehicle-treated samples were subjected to SDS-PAGE. $^{125}$I-Aβ that was covalently cross-linked to αM-MA (bound) and free $^{125}$I-Aβ (free), which includes free Aβ and Aβ that was bound to αM-MA but not covalently linked, were quantitated by PhosphorImager analysis. Results were analyzed according to the following equation (12).

$$\text{free/bound} = (K_d/z)(1/\alpha\text{M-MA}) + 1/z - 1$$  (Eq. 1)

The cross-linking efficiency, $z$, is a constant, derived from the $y$ intercept, for each set of proteins and conditions (12). $z$ is referred to as the BS3-cross-linking efficiency but may also be affected if a fraction of the radiiodinated protein is incapable of binding to the αM. The apparent $K_d$ was determined from the slope when free/bound was plotted against $1/(\alpha\text{M-MA})$. This value is based on the assumption that there is a single binding site for Aβ in αM. Assuming one Aβ-binding site/αM subunit, as suggested by our data, then the $K_d$ must be multiplied by the apparent $K_d$ by a factor of four.

**Ligand Blotting**—This method has been previously used to demonstrate specific and saturable binding of growth factors to denatured αM subunits, αM fragments, and GST-αM-peptide fusion proteins (17–19). Protein preparations were denatured in 2% SDS or treated with 1 mM DTT in 2% SDS and then with 5 mM iodoacetamide for 2 h, as previously described (17). Samples were then subjected to SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% milk in 20 mM sodium phosphate, 150 mM NaCl, 0.1% Tween 20, pH 7.4, and probed for 2 h with $^{125}$I-Aβ or biotinylated-Aβ. $^{125}$I-Aβ ligand blots were washed and subjected to PhosphorImager analysis. Biotinylated Aβ ligand blots were probed with horseradish peroxidase-conjugated avidin (1:5000 dilution). The membranes were then subjected to enhanced chemiluminescence (ECL) and densitometry. Equivalent loading and transfer of protein were demonstrated by Coomassie Blue staining or, when applicable, by immunoblot analysis with GST-specific antibody (17).

**Aβ-peptide Immuno blotting**—Denatured αM subunits and BSA were treated with 1 mM DTT and then with 5 mM iodoacetamide to block free sulfhydryl groups, subjected to SDS-PAGE, and electrotransferred to PVDF. The membranes were blocked with 5% milk. Unlabeled Aβ1–40 or Aβ1–42 was incubated with the immuno-labeled αM and BSA in PBS-T at 37°C for 2 h. The membranes were then washed extensively and probed with rabbit Aβ-specific IgG (1:4000 in PBS-T and 0.1% milk (v/v), followed by anti-rabbit IgG-horseradish peroxidase conjugate (1:10,000). Membranes were analyzed by ECL and densitometry.

**Plasma Clearance Experiments in Mice**—$^{125}$I-αM-MA (20 nmol) was incubated with 20 μM Aβ or with vehicle for 2 h at 37°C. The $^{125}$I-αM-MA (2.5 nM) was then injected, in the presence and absence of GST-RAP (40 or 80 μg), into the lateral tail veins of CD-1 female mice (30 g). Blood samples (40 μl) were withdrawn from the retro-orbital venous plexus, using heparinized capillary tubes, at the designated times (0.5–30 min). The radioactivity in each sample was determined using a gamma counter and expressed as a fraction of that present in the 0.5-min time point.

**RESULTS**

**Aβ Binding to αM** Is αM Conformation-dependent—Aβ1–40 and Aβ1–42 function differently in the initiation and progression of AD; however, both forms of Aβ bind to αM equivalently (33). Thus, we conducted our analysis of Aβ binding to αM and its derivatives using one form of Aβ (Aβ1–40). To determine whether Aβ binding to αM is αM conformation-specific, as has been demonstrated with growth factors (12, 13), $^{125}$I-Aβ (2.5 nM) was incubated with native αM or αM-MA (each at 0.02 μM) in solution. The products were analyzed by non-denaturing PAGE, which preserves non-covalent interactions. As shown in Fig. 2A, $^{125}$I-Aβ bound to αM-MA, whereas binding was not detected with native αM. Free $^{125}$I-Aβ migrated near the dye front. These results suggest that Aβ binds selectively to αM-MA that has undergone conformational change.

