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Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis

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ABSTRACT **Extracellular deposition of amyloid fibrils is responsible for the pathology in the systemic amyloidoses and probably also in Alzheimer disease [Haass, C. & Selkoe, D. J. (1993) *Cell* 75, 1039–1042] and type II diabetes mellitus [Lorenzo, A., Razzaboni, B., Weir, G. C. & Yankner, B. A. (1994) *Nature (London)* 368, 756–760]. The fibrils themselves are relatively resistant to proteolysis *in vitro* but amyloid deposits do regress *in vivo*, usually with clinical benefit, if new amyloid fibril formation can be halted. Serum amyloid P component (SAP) binds to all types of amyloid fibrils and is a universal constituent of amyloid deposits, including the plaques, amorphous amyloid β protein deposits and neurofibrillary tangles of Alzheimer disease [Coria, F., Castano, E., Prelli, F., Larrondo-Lillo, M., van Duinen, S., Shelanski, M. L. & Frangione, B. (1988) *Lab. Invest.* 58, 454–458; Duong, T., Pommier, E. C. & Scheibel, A. B. (1989) *Acta Neuropathol.* 78, 429–437]. Here we show that SAP prevents proteolysis of the amyloid fibrils of Alzheimer disease, of systemic amyloid A amyloidosis and of systemic monoclonal light chain amyloidosis and may thereby contribute to their persistence *in vivo*. SAP is not an enzyme inhibitor and is protective only when bound to the fibrils. Interference with binding of SAP to amyloid fibrils *in vivo* is thus an attractive therapeutic objective, achievement of which should promote regression of the deposits.**

Amyloid fibrils are composed only of autologous proteins and glycosaminoglycans (1), and they should therefore be degradable *in vivo*. Indeed, when production of the protein precursors of amyloid fibrils can be sufficiently reduced, specific noninvasive scintigraphic imaging of amyloid shows that the deposits do regress slowly over months or years (2–4). There is also *in vitro* and neuropathologic evidence of a dynamic balance between deposition and resolution of the cerebral amyloid β protein (Aβ) lesions in Alzheimer disease (5, 6). The usual apparent irreversibility of amyloid in clinical practice thus largely reflects the progressive nature of the incurable conditions that underlie most forms of amyloidosis. However, there are also likely to be mechanisms other than the intrinsic properties of the fibrils that contribute to their persistence *in vivo*.

Serum amyloid P component (SAP) is a universal constituent of amyloid deposits *in vivo* (1), including the plaques, amorphous Aβ deposits, and neurofibrillary tangles of Alzheimer disease (7–14). It undergoes reversible calcium-dependent binding to all types of amyloid fibrils *in vitro* (15) and comprises up to 15% of the mass of amyloid deposits *in vivo* (16). This remarkable specific concentration of a trace plasma protein (20–30 mg/liter) contrasts with the very small quantities in amyloid deposits of other, much more abundant, plasma and extracellular fluid proteins: apolipoprotein E (17), α_1-antichymotrypsin (18), and some complement components (19). Although the physiological role of SAP is not known, we have previously proposed that SAP might protect amyloid from degradation *in vivo* by masking the abnormal fibrillar conformation that would otherwise be expected to trigger phagocytic clearance mechanisms (20–22). Indeed, SAP isolated from amyloid deposits is identical to circulating SAP (23). Furthermore, SAP molecules deposited in amyloid are not catabolized and are broken down only when they return to the circulation (24). The only significant site of *in vivo* catabolism of SAP is the hepatocyte (25), suggesting that other cells, especially phagocytes, lack receptors for SAP. In addition, SAP is highly resistant to proteolysis (26), probably because of its flattened β-jelly roll structure in which the β-strands are joined by compact loops tightly bonded to the body of the oligomeric assembly (27).

Prompted by these recent findings, we have investigated the capacity of SAP to prevent proteolytic digestion of amyloid fibrils and used the cyclic pyruvate acetal, methyl 4,6-*O*-[(*R*)-1-carboxyethylidene] β-D-galactopyranoside (MOβDG), to analyze its mode of action. MOβDG is a specific ligand for SAP that inhibits binding of SAP to amyloid fibrils and dissociates bound SAP (20, 28).

