The Serpin-Enzyme Complex Receptor Recognizes Soluble, Nontoxic Amyloid-β Peptide but Not Aggregated, Cytotoxic Amyloid-β Peptide*

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There is now extensive evidence that amyloid-β peptide is toxic to neurons and that its cytotoxic effects can be attributed to a domain corresponding to amyloid-β 25–35, GSNGKAIIGLM. We have shown recently that the serine proteinase inhibitor (serpin)-enzyme complex receptor (SEC-R), a receptor initially identified for binding of α1-antitrypsin (α1-AT) and other serine protease inhibitors, also recognizes the amyloid-β 25–35 domain. In fact, by recognizing the amyloid-β 25–35 domain, SEC-R mediates cell surface binding, internalization, and degradation of soluble amyloid-β peptide. In this study, we examined the possibility that SEC-R mediates the neurotoxic effect of amyloid-β peptide. A series of peptides based on the sequences of amyloid-β peptide and α1-AT was prepared soluble in dimethyl sulfoxide or insoluble in water and examined in assays for SEC-R binding, for cytotoxicity in neuronal PC12 cells and murine cortical neurons in primary culture, and for aggregation in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The results show that amyloid-β peptide 25–35 and amyloid-β peptide 1–40 prepared soluble in dimethyl sulfoxide compete for binding to SEC-R, are nontoxic, and migrate as monomers in SDS-PAGE analysis. In contrast, the same peptides aged in water did not compete for binding to SEC-R but were toxic and migrated as aggregates in SDS-PAGE. An all-D-amyloid-β 25–35 peptide was not recognized at all by SEC-R but retained full toxic/aggregating properties. Using a series of deleted, substituted, and chimeric amβlα-1AT peptides, toxicity correlated well with aggregation but poorly with SEC-R recognition. In a subclone of PC12 cells which developed resistance to the toxic effect of aggregated amyloid-β 25–35 there was a 2.5–3-fold increase in the number of SEC-R molecules/cell compared with the parent PC12 cell line. These data show that SEC-R does not mediate the cytotoxic effect of aggregated amyloid-β peptide. Rather, SEC-R could play a protective role by mediating clearance and catabolism of soluble, monomeric amyloid-β peptide, if soluble amyloid-β peptide proves to be an in vivo precursor of the insoluble, toxic peptide.

The serpin1-enzyme complex receptor (SEC-R) was identified originally as a receptor of human mononuclear phagocytes and liver cells which recognized α1-antitrypsin (α1-AT)-elastase complexes and mediated feedback up-regulation of α1-AT biosynthesis (1). The receptor was characterized with a radioiodinated synthetic peptide, 125I-peptide 105Y (SIPPEVKFNK-PFYVL), bound on the surface of a candidate receptor binding domain in the carboxyl-terminal tail of α1-AT. This peptide mediated an increase in synthesis of α1-AT in monocytes and human hepatoma HepG2 cells, and binding studies showed that it described a receptor with a K_d of ~40 nM and ~450,000 plasma membrane receptors per cell (1). Binding of radioiodinated peptide 105Y was blocked by unlabeled α1-AT-elastase complexes, and binding of radioiodinated α1-AT-elastase complexes was blocked by unlabeled peptide 105Y (1), thus providing evidence that SEC-R was indeed a binding site for α1-AT-elastase complexes.

SEC-R is now known to have a ligand-binding subunit of ~84 kDa (2) which is expressed on a diverse array of cell types. It mediates endocytosis and intracellular degradation of α1-AT-elastase complexes (3) and mediates directed migration of neutrophils toward this ligand (4). In studies designed to define the minimal requirements for binding, we showed that a pentapeptide at the carboxyl-terminal aspect of peptide 105Y was sufficient for binding to SEC-R (5). This pentapeptide is highly conserved among members of the serpin supergene family. Several of the other serpins, such as α1-antichymotrypsin, antithrombin III, heparin cofactor II, and, to a lesser extent, C1 inhibitor and plasminogen activator inhibitor 1, when in complex with their cognate serine protease, compete for binding to SEC-R (1, 6). A similar pentapeptide sequence was identified in several tachykinins and amyloid-β peptide. In fact, competitive binding studies have shown that: (i) soluble amyloid-β peptide binds to SEC-R on HepG2 cells (2); (ii) soluble amyloid-β peptide binds to SEC-R on neutrophils, mediating chemotactic activity and conferring homologous desensitization to the chemotactic activity of peptide 105Y (4); (iii) soluble amyloid-β peptide binds to SEC-R on neuronal cells (7); (iv) the region corresponding to amyloid-β peptide 25–35, and particularly amyloid-β 31–35, is critical for binding to SEC-R on any of these cell types (7); (v) amyloid-β 1–39, amyloid-β 1–40, and amyloid-β 1–42, when prepared in soluble form in Me2SO, present this binding domain to SEC-R to an equivalent extent (7); (vi) SEC-R mediates endocytosis and catabolism of soluble amyloid-β peptide in hepatocytic and neuronal cell types (7).