To estimate the $K_p$ for Aβ binding to αM-MA, we used the BS3-rapid cross-linking method, which has been used extensively to determine binding affinities for αM and growth factors (12, 13, 50). A major advantage of this method is that it is not necessary to resolve free and bound Aβ, which typically involves the use of steps, such as chromatography or PAGE, that promote dissociation of non-covalent protein complexes. A representative study in which $^{125}$I-Aβ was incubated with increasing concentrations of αM-MA is shown in Fig. 2B. The exact fraction of the non-covalent αM-MA complex, which was cross-linked by BS3 ($z$), was determined from the y intercept, as previously described (12). In three separate experiments, $z = 0.06 – 0.14$, compared with $z$ values that are typically in the range of 0.15–0.40 for the binding of growth factors to αM (12). $z$ may be decreased if a fraction of the $^{125}$I-Aβ was incapable of binding to αM-MA or if the Aβ, which bound to αM-MA, multimerized so that individual Aβ monomers could not be cross-linked to the αM-MA. Neither of these effects would be expected to influence the calculated apparent $K_p$.

The apparent $K_p$ for the binding of Aβ to αM-MA was 0.29 ± 0.02 μM (n = 3). This value is based on the assumption that each molecule of αM has one binding site for Aβ. If each αM subunit has a distinct Aβ-binding site, as the evidence to be presented will suggest, then the $K_p$ for the binding of Aβ to the individual binding site is 1.2 μM. Although this is a low affinity interaction, due to the homotetrameric structure of αM, the plasma concentration of αM subunits is 12–20 μM (11).

**Binding of $^{125}$I-Aβ to Denatured αM Subunits**—Native αM was denatured in SDS and DTT and treated with iodoacetamide. A similar protocol was executed with αM-MA and with BSA. The preparations were then subjected to SDS-PAGE. Coomassie Blue staining revealed the 180-kDa αM subunit as the major band in both the native αM and αM-MA preparations, as anticipated (Fig. 3). Faint bands with apparent masses of 120 and 60 kDa were observed in the native αM lane. These bands correspond to the αM heat fragmentation products that result from an internal reaction involving the thiol ester bonds at 100°C, as previously described (48). αM-MA does not undergo heat fragmentation, because the thiol esters have already undergone aminolysis (46, 47).
To determine whether $A_\beta$ bound to denatured $\alpha_\text{M}$ subunits that were electro-transferred to PVDF membranes. No difference in $^{125}$I-$A_\beta$ binding was observed with native $\alpha_\text{M}$ and $\alpha_\text{M}$-MA, as was anticipated, because the difference in structure between these two forms of $\alpha_\text{M}$ is mainly conformational. $^{125}$I-$A_\beta$ did not bind to BSA, suggesting that the interaction with $\alpha_\text{M}$ is specific. The interaction of $^{125}$I-$A_\beta$ with $\alpha_\text{M}$, in the ligand blotting system, suggests that the individual $\alpha_\text{M}$ subunit binds $A_\beta$ and that $\alpha_\text{M}$ tertiary and quaternary structure are not necessary. In this respect, $A_\beta$ binding to $\alpha_\text{M}$ resembles the interaction observed with growth factors (17) but not with proteinases (11, 15).

To confirm that the interaction of $A_\beta$ with $\alpha_\text{M}$ was not dependent on an unanticipated modification occurring during $A_\beta$ radiiodination, we developed alternative methods for detecting $A_\beta$ binding to PVDF-immobilized $\alpha_\text{M}$ subunits. In the first protocol, unlabeled $A_\beta$ was used to probe the PVDF membranes. $\alpha_\text{M}$-associated $A_\beta$ was then detected by immunoblot analysis. In the second protocol, biotinylated $A_\beta$ was substituted for $^{125}$I-$A_\beta$. In both cases, binding of $A_\beta$ to PVDF-immobilized native $\alpha_\text{M}$ and $\alpha_\text{M}$-MA was detected whereas $A_\beta$ binding to BSA was not.