MATERIALS AND METHODS

Reagents. Amyloid fibrils were isolated from amyloidotic spleens of patients with amyloid A (AA), monoclonal immunoglobulin light chain (29), and apolipoprotein AI Arg-60 amyloidosis (30) by water extraction (31) after repeated homogenization of the tissue in 10 mM Tris·HCl/138 mM NaCl/10 mM EDTA/0.1% NaN_3, pH 8.0 (TE buffer) to remove endogenous SAP (32), and some were radiolabeled with ^{125}I (33). Aβ peptide (residues 1–40) purified by HPLC (California Peptide Research, Napa, CA) was dissolved in sterile pure water (1 mmol/liter; ≈4 mg/ml) and kept at 4°C or aged at 37°C for 7 days. After aging, typical fibrils were demonstrated electron micrographically and by green birefringent Congophilia. *Ex vivo* Aβ fibrils isolated from the cerebrovascular amyloid lesions of patients with Alzheimer disease were provided by G. Glenner (34). MOβDG was synthesized as reported (28) and freshly dissolved in 10 mM Tris·HCl/138 mM NaCl, pH 8.0 (TN buffer). SAP and C-reactive protein (CRP) were isolated in pure form as described (35–37). In the absence of physiological concentrations of albumin or of a specific ligand, isolated SAP precipitates in the presence of calcium (38). It was therefore kept in TN buffer before mixing with amyloid fibrils in the same solvent containing 2 mM $CaCl_2$ (TC buffer), with or without MOβDG, and with extra $CaCl_2$ to 2 mM final concentration. CRP was in solution in TC buffer throughout. The proteinases used were porcine pancreatic

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Abbreviations: AA, amyloid A protein; Aβ, amyloid β protein of Alzheimer disease; BSA, bovine serum albumin; CRP, C-reactive protein; MOβDG, methyl 4,6-*O*-[(*R*)-1-carboxyethylidene] β-D-galactopyranoside; SAP, serum amyloid P component; TCA, trichloroacetic acid.

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trypsin (type II; Sigma), bovine pancreatic chymotrypsin (type VII; Sigma), Pronase (*Streptomyces griseus*; Boehringer Mannheim); human neutrophil elastase (>98% pure by SDS/PAGE; Calbiochem–Novabiochem), human neutrophil cathepsin G (>95% pure by SDS/PAGE; Calbiochem–Novabiochem), and collagenase type VII (*Clostridium histolyticum*; Sigma). Human and bovine serum albumin were from Sigma.

Binding of SAP to Amyloid Fibrils. AA fibrils (8.5 μg), ^{125}I-labeled SAP (^{125}I-SAP) (24) (0.55 μg), and MOβDG were incubated in 200 μl of TC buffer containing 4% (wt/vol) bovine serum albumin (BSA) for 1 h at 21°C on a plate shaker in Multiscreen 96-well filtration plates with a 0.22-μm low protein binding hydrophilic Durapore membrane (Millipore). The fibrils were then harvested by filtration (multiscreen assay system; Millipore) and washed three times with TC buffer containing 1% BSA, and bound radioactivity was determined. Aged Aβ was incubated with mixing for 90 min at 21°C with ^{125}I-SAP in 605 μl of TC buffer containing 4% BSA, centrifuged (13,000 × *g*, 5 min, 4°C), and washed with 1.5 ml of TC buffer containing 4% BSA, and residual radioactivity in the pellet was determined.

Digestion of AA Fibrils. Lyophilized AA fibrils (100 μg in TC buffer) were preincubated with shaking (30 min at 37°C) with and without SAP (10 and 50 μg) or equimolar amounts of CRP (4.5 and 23 μg). Replicate incubations included MOβDG at 7 mM. Trypsin or chymotrypsin (10 μg in TC buffer) was added and the final 112-μl mixtures were incubated with shaking at 37°C for 6 h. Digestion was stopped by boiling for 10 min in SDS sample buffer and residual AA protein was estimated densitometrically in SDS/8–18% gradient polyacrylamide gel (ExcelGel; Pharmacia) stained with Brilliant Blue R-350 (see Fig. 1*A*). In other experiments ^{125}I-labeled AA (^{125}I-AA) fibrils were preincubated at 37°C for 1 h with various concentrations of SAP and then with Pronase at 50 mg/liter (final concentration) in TC buffer. Release of radioactivity soluble in 10% (vol/vol) trichloroacetic acid (TCA), representing low molecular weight products of digestion, was determined. Analysis of digestion by SDS/15% PAGE and autoradiography or immunoblotting (data not shown) confirmed degradation of AA protein and protection by SAP. Loss of the AA band was more complete than suggested by the variable partial release of TCA-soluble activity, but the protective effect of SAP and its abrogation by MOβDG were consistent. The effect of MOβDG was investigated in this same protocol with SAP at 50 mg/liter and by stopping digestion at 4 h (see Fig. 1*B*). Digestion by activated monocytes was tested in microtiter plate wells (Nunc Immunobind II; GIBCO) that had been coated with ^{125}I-AA fibrils by evaporating 200 μl of fibril suspension to dryness at 37°C and then washed twice with 200 μl of PBS at 37°C for 3 h. Each coated well was preincubated for 1 h at 37°C with or without SAP (10 μg) or CRP (4.5 μg) in Iscove's medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and $CaCl_2$ (final concentration, 2 mM). Human peripheral blood monocytes (39) (5 × 10^4 cells and >90% pure) in Iscove's medium were added to 2 ng of phorbol 12-myristate 13-acetate in each well in a final vol of 200 μl and incubated at 37°C for 5 h in 5% CO_2/95% air. TCA-soluble activity in the supernatants, representing digested fibril protein, was then determined.