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1 The abbreviations used are serpin, serine proteinase inhibitor; SEC-R, serpin-enzyme complex receptor; α1-AT, α1-antitrypsin; Me2SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; amyil, amyloid β.
The data regarding amyloid-β binding to SEC-R are particularly interesting in that neurotoxic effects have been attributed to this peptide and have been considered in the common final pathway for neuronal degeneration and presenile dementia of Alzheimer’s disease and Down’s syndrome (8, 9). Although the minimal peptide sequence for the neurotoxic effect of amyloid-β, amyloid-β 25–35 correlates with the minimal peptide sequence for binding to SEC-R, several recent studies have suggested that amyloid-β 25–35 must be aggregated to induce its toxic effects (9–16). In this study we compared monomeric, soluble amyloid-β peptide with its aggregated form to determine the correlation between SEC-R binding and cytotoxic activities.

EXPERIMENTAL PROCEDURES

Materials—Amyloid-β 1–40 was purchased from Bachem California. Peptide 105Y, amyloid-β 25–35, amyloid-β 31–35, Yαmį 25–35, and all other deleted, substituted, swapped, and chimeric peptides were synthesized by the solid phase method, purified, and subjected to amino acid composition and sequence analysis as described previously (5). Peptides were prepared by dissolving in Me2SO at 8 mg/ml or aging in water at 4 °C for 5 days. These conditions have been shown, in previous studies (7–13, 15–17), to result in predominant monomeric peptide and a predominately aggregated peptide, respectively, as evidenced by sedimentation analysis, spectrophotometric analysis of turbidity, light microscopy, electron microscopy, circular dichroism, as well as by SDS-PAGE analysis.

Cell Lines—Maintenance of HepG2 cells has been described previously (3). PC12 cells were kindly provided by Drs. Eugene J. Johnson and Karen O’Malley (St. Louis, MO). Human glioblastoma U373MG cells, HeLa cells, and Chinese hamster ovary cells were purchased from ATCC.

Murine Cortical Cultures—Dissected murine cortical cultures containing both neurons and glia were prepared from fetal (E15) Swiss Webster mice and subcultured in 24-well plates on an established monolayer of glial cells (15–30 days in vitro) as described previously (18). Glial cell division was inhibited after 3–5 days by a 48-h incubation in medium supplemented with cysteine arabinoside (10 μM).

Determination of Cell Surface Receptor Binding—Peptides 105Y and Yαmį 25–35 were radioiodinated by the chloramine-T method (1). 125I-β amyloid-β 25–35, and all other deleted, substituted, swapped, and chimeric peptides were purified on Sephadex G-10. Specific radioactivity varied from 25,000 to 35,000 cpm/ng. For binding studies in HepG2 cells, separate monolayers in 24-well plates were dunked in phosphate-buffered saline and incubated for 2 h at 4 °C in 125I-labeled ligand in the absence or presence of 1000-fold molar excess unlabeled competitor. Nonspecific binding did vary from 8 to 30% defined by an increase in LDH release, decrease in [3H]thymidine incorporation, and abnormal morphology (19, 20). First, we examined the effect of amyloid-β 25–35 (19), was selected. Toxicity was measured by MTT reduction assay. Inhibition of MTT reduction by PC12 cells has been shown to be an early indicator of cell death as defined by an increase in lactate dehydrogenase (LDH) release, DNA fragmentation, and abnormal morphology (19, 20). First, we examined the effect of amyloid-β 25–35 and other amyloid peptides on MTT reduction in PC12 cells (Fig. 1). In each case the peptides were prepared in water and incubated at 37 °C for 5 days to optimize formation of aggregates. The results showed a concentration-dependent inhibition of MTT reduction by amyloid-β 25–35 and amyloid-β 1–40, but not by amyloid-β 1–16 or amyloid-β 1–28 (Fig. 1A). The effect of amyloid-β 25–35 on MTT reduction by PC12 cells is half-maximal at 8–10 nM. Next, we examined the possibility that the neurotoxic effect of amyloid-β 25–35 involved an apoptotic mechanism. PC12 cells were incubated with actinomycin D or cycloheximide and then subjected to electrophoresis (Fig. 1B). The mechanism of amyloid-induced neurotoxicity was also examined by agarose gel electrophoresis (Fig. 1C). There was evidence for intranuclear DNA fragmentation in response to amyloid-β 25–35 (left lane). This DNA fragmentation was similar to that induced by a positive control, serum starvation (middle lane). For a negative control, we examined glutamate (right lane). Glutamate is known to induce a toxic, nonapoptotic effect in primary culture of cortical...
neurons (22). At 1 μM it also has a toxic effect on PC12 cells as reported in Ref. 23 and determined by us in the MTT assay (data not shown). However, it did not cause DNA fragmentation in PC12 cells (right lane). Thus, the effect of amyloid-β 25–35 on DNA fragmentation in PC12 cells is specific. These data provide evidence for a specific apoptotic effect of amyloid-β 25–35 in a neuronal cell line using a relatively simple and reproducible assay. We could now examine whether this effect was mediated by the SEC-R.