$A_\beta$ Binding to $\alpha_\text{M}$ Heat Fragments—When $\alpha_\text{M}$ is heated in the presence of denaturant, the thiol ester bonds, which are formed from the side chains of Cys-949 and Gln-952, react internally with the $\alpha_\text{M}$ polypeptide backbone, causing scission of the $\alpha_\text{M}$ subunit at residue 952 (46–48). The N-terminal 120-kDa fragment includes the bait region and the growth factor binding sequence. The C-terminal 60-kDa fragment includes the LRP recognition sequence (38–42) and the region identified by Hughes et al. (37) as a candidate $A_\beta$-binding site. To determine whether $A_\beta$ binding activity is localized to either or both of these denatured $\alpha_\text{M}$ fragments, $\alpha_\text{M}$ was subjected to heat fragmentation and analyzed by $^{125}$I-$A_\beta$-ligand blots. Only the 60-kDa $\alpha_\text{M}$ heat fragment bound $^{125}$I-$A_\beta$ (Fig. 4). The 120-kDa $\alpha_\text{M}$ heat fragment was without activity. This result provides evidence that a specific sequence is responsible for the interaction of $\alpha_\text{M}$ with $A_\beta$. Furthermore, this result suggests that the $\alpha_\text{M}$ growth factor-binding site and the $A_\beta$-binding site are distinct.

The 18-kDa RBF Competes with $\alpha_\text{M}$-MA for $A_\beta$ Binding—A second method for defined fragmentation of $\alpha_\text{M}$ involves pain treatment of the activated conformation under mildly acidic conditions. An 18-kDa fragment, which retains LRP binding activity, is dissociated from the C terminus of each $\alpha_\text{M}$ subunit (aa 1314–1451) (38–40). The residual 600-kDa fragment retains the major structural characteristics of $\alpha_\text{M}$-MA, as determined by electron microscopy (45). The 18- and 600-kDa $\alpha_\text{M}$ fragments were purified and assessed for their ability to bind $A_\beta$ without prior denaturation. When $^{125}$I-$A_\beta$ was incubated with the 600-kDa fragment, in solution, binding was not detected by non-denaturing PAGE (Fig. 5A). Under equivalent conditions, binding was readily detected with intact $\alpha_\text{M}$-MA.

In separate experiments, 2.5 nM $^{125}$I-$A_\beta$ was incubated with 0.3 μM $\alpha_\text{M}$-MA and increasing concentrations of purified 18-kDa RBF in solution. $^{125}$I-$A_\beta$ binding to $\alpha_\text{M}$-MA was decreased in the presence of the 18-kDa RBF, and the magnitude of the effect was dependent on the RBF concentration (Fig. 5B). The IC$_{50}$ was 0.7 μM. Because of the relatively high concentration of $\alpha_\text{M}$-MA, the IC$_{50}$ for $A_\beta$ binding to the 18-kDa RBF was ~2-fold lower (0.3–0.4 μM) than the IC$_{50}$. These results provide further evidence that an $A_\beta$-binding site is localized near the C terminus of the $\alpha_\text{M}$ subunit and that this site is distinct from the growth factor-binding sequence.

$A_\beta$ Binding to GST-$\alpha_\text{M}$-peptide Fusion Proteins—To comprehensively analyze the $\alpha_\text{M}$ sequence with regard to $A_\beta$ binding, we utilized ligand blotting to screen a series of six previously described $\alpha_\text{M}$-peptide-GST fusion proteins (FP1–FP6) (18). In the intact $\alpha_\text{M}$ subunit, the bait region and growth factor-binding site are located in FP3. The residues that comprise the thiol ester bond are located in FP4, and the LRP recognition sequence is in FP6. To assess $A_\beta$ binding, the fusion proteins were treated with iodoacetamide, without prior reduction, and subjected to SDS-PAGE. After electrotransfer to PVDF, only FP6 bound $^{125}$I-$A_\beta$ (Fig. 6A).