Digestion of Aβ Fibrils. Aliquots of fresh and aged Aβ (10 μg in TC buffer) were preincubated with shaking at 37°C for 1 h with and without SAP, CRP, and MOβDG in a final $CaCl_2$ concentration of 2 mM. Pronase (0.1 or 1.0 μg in TC buffer) was added and the 100-μl reaction mixture was incubated with shaking for 1 h at 37°C. Digestion was stopped by boiling for 5 min with SDS sample buffer. Remaining protein was estimated densitometrically in SDS/15% PAGE stained with silver (Pharmacia). In other experiments, Aβ remaining after digestion was estimated by dot-blot immunoassay on 0.2-μm nitrocellulose membrane (Schleicher & Schuell) using anti-Aβ monoclonal antibodies (Dako) and horseradish peroxidase-labeled anti-mouse IgG (Dako) detected by ECL (Amersham).

RESULTS

Digestion of Amyloid Fibrils. Isolated *ex vivo* AA (Fig. 1), monoclonal immunoglobulin light chain, and apolipoprotein AI Arg-60 amyloid fibrils (data not shown) were digested *in vitro* by the pancreatic proteinases trypsin and chymotrypsin, by the microbial enzyme Pronase, and by the human neutrophil proteinases elastase and cathepsin G. Cathepsin G was much more effective than elastase, while collagenase had no effect at all (data not shown). In parallel experiments, amyloid fibrils from the cerebrovascular deposits of Alzheimer disease, specifically the Aβ peptide subunits, were also digested by Pronase, as shown in silver-stained SDS/PAGE. Amyloid fibrils formed *in vitro* by aging synthetic Aβ peptide (residues 1–40) were much more susceptible to proteolysis than *ex vivo* fibrils and were digested by cathepsin G and by Pronase (Fig. 2).

Binding of SAP to Amyloid Fibrils. SAP showed reversible calcium-dependent binding to *ex vivo* amyloid fibrils and this was inhibited by MOβDG, as reported (15, 28) (Fig. 3). SAP also showed saturable binding to synthetic Aβ fibrils, reaching a plateau at a molar ratio of ≈1 mol of SAP (M_r, 254,620) per 500 mol of Aβ (M_r, 4330), compatible with interaction between decameric SAP and fibrillar aggregates of Aβ (Fig. 4). The mass of SAP present at saturation corresponded to ≈12% that of the fibrils and was comparable to their relative proportions in amyloid deposits *in vivo* (16). In the presence of 10 mM EDTA, to chelate calcium, or of 7 mM MOβDG