**Cell Type Specificity of Amyloid-β-induced Cytotoxicity**—We examined whether the toxicity of amyloid-β 25–35 was confined to cell types that express SEC-R (Fig. 2). Three cell lines that express SEC-R, neuronal cell line PC12, hepatoma cell line HepG2, and glial cell line U373MG, and two cell lines that do not express SEC-R, HeLa and Chinese hamster ovary, were subjected to MTT assay after incubation in amyloid-β 25–35 under conditions associated previously with toxicity in PC12 cells, as shown in Fig. 1. The results show some variability in susceptibility to the neurotoxic effect among these cell lines with PC12 cells being most susceptible. However, there was inhibition of MTT reduction in all five cell lines, suggesting that the toxic effect is independent of SEC-R expression.

**Correlation among SEC-R Binding, Toxicity, and Aggregation of Amyloid-β Peptides**—We used a panel of assays to examine the correlation of SEC-R binding, toxicity, and aggregation of amyloid-β peptides. For SEC-R binding, we examined the competition of unlabeled amyloid-β peptides for binding of 125I-peptide 105Y to HepG2 cells. In each case, with a limited number of replicate samples, the results were almost identical for binding of 125I-Yamβ 25–35 to HepG2 cells or for binding of 125I-peptide 105Y or 125I-Yamβ 25–35 to PC12 cells (data not shown). For toxicity we used the MTT reduction assay in PC12 cells. For assessment of peptide aggregation, we used SDS-PAGE analysis. Each peptide was examined in presumed soluble form by dissolving it in Me2SO and in presumed aggregated form by aging it in water for 5 days at 37 °C as described...
Cytotoxic and SEC Receptor Binding Properties of Amyloid-β Peptide

Fig. 2. Cell type specificity of amyloid-β 25–35-induced toxicity. Human hepatoma HepG2, glioblastoma U373MG, neuronal PC12, epidermal HeLa cells, and Chinese hamster ovary (CHO)-K1 cells at a density of 1 x 10⁴ cells/ml were incubated with amyloid-β 25–35 (aged in water) at several different concentrations and then subjected to MTT assay exactly as described in the legend for Fig. 1.

by previous studies (7–15).

First, we examined amyloid-β 25–35 (Fig. 3A). The data showed that amyloid-β 25–35 prepared in Me2SO was much better than amyloid-β 25–35 aged in water as a competitor for binding to SEC-R (left panel). However, amyloid-β 25–35 1–40 prepared in Me2SO, was toxic (right panel). The results were almost identical when amyloid-β 1–40 was subjected to this comparison (Fig. 3B). Amyloid-β 1–40 prepared in Me2SO was a much better competitor than amyloid-β 1–40 aged in water for binding to SEC-R (left panel). However, amyloid-β peptide prepared in Me2SO had minimal toxicity, and that minimal toxicity was elicited at a significantly higher concentration than the potent toxic effect of amyloid-β 1–40 aged in water (right panel). There was ~15% inhibition of MTT reduction at 10 μM amyloid-β 1–40 prepared in Me2SO but almost 60% inhibition of MTT reduction at 1 μM amyloid-β 1–40 aged in water. For each of these experiments the amyloid-β peptide was prepared in Me2SO in such a way that the final concentration of Me2SO was 0.12% or less. Me2SO at 0.2% did not alter SEC-R binding for amyloid-β 25–35 (Fig. 3C). The results showed that amyloid-β 25–35 prepared in Me2SO migrated predominantly as a single band at the leading edge, and soluble amyloid-β 1–40 migrated predominantly as a single band at ~4.5 kDa. Almost all of the amyloid-β 25–35 prepared in Me2SO was retained at the top of the gel. A significant proportion of the amyloid-β 1–40 preparation that had been aged in water was retained at the top of the gel or spread at slower electrophoretic mobility. These results are similar to those shown in many previous studies (9, 10, 12, 14, 16). It was therefore possible to call the peptides prepared in Me2SO predominantly soluble or predominantly monomeric and the same peptides aged in water predominantly aggregated. It was also possible to conclude that these peptide preparations were similar to those of previous studies in which amyloid-β peptide neurotoxicity has been characterized. Taken together, these data suggest that soluble, predominantly monomeric amyloid-β peptide is recognized by SEC-R and is nontoxic, whereas aggregated amyloid-β peptide is poorly recognized by SEC-R and is toxic.

Previous studies have shown that human amylin, a peptide that is unrelated to amyloid-β peptide in primary sequence but can form a β-helical fibrillar structure, is similar to amyloid-β peptide in cytotoxic effects (11, 15). We examined the capacity for human amylin to compete for SEC-R binding and to mediate a cytotoxic effect in our system. The results showed that amylin does not compete for binding of peptide 105Y, even when prepared soluble in Me2SO, but was instead as toxic as amyloid-β peptide when aged in water or prepared soluble in Me2SO (data not shown).

We also compared amyloid-β 25–35 with the original α1-AT peptide, peptide 105Y, in terms of SEC-R binding properties and toxicity (Fig. 4A). When prepared in Me2SO both are effective competitors for SEC-R binding (left panel) and are nontoxic (right panel). When aged in water, however, there is a marked reduction in SEC-R binding for amyloid-β 25–35 (left panel), even though it is now quite toxic (right panel). When aged in water, there is no reduction in the capacity of peptide 105Y to compete for binding to SEC-R (left panel) and no development of toxic properties (right panel).