Because intact disulfide bonds may allow partial restoration of non-denatured structure following protein electrophoresis to
Fig. 5. Aβ binding to α,M fragments derived by papain treatment. The 600- and 18-kDa papain fragments of α,M-MA were prepared and purified. A, 125I-Aβ was incubated with α,M-MA or the 600-kDa fragment (1.0 μM), under non-denaturing conditions, for 2 h at 37°C. The samples were subjected to non-denaturing PAGE. 125I-Aβ was detected by PhosphorImager analysis. B, 125I-Aβ was incubated with α,M-MA (0.3 μM) and increasing concentrations of the 18-kDa RBF for 2 h at 37°C. The samples were then subjected to non-denaturing PAGE and PhosphorImager analysis. The figure shows the fraction of the 125I-Aβ that was associated with α,M-MA, compared with the amount observed in the absence of 18-kDa RBF.

Fig. 6. Ligand blot analysis of Aβ binding to GST-α,M fusion proteins. A, PVDF membranes with FP1–FP6, which had been denatured in the absence of reductant and treated with iodoacetamide, were probed with 125I-Aβ. B, a representative study in which FP3 and FP6 were denatured in the presence of DTT, treated with iodoacetamide, and subjected to ligand blot analysis with 125I-Aβ. C, FP3, FP4, and FP6 were denatured in 2% SDS and DTT, treated with iodoacetamide, subjected to SDS-PAGE, and electrotransferred to PVDF membranes. The membranes were probed with biotinylated Aβ or subjected to immunoblot analysis with GST-specific antibody.

PVDF membranes, ligand-blotting experiments were also performed using FP3 and FP6 that were reduced with DTT and then alkylated with iodoacetamide. In these experiments, 125I-Aβ binding was still detected only with FP6 and not with FP3 (Fig. 6B). Equivalent results were obtained when biotinylated Aβ was substituted for 125I-Aβ (Fig. 6C). Based on these results, a model emerges in which the structure of α,M includes at least two distinct protein interaction sites with differing specificity. A site located near the center of the α,M subunit is responsible for the binding of growth factors whereas a separate site near the C terminus is exclusively responsible for the binding of Aβ.

Resolution of the LRP- and Aβ-binding Sites in α,M—In intact human α,M, an 1370–1377 constitute an α helix that is the center of the LRP recognition site (41, 42). The α helix is anchored in position by a β sandwich so that the side chains of two critical Lys residues (aa 1370 and 1374) protrude at 45° angles and are surrounded by hydrophobic surface residues (41). This complex secondary and tertiary structure may explain why the 18-kDa RBF is recognized by LRP, whereas cryptic peptides corresponding to the same region and partially denatured forms of the 18-kDa RBF are not (38, 41).

Because our ligand blotting results demonstrated that tertiary structure is not necessary for Aβ binding to FP6, we generated a new set of fusion proteins to explore the relationship between the LRP- and Aβ-binding sites in α,M. FP6c included all of the amino acids that form the LRP recognition α helix, five amino acids N-terminal to the α helix and the entire sequence C-terminal to the α helix; however, FP6c did not bind Aβ (Fig. 7). FP6a, which included the N-terminal segment of FP6 but terminated five amino acids before the start of the α helix, bound Aβ, albeit at lower levels than FP6. These results suggest that the Aβ-binding site is located N-terminal to the LRP binding α helix. FP6b, which was equivalent to FP6a but extended through the α helix to aa 1400, bound slightly increased levels of Aβ, suggesting that amino acids 1365–1400 may impact positively on this interaction; however, ligand blotting results provide only an approximation of differences in binding affinity.

Fusion proteins, which correspond exactly to the sequence of the 18-kDa RBF, were generated and mutated to essentially eliminate the LRP-binding site (FP6d-AR) (39) or substantially reduce binding to LRP while eliminating binding to the previously described α,M signaling receptor (FP6d-AA) (40). By

Resolution of the Aβ- and LRP-binding Sites in α,M—These experiments were carried out with the 18-kDa RBF, which was subjected to non-denaturing PAGE and PhosphorImager analysis. The figure shows the fraction of the 125I-Aβ that was associated with α,M-MA, compared with the amount observed in the absence of 18-kDa RBF.
ligand blotting, both forms of FP6d retained Aβ binding activity, supporting our hypothesis that the Aβ and α2M receptor recognition sequences in α2M are non-identical. A slight decrease in the binding of Aβ to FP6-AA and FP6-AR, compared with FP6, may indicate that the mutated Lys residues, although non-essential, impact positively on the interaction.

**Determination of the KD for Aβ Binding to FP6**—In the ligand blotting experiments, the concentration of 125I-Aβ, used as probe, was substantially lower than the likely KD value for 125I-Aβ binding to any of the fusion proteins. Thus, assuming equivalent load and insignificant contributions from “low affinity” or “nonspecific” binding sites, the amount of binding observed is inversely proportional to the KD for each interaction. To more accurately assess the binding affinity of Aβ for FP6 and FP6d-AA, specific binding experiments were performed.

FP6 and FP6d-AA were purified to homogeneity; however, unlike intact α2M and the 18-kDa RBF, the fusion proteins did not bind Aβ in solution, even when refolding protocols were executed. Possible explanations for this observation are provided under “Discussion.” As an alternative approach, we immobilized purified FP6, FP6d-AA, and FP3 on PVDF after exposure to SDS and probed the membranes with 125I-Aβ (0.1 μM) and increasing concentrations of unlabeled Aβ. Nonspecific binding was defined by the level of 125I-Aβ binding observed in the presence of 30 μM unlabeled Aβ. As shown in Fig. 8, specific binding of 125I-Aβ to both FP6 and FP6d-AA was detected. The KD values were 2.4 ± 0.8 and 5.2 ± 0.6 μM, respectively (mean ± S.E., n = 3 with internal triplicate replicates). The Bmax, which is not an informative value when this method is used, was 20–25% higher with FP6. Importantly, the binding of 125I-Aβ to FP3 was entirely nonspecific.

**Effects of Aβ on α2M Recognition by LRP**—Others have demonstrated that LRP mediates cellular uptake and degradation of Aβ by binding Aβ-α2M complexes (33–35). Because of the close proximity of the Aβ and LRP recognition sequences in α2M, we considered the possibility that Aβ might inhibit binding of α2M to LRP even though the binding sites are non-identical. Each α2M tetramer has four independent LRP recognition sequences (38). Thus, even if some of the LRP-binding sites were blocked by Aβ, receptor recognition may still occur. To address this question, we examined the plasma clearance of α2M-MA in mice. In this well characterized system, conformationally modified forms of α2M, such as α2M-MA, clear from the plasma as a first-order process with a t1/2 of 3–5 min, and clearance competition is observed when 125I-α2M-MA is injected in the presence of excess unlabeled α2M-MA (51). The rapid plasma clearance of α2M-MA represented a clear advantage for our experiments with Aβ, compared with binding or endocytosis experiments performed in vitro, because of the opportunity to minimize the time period during which dissociation of Aβ-α2M-MA complex may occur.

Fig. 9 shows the plasma clearance of 125I-α2M-MA (n = 4) in the absence of competing ligand and in the presence of 40 or 80 μg of GST-RAP. The GST-RAP inhibited the clearance of 125I-α2M-MA from the plasma, as anticipated due to competition for plasma-accessible LRP, which is mainly located in the liver (51). To determine whether Aβ inhibits α2M-MA binding to LRP, 125I-α2M-MA was preincubated with a nearly saturating concentration of Aβ (20 μM, 16-fold the KD) for 2 h at 37 °C and then injected intravenously in mice. The rate of 125I-α2M-MA clearance was completely unchanged. We cannot rule out the possibility that Aβ dissociated from the 125I-α2M-MA after the preparation was injected intravascularly, due to dilution in the bloodstream; however, given the rapid timeframe of the plasma clearance experiments, our results demonstrate that α2M-associated Aβ either does not interfere with LRP recognition or rapidly dissociates from some sites to free up LRP recognition sequences and allow uptake of the remaining Aβ.

**DISCUSSION**

LRP and its ligands, α2M, apolipoprotein E4, and Kunitz-proteinase inhibitor domain-containing isoforms of APP, form an intriguing functional family, the members of which have been implicated in familial or late-onset AD (52). Deciphering the mechanisms whereby these proteins affect AD may be difficult because of their multifunctional nature. For example, LRP may mediate the clearance of Aβ in association with α2M and may be essential for Aβ transport across the blood-brain barrier (53). However, LRP may also mediate the transfer of APP into intracellular compartments where there is increased access to amyloidogenic proteinases (54).