These results could be interpreted in two ways. The most likely explanation is that the toxic effect of amyloid-β 25–35 requires that it assume a particular conformation that is not recognized by SEC-R and thus is not mediated by SEC-R. Because previous studies have shown that the carboxyl-terminal pentapeptides of peptide 105Y and soluble amyloid-β 25–35 are essential for SEC-R binding (5, 7), there was still a remote possibility that toxicity required both the binding properties of the carboxyl-terminal pentapeptide and the signal transduction properties conferred by the amino-terminal domain of amyloid-β 25–35, but not by the corresponding domain of peptide 105Y. To exclude this possibility we generated two chimeric α1-AT/amylin amino-terminal and carboxyl-terminal domains (Fig. 4B). The results showed that the α1-AT/amylin chimeric peptide competed effectively for SEC-R binding (Fig. 4A, left panel) but was nontoxic (right panel) when aged in water or prepared in Me2SO. The α1-AT/chimeric peptide did not compete for SEC-R binding when aged in water and was a poor competitor when prepared in Me2SO (left panel). However, the amylin/am1-AT chimeric peptide was toxic as amyloid-β 25–35 when aged in water and was almost as toxic when prepared in Me2SO (right panel). To determine whether there was a correlation among loss of recognition by SEC-R, development of toxic properties, and formation of aggregates, we subjected peptide 105Y, α1-AT/amylin, and α1-AT/chimeric peptides to SDS-PAGE (Fig. 4B). The results show that the nontoxic peptides α1-AT/amyl and 105Y migrate predominantly as monomers, whereas the toxic peptides α1-AT/chimeric is retained predominantly at the top of the gel in a manner identical to that of the amyloid-β 25–35 peptide. The α1-AT/chimeric peptide was the only peptide that was toxic when prepared in Me2SO. It was also retained predominantly at the top of SDS gels, whereas peptide 105Y Me2SO, amyloid-β 25–35 Me2SO, and α1-AT/amyl Me2SO migrated predominantly as monomers (Fig. 4C). The results of this SDS-PAGE analysis are similar to results for typical predominantly monomeric and predominantly aggregated peptides in previous studies (9, 10, 12, 14, 16).

We also examined the amino-terminal and carboxyl-terminal domains of amyloid-β 25–35 as isolated peptides (Fig. 4D). The results show that amyloid-β 31–35 competes for binding to...
SEC-R (left panel), confirming previous results (7). Amyloid-β 25–30 does not compete for binding to SEC-R (left panel), and neither peptide is toxic by itself (right panel) or when added together (data not shown).

To exclude the possibility that these results were unique to the PC12 neuronal cell line and the MTT assay, we examined the same peptides for toxicity in primary cultures of murine cortical neurons using morphology and LDH release assays (Fig. 5). After 9 days in culture, separate cultures were incubated for 48 h in medium alone or medium supplemented with...
specific synthetic peptides. By morphologic criteria there was degeneration of neurons mediated by amyloid-β aged in water and by the amβ/a1-AT chimera, but not by medium alone, by the amβ/a1-AT chimera aged in water, peptide 105Y aged in water, or the amβ/a1-AT chimera prepared in DMSO (panels A–F). By LDH assays, there was neuronal cell death mediated in a concentration-dependent manner by amyloid-β, amβ/a1-AT chimera prepared in water, amβ/a1-AT chimera aged in water, or amβ/a1-AT chimera prepared in DMSO. By MTT assay, there was no cell death in response to amβ/a1-AT chimera, peptide 105Y aged in water, or the amβ/a1-AT chimera aged in water (panel A–F). The concentration of peptide required for toxic effects (half-maximal at ~20 μM) is very similar to that reported previously by other laboratories using morphology and LDH assays in primary culture (8, 10–16). These results provide further evidence for the high degree of correlation between the MTT assay in PC12 cells and more conventional assays for toxicity in neurons in primary culture. In fact, the MTT assay is apparently more sensitive than either morphologic or LDH assays. In all three assay systems the amβ/a1-AT chimera has the most potent toxic effect on neuronal cells.