Like LRP, α2M expresses multiple activities that may be involved in AD progression. Because α2M is a broad-spectrum proteinase inhibitor, which reacts with proteinases from all four major mechanistic classes (15, 55), various proteinases that are involved in Aβ catabolism, such as neprilysin, insulin- and plasmin (56–59), may be α2M targets. The possibility that α2M regulates the activity of proteinases involved in APP processing has also been considered; however, De Strooper et al. (60) demonstrated that this does not occur. The lack of an effect of α2M on APP processing is consistent with other studies demonstrating that α2M is poorly reactive with proteinases functioning at or near the cell surface (61).
The ability of α₂M to function as a carrier and deliver proteins to LRP for catabolism was first demonstrated with TGF-β and PDGF-BB (62, 63). Only activated α₂M is functional in this capacity, because native α₂M is not recognized by LRP (51). Growth factors, such as TGF-β, bind to native α₂M, as well as activated α₂M, and the resulting effects on growth factor activity are complicated. When bound to native α₂M, growth factors are typically inactive; however, because this interaction is reversible, α₂M-associated growth factors may provide a reservoir, buffering against changes in the free growth factor concentration (13, 64).

In our experiments, Aβ bound selectively to the LRP-recognized or activated form of α₂M, confirming the work of Narita et al. (33). To activate α₂M, we reacted the protein with methy­lamine. This reaction induces a conformational change in α₂M that is equivalent to the structural rearrangement induced by proteasines (11, 15, 44). In the ligand binding system, denatured α₂M subunits retained Aβ binding activity. This result suggests that α₂M tertiary and quaternary structures are not necessary for Aβ binding. Instead, we hypothesize that α₂M activation reveals an otherwise cryptic linear sequence of amino acids that constitute a binding site for Aβ. α₂M conformational change is also necessary for recognition by LRP; however, this interaction apparently requires retention of secondary and tertiary structure in the α₂M RBF (38, 41).

Although the primary sequence in α₂M that is responsible for the binding of growth factors is fairly promiscuous, this sequence did not interact with Aβ and apparently did not contribute to the Aβ binding activity of intact α₂M. Instead, a distinct protein-interaction site was identified, and our analysis of GST fusion proteins suggests that the center of this site is located between amino acids 1314 and 1365. The Aβ binding sequence identified in our experiments may be equivalent to the candidate Aβ-binding site identified by yeast-two-hybrid screen (37). Based on these results, we now propose that α₂M contains at least two distinct protein-interaction sequences that are functional even when higher order α₂M structure is eliminated. These two binding sites demonstrate distinct ligand binding specificities, because the growth factor-binding site in FP3 does not bind Aβ, and the Aβ-binding site in FP6 does not bind TGF-β, PDGF-BB, or NGF-β (18).

The LRP recognition sequence includes two Lys residues within an α helix that includes amino acids 1370–1377 (41, 42). In the intact three-dimensional structure of the α₂M RBF, the two Lys residues are oriented so that the side chains are readily available for interaction with LRP. Furthermore, the Lys residues are surrounded by a high density of hydrophobic surface residues. All of our evidence indicates that the Aβ-binding site and the LRP recognition sequence are adjacent but distinct. FP6c, which includes the entire α helix, did not bind Aβ, whereas FP6a, which lacks the α helix, did. Mutants of the RBF, which have been previously shown to not bind LRP (39, 40), still bound Aβ. Furthermore, a saturating concentration of Aβ did not inhibit the plasma clearance of α₂M-MMA, which is mediated by LRP (51). We propose that the center of the Aβ-binding sequence is located on the N-terminal side of the LRP recognition α helix. Comparison of the structure of RBFs from various α-macroglobulins has demonstrated that one surface of the RBF is highly conserved (41). Jenner et al. (41) proposed that the conserved surface is divided into two patches, one of which constitutes the LRP recognition site. Only speculation was offered regarding the function of the second patch, which includes amino acids from our fusion proteins that bind Aβ. The possibility that this second conserved surface patch represents an Aβ-binding site merits consideration.

Determining the binding affinity of Aβ for α₂M and its de-