Taken together, these data are most likely explained by the capacity of amyloid-β 25–35, when aged in water, and amβ/a1-AT, when aged in water or prepared in DMSO, to aggregate into a conformation that is not recognized by SEC-R and, thus, not delivered into the endocytic pathway for catabolism. This conformation also confers cytotoxic properties on the peptide. The amino-terminal domain, amyloid-β 25–30, is critical but not sufficient for conferring the aggregating/toxic properties.
Fig. 5. Toxic properties of α1-AT peptides, amyloid-β peptides, and chimeric α1-AT/amyloid-β peptides in cultured murine cortical neurons. Panels A–F, photomicrographs of cortical cultures. After 9 days in culture, separate monolayers were incubated for 48 h in medium alone (panel A) or medium supplemented with peptides at a final concentration of 20 μM. Panel B, amyloid-β 25–35 aged in water. Panel C, α1-AT/amyloid-β chimeric peptide aged in water. Panel D, α1-AT/amyloid-β chimeric peptide aged in water. Panel E, peptide 105Y aged in water. Panel F, amyloid-β 25–35 prepared in Me_2SO. Arrows point to degenerated neurons in panels B and C. The bar in the right lower corner of panel F represents 50 μm. Panel G, dose-response curves of neuronal degeneration induced by exposure to the synthetic peptides as assessed by LDH release. Cultures were incubated for 48 h in medium alone, medium supplemented with N-methyl-D-aspartic acid (NMDA; 500 μM), or medium supplemented with...
\(a\)-1-AT-derived peptide 105Y and amyloid-\(\beta\) 25-35, we again examined the sequences of these two peptides (Fig. 4A, top). First, compared with the native \(a\)-1-AT sequence (shown in the top line), there are two substitutions in peptide 105Y: Phe at amino acid 372 was replaced by Tyr for iodination, and Met at amino acid 372 was replaced by Ile for ease of synthesis. However, neither of these substitutions affected the properties of the peptide because a new peptide, which was identical to the \(a\)-1-AT sequence in the corresponding region, had identical properties in binding, toxicity, and SDS-PAGE assays (data not shown). Second, compared with amyloid-\(\beta\) 25-35 (Fig. 4A, top), peptide 105Y has five additional amino acids (SIPPE) at its amino terminus. These additional amino acids do not affect the properties of peptide 105Y because peptide 105BC, which is identical to peptide 105Y except that it is missing the five amino-terminal amino acids (Fig. 6, top), had properties identical to those of peptide 105Y in binding, toxicity, and SDS-PAGE assays (Fig. 6 and data not shown). Peptide 105BC prepared in \(\text{Me}_2\text{SO}\) or aged in water competes for binding to SEC-R, is nontoxic, and migrates as a monomer in SDS-PAGE.

Next, we compared the sequences in the amino-terminal domains of peptides 105BC and amyloid-\(\beta\) 25-35 (Fig. 6, top) and noticed an NK sequence in both. In amyloid-\(\beta\) 25-35 it is separated by one amino acid, P. We examined the effect of swapping these two domains on SEC-R binding, toxicity, and aggregation (Fig. 6). In the case of \(a\)-1-AT, the insertion of GA for P (swap 2) did not affect its properties. The swapped peptide competed effectively for SEC-R binding (left panel), was not toxic (right panel), and migrated as a monomer on SDS-PAGE (data not shown). However, for amyloid-\(\beta\) 25-35, the substitution of P for GA (swap 1) made the peptide an effective competitor for SEC-R (left panel), abrogated toxicity (right panel), and it migrated as a monomer when in \(\text{Me}_2\text{SO}\) or aged in water (data not shown). These data indicate that the GA sequence at amyloid-\(\beta\) 29-30 is important for the toxic/aggregating properties of amyloid-\(\beta\) 25-35. We also examined the importance of the II sequence at amyloid-\(\beta\) 31-32. Replacement of these two residues by TT (amyloid-\(\beta\) 25-35 TT) had interesting effects. There was a marked reduction in both competitive binding efficacy of amyloid-\(\beta\) 25-35 TT (left panel) and in its toxic properties (right panel) whether prepared in \(\text{Me}_2\text{SO}\) or aged in water. In SDS-PAGE analysis, there was no evidence for aggregation of amyloid-\(\beta\) 25-35 TT even when aged in water (data not shown). These data suggest that amyloid-\(\beta\) 31-32 II is important for SEC-R binding of peptide dissolved in \(\text{Me}_2\text{SO}\) and important for toxic/aggregating properties of peptide aged in water.

Next, we examined the effect of deletions on the cell surface binding properties and toxicity of amyloid-\(\beta\) peptide (Fig. 7A). The results show that deletion of two or three carboxyl-terminal residues, as exemplified by peptides amyloid-\(\beta\) 25-33 and Yam\(\beta\) 22-32, is associated with loss of both SEC-R binding and toxicity. This effect is not due to reduction in the length of the peptide as shown by peptide Yam\(\beta\) 22-32, in which there is deletion of three carboxyl-terminal residues, but inclusion of four additional residues, derived from the sequence of amyloid-\(\beta\) peptide, at the amino terminus. This peptide does not compete for binding to SEC-R and does not have toxic effects. Deletion of two amino-terminal residues in peptide amyloid-\(\beta\) 27-35 has no effect on binding to SEC-R but is associated with loss of toxic effects. Amyloid-\(\beta\) 27-35 prepared in water and aged to optimize aggregate formation still competes for binding to SEC-R (left panel), has minimal toxicity (right panel), and migrates as a monomer on SDS-PAGE (data not shown). These data suggest that deletion of residues at either end of amyloid-\(\beta\) 25-35 prevents aggregate formation and toxicity, but only deletion of the carboxyl-terminal residues, within the SEC-R binding pentapeptide domain, affects SEC-R binding.

We also examined the effect of substitutions on the cell surface binding properties and toxicity of amyloid-\(\beta\) peptide (Fig. 7B). The results show that substitution of alanine for the carboxyl-terminal methionine (amyloid-\(\beta\) 25-35 35A) reduces competitive binding efficacy, but substitution of alanine for

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**Fig. 6.** Cell surface binding and toxic properties of \(a\)-1-AT peptides and amyloid-\(\beta\) 25-35 peptides with swapping or substitutions in central regions of the linear sequence. Methods are exactly as described in the legend to Fig. 3.
amino-terminal glycine (amyloid-β 25–35 25A) or substitution of alanine for serine 26 (amyloid-β 25–35 26A) does not reduce SEC-R binding when peptide is prepared in Me 2SO (left panel). These results are again consistent with the importance of the carboxyl-terminal domain, and particularly the carboxyl-terminal methionine, in recognition by SEC-R (2, 5). There is a marked reduction in SEC-R binding of all these substituted peptides when they are prepared in water and aged (left panel). All three substituted peptides retain some toxicity when they are prepared in water and aged (left panel), and this correlates with marked slowing of electrophoretic mobility on SDS-PAGE (data not shown). The rank order of cytotoxic potency is amyloid-β 25–35 > amyloid-β 25–35 25A > amyloid-β 25–35 26A > amyloid-β 25–35 35A. Toxicity is reduced markedly when these peptides are prepared soluble in Me 2SO (right panel), and this correlates with migration as monomers on SDS-PAGE (data not shown). These data again show that toxicity correlates most closely with aggregating ability as determined by SDS-PAGE. The carboxyl-terminal methionine residue is critical for SEC-R binding of amyloid-β 25–35. Substitution of the carboxyl-terminal methionine and the amino-terminal glycine and serine residues by alanine has minimal effects on aggregating and toxic properties.

Finally, we examined the SEC-R binding and toxicity of All-D-amyloid-β 25–35 (Fig. 8). The results show that D-amyloid-β 25–35 does not compete for binding to SEC-R in either Me 2SO or water but remains a potent toxin when aged in water and, to a lesser extent, when prepared in Me 2SO. Substitution of the II sequence of D-amyloid-β 25–35 residues 31–32 with TT completely abrogated toxicity. The toxicity of All-D-amyloid-β 25–35 peptide correlated exactly with aggregation as determined by SDS-PAGE (data not shown). Results of studies in murine cortical neurons using morphologic and LDH assays also indicated that the All-D-amyloid-β 25–35 peptide was toxic, although somewhat less toxic than the L-enantiomer. Substitution of the II sequence at residues 31–32 with TT completely abrogated toxicity (data not shown).

These data provide strong evidence that SEC-R does not mediate the toxic effect of amyloid-β 25–35. Rather, the toxic effect is conferred by amyloid-β peptide, which is capable of forming aggregates and, in this state, is no longer recognized by SEC-R. Although the D-enantiomer of amyloid-β 25–35 is ca-
pable of conferring toxic/aggregating properties, there is sequence specificity required for this effect in that replacement of amyloid-β 31–32II by TT completely reverses these properties.

Effect of Blocking SEC-R on the Toxic Properties of Amyloid-β 25–35—Next, we examined the effect of blocking SEC-R with nontoxic ligand peptide 105Y on the cytotoxicity of aggregated amyloid-β peptide. Initially, we found that preincubation and/or coincubation of PC12 cells with peptide 105Y did not decrease the toxic effect of amyloid-β 25–35 aged in water. If anything, peptide 105Y potentiated the toxic effect of amyloid-β 25–35 (data not shown). To provide further evidence for this potentiation effect, we used the MTT assay (Fig. 9) on PC12 cells that were preincubated with peptide 105Y, irrelevant peptide, or no peptide. The PC12 cells were then incubated with the amß4α1-AT chimeric peptide prepared in Me2SO or the amyloid-β 25–34A peptide aged in water. Each of these peptide preparations is suboptimal for toxicity and, therein, would allow potentiation of toxicity to be detected. The results showed that preincubation with peptide 105Y, but not irrelevant control peptide, potentiated the toxicity in each case even though it had no toxic effect by itself. The mechanism for this potentiation is not yet known.

Binding to SEC-R in an Amyloid-β Peptide-resistant PC12 Cell Line—Behl et al. (11) have shown that PC12 cells become resistant to the toxic effect of amyloid-β peptide if they are grown in the presence of aggregated amyloid-β 25–35 for a number of passages. We examined the possibility of establishing such a cell line and determining its SEC-R binding characteristics. PC12 cells were incubated with 20 μM amyloid-β 25–35 aged in water for 10 passages. An aliquot of these cells was then grown in the absence of amyloid-β 25–35 for one passage. MTT assays showed that these cells were, indeed, resistant to the toxic effects of aggregated amyloid-β 25–35 (Fig. 10A). The same cells were then subjected to SEC-R binding studies (Fig. 10B). In the amyloid-β- resistant subclone there was specific and saturable binding of 125I-peptide 105Y with a plateau reached at 60 nM (left panel). Binding of 125I-peptide 105Y to the resistant subclone was then compared with that of the parent cell line (right panel). The results show that SEC-R binding does not decrease in the PC12 cell line that has become resistant to the toxic effect of aggregated amyloid-β 25–35. In fact, Scatchard plot analysis indicates that there are \(-1.1 \times 10^6\) SEC-R molecules/cell in the amyloid-β-resistant subclone, 2.5–3-fold higher than the 4.33 \(\times 10^5\) SEC-R molecules/cell in the parental PC12 cells. The \(K_d\) for binding of 125I-peptide 105Y in the amyloid-β-resistant PC12 subclone was 48.3 nM compared with 43.7 nM for the parental PC12 cells (7). There was no change in the specificity of SEC-R binding in the amyloid-β-resistant subclone of PC12 in that binding of 125I-peptide 105Y to these cells was blocked by amyloid-β 25–35 prepared in Me2SO, but not by amyloid-β 25–35 aged in water (data not shown).

We also examined the effect of human amylin on cytotoxicity in the amyloid-β-resistant subclone of PC12 compared with the parent PC12 cell line (Fig. 10C). Although amylin had a potent toxic effect on the parent, it did not alter the subclone at all. Taken together, these data provide initial evidence that the development of resistance to amyloid-β cytotoxicity is a general rather than a specific phenomenon.

DISCUSSION

A number of studies have shown that amyloid-β peptide is toxic to neurons and that its toxic properties may be fundamentally important in the common final pathway for neuronal degradation in Alzheimer’s disease (8, 9). Moreover, the amyloid-β 25–35 region and an aggregated state appear to be required for full neurotoxic potential (9–16). In this study, we examined the role of SEC-R in the neurotoxic effects of amyloid-β peptide. SEC-R is expressed on many cell types, including neurons and glial cells. It mediates endocytosis and degradation of soluble amyloid-β peptide by recognizing the amyloid-β 25–35 region in a sequence-specific manner (7). Using an assay for cellular redox activity which is indicative of amyloid-β toxicity and an assay for DNA fragmentation in the neuronal cell line PC12 as well as conventional morphologic and LDH release assays in primary cultures of murine cortical neurons, our results confirm previous studies indicating that amyloid-β peptide must be aggregated to induce its neurotoxic effects. SEC-R does not recognize amyloid-β peptides that are aggregated and toxic and, therefore, cannot be implicated in mediating the neurotoxicity of amyloid-β peptide. SEC-R does recognize the same peptides when in a soluble, nontoxic state.

Our conclusions are based on several major results. First, aggregated amyloid-β 25–35 is cytotoxic in two cell lines that do not express SEC-R, or at most, have negligible levels of SEC-R expression (Fig. 2). Second, amyloid-β 25–35 and amyloid-β 1–40 are recognized by SEC-R, are nontoxic, and migrate in SDS-PAGE as monomers when prepared in Me2SO, but are
not recognized by SEC-R, are toxic, and are aggregated when aged at 37 °C for 5 days in water (Fig. 3). Third, the D-enantiomer of amyloid-β 25–35 is not recognized by SEC-R when prepared in either Me2SO or water but is toxic when prepared as an aggregate in water (Fig. 8).

Substitutions, deletions, and swapping of domains within the amyloid-β 25–35 peptide also demonstrate multiple examples of dissociation of SEC-R binding from toxic properties. For example, substitution of the amino-terminal domain of amyloid-β 25–35 with the corresponding domain of α1-AT results in a peptide, the α1-AT/amloid peptide, which is now well recognized by SEC-R, is no longer toxic, and behaves as a monomer in SDS-PAGE even when aged in water at 37 °C for 5 days (Figs. 4 and 5). The opposite, the amloid peptide, is poorly recognized by SEC-R because it aggregates and induces a potent toxic effect on PC12 cells. A second example, the α1-AT peptide 105Y, is soluble and behaves as a monomer in either Me2SO or aged in water. It binds well to SEC-R but is not toxic (Figs. 4 and 5). Third, deletion of the amino-terminal domain of amyloid-β 25–35 (peptide 11GLM) minimally reduces SEC-R binding but completely abrogates toxicity (Fig. 4D). Fourth, swapping of the central glycine-alanine domain of amyloid-β 25–35, amyloid-β 29–30, for the corresponding domain of α1-AT, prolinc 369, results in a peptide that is recognized by SEC-R but nontoxic and migrates as a monomer in SDS-PAGE (Fig. 6). Fifth, deletion of amyloid-β 25–26 results in a peptide, amyloid-β 27–35, which still competes for SEC-R binding but is nontoxic and migrates as a monomer on SDS-PAGE (Fig. 7). Sixth, replacement of amyloid-β 35 methionine by alanine markedly reduces SEC-R binding but only minimally affects toxicity. The effect of this substitution is highly specific since replacement of amyloid-β 25 or 26 by alanine has minimal effects on SEC-R binding or toxicity (Fig. 7).

Several other experimental results show that SEC-R does not recognize aggregated amyloid peptide and does not mediate its cytotoxic effect. Blocking of SEC-R with the nontoxic ligand peptide 105Y does not block the toxic effect of aggregated amyloid-β 25–35 (Fig. 9). In fact, it potentiated the toxic effect of amyloid-β peptide preparations. Finally, when compared with the parental PC12 cell line there was an increase in the number of SEC-R molecules/cell for a subclone of PC12 cells which had developed resistance to the cytotoxic effect of amyloid-β 25–35 (Fig. 10).

The results of this study also provide further evidence that the neurotoxic effects of amyloid-β peptide depend on its capacity to form aggregates. There is excellent correlation between toxicity as defined by the MTT assay and migration as an aggregate in SDS-PAGE. Moreover, acquisition of toxic and aggregating properties, as defined by these assays, is closely correlated with loss of recognition by SEC-R. By virtue of the correlation between these different types of assays, the results also provide internally consistent information about the structural requirements for the toxic/aggregating properties of amyloid-β peptide. For example, deletion of two amino acids at either the amino or carboxyl terminus is associated with a loss of toxic/aggregating properties (Fig. 7). This is not simply an issue of length, however, since Yαmβ 22–32 does not aggregate or have toxic effects. Replacement of amyloid-β 25, 26, or 35 with alanine is associated with a mild reduction in toxic/aggregating properties. Second, replacement of amyloid-β 31–32 I1 with TT is associated with loss of toxic/aggregating properties (Fig. 6). Third, replacement of amyloid-β 25–30 GSNKGA with the α1-AT sequence VKFNFKP is associated with a loss of aggregation, whereas replacement of amyloid-β 31–35 I1GLM with the α1-AT sequence FVFLM does not affect toxic/aggregating properties (Figs. 4 and 5). In fact, the αm/α1-AT chimeric peptide has an even greater tendency toward aggregation/toxicity than amyloid-β 25–35 itself. The amyloid-β 29–30 GA sequence may be particularly important. Replacement by P, which is in the corresponding position in α1-AT, abrogates toxic/aggregating properties. However, insertion of GA in the middle of the corresponding α1-AT sequence did not confer new aggregating/toxic properties on it. The structural requirements for the toxic/aggregating properties of amyloid-β peptide defined by our data using the MTT assay in PC12 cells are similar to those defined by morphometry and LDH assays in primary cultures of neurons in this study (Fig. 5) and in the studies of
Several lines of evidence suggest that these only represent the requirements for formation of the aggregated/fibrillar conformation and not for the toxic interaction with cells. For example, the observation that All-D-amyloid-β25–35 forms aggregates and elicits a toxic effect implies that the toxic interaction with cells is not sequence-specific. Lorenzo et al. (15) showed similar toxic effects in neurons and pancreatic islet cells using amylin, a peptide with a completely different sequence but with a tendency to aggregate. Our data also suggest that the toxic effect of amyloid-β peptide is not cell type-specific. So far there is evidence for toxic effects in every cell type that we have exposed to aggregated amyloid-β25–35.

Using model cell systems and conditions designed to exaggerate the soluble and aggregated states of amyloid-β peptide in this and our previous study (7), we could clearly distinguish the fate of soluble and aggregated forms of this peptide. The soluble form of amyloid-β peptide is recognized by SEC-R and delivered by endocytosis to an intracellular vesicular compart-

Fig. 10. Cytotoxicity and SEC-R binding in an amyloid-β peptide-resistant PC12 subclone. PC12 cells were incubated with 20 μM aggregated amyloid-β 25–35 for 10 passages. An aliquot of these cells was then grown in the absence of amyloid-β 25–35 for one passage. Panel A, toxic effect of aggregated amyloid-β 25–35 in the amyloid-β-resistant subclone compared with the parent PC12 cells. Results are shown as the mean ± 1 S.D. for four separate determinations at each point. Panel B, binding of 125I-peptide 105Y to the amyloid-β-resistant subclone of PC12 cells. Total, specific, and nonspecific binding are shown in the left panel. The subclone is compared with its parent for specific binding alone in the right panel. Panel C, toxic effect of human amylin in the amyloid-β resistant-subclone compared with the parent PC12 cells. Results are shown as the mean ± 1 S.D. for four separate determinations at each point.
ment for degradation. The same peptide, when aggregated, is not recognized by SEC-R but can interact with the plasma membrane of the target cell in a relatively specific manner to elicit a completely different biologic effect, apoptosis. However, these studies do not address the relationship between soluble and aggregated forms of amyloid-β peptide and SEC-R under physiologic and pathophysiologic conditions, which are likely to be far more complex. In future studies, it will be essential to provide quantitative information about the conversion of soluble amyloid-β peptide to its aggregated form, about the relative reversibility of this conversion, about the relationship of this conversion to the kinetics (on-rate and off-rate) with which soluble amyloid-β peptide binds to SEC-R, and about the relationship of this conversion to the kinetics and specificity with which aggregated amyloid-β peptide interacts with the target cell membrane in a SEC-R-independent manner. Finally, it will be essential to consider other factors such as apolipoprotein E (24, 25), α1-antichymotrypsin (26, 27), transthyretin (28), zinc (29), and the nonamyloid components of Alzheimer's disease amyloid (30), which are likely to regulate these relationships in physiologic and pathophysiologic states in vivo.

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