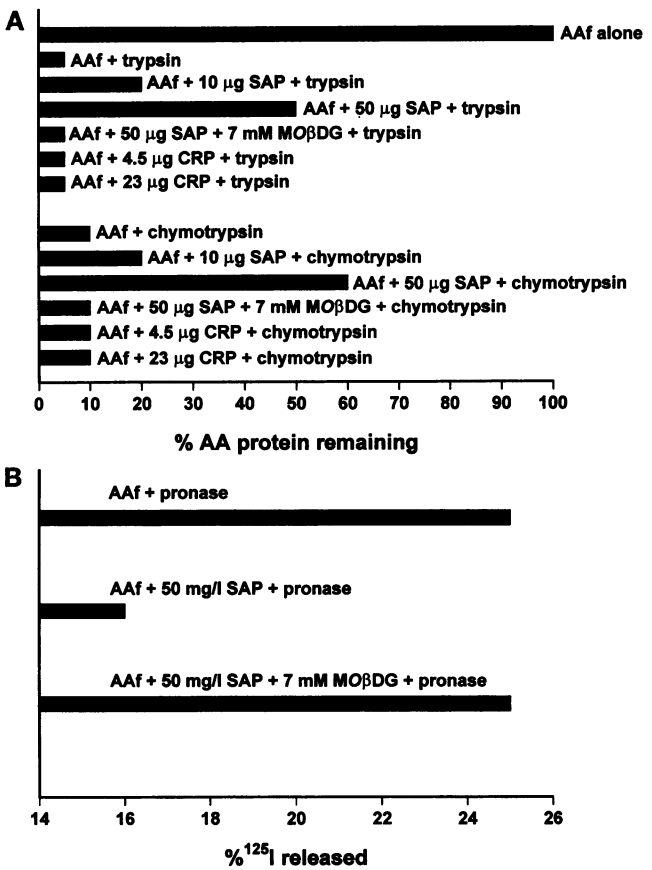


FIG. 1. SAP prevents proteolysis of AA amyloid fibrils (AAf) and is inhibited by MOβDG. (*A*) Digestion by trypsin and chymotrypsin monitored by densitometry after reduced SDS/PAGE. (*B*) Digestion by Pronase of ^{125}I-labeled fibrils, monitored by release of TCA-soluble radioactivity.

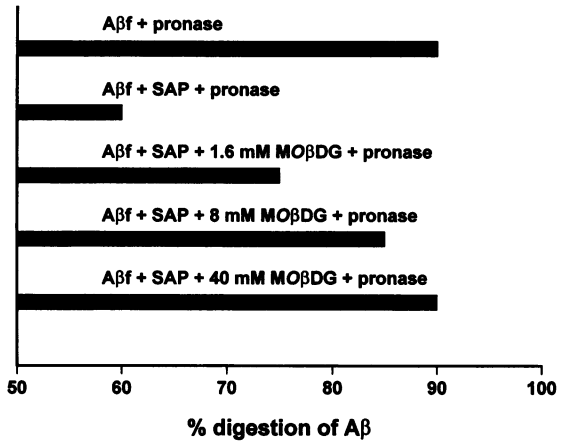


FIG. 2. SAP prevents proteolysis of Aβ fibrils (Aβf) and is inhibited by MOβDG. Digestion by Pronase monitored by densitometry after reduced SDS/PAGE.

in the presence of calcium, binding of SAP to Aβ fibrils was reduced to background levels (7–8 fmol in the experiment illustrated in Fig. 4).

Inhibition by SAP of Proteolysis of Amyloid Fibrils. Physiological concentrations of SAP dose-dependently inhibited digestion by Pronase, trypsin, and chymotrypsin of all types of amyloid fibrils tested, including *ex vivo* AA fibrils (Figs. 1 and 5), *ex vivo* Aβ fibrils from the cerebrovascular amyloid deposits of Alzheimer disease (data not shown), and synthetic Aβ fibrils (Fig. 6). In contrast, neither CRP nor human serum albumin had any effect on fibril digestion (Figs. 1*A*, 7, and 8). When fresh Aβ that had not formed fibrils was used as substrate, SAP had little protective effect against digestion by Pronase (Figs. 6 and 9).

Amyloid regression *in vivo* is likely to be mediated by phagocytic cells, and both monocytes (Fig. 10) and neutrophils (data not shown) degraded AA fibrils *in vitro*. This was significantly inhibited by SAP while CRP and albumin had no effect.

Effect of MOβDG. When SAP was prevented from binding to the fibrils by MOβDG, its inhibitory effect on their proteolysis was completely abrogated (Figs. 1, 2, 7, and 8). At the same time, the presence of MOβDG conferred on SAP itself dose-dependent protection against digestion by Pronase (Fig. 7). This reflects the fact that the surface loops of SAP that comprise the calcium-dependent ligand binding site, and that are the most proteinase-sensitive part of the SAP sequence

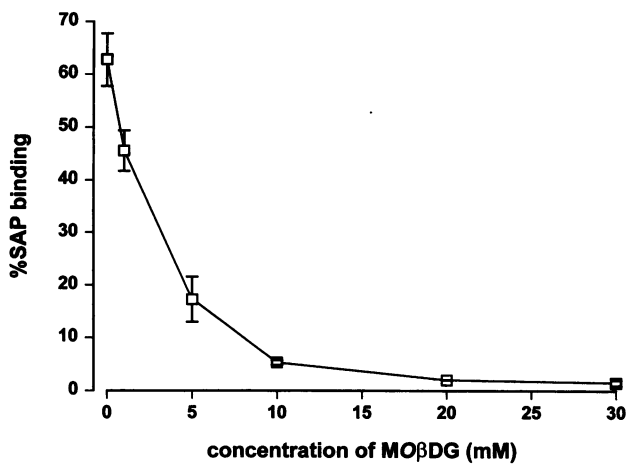


FIG. 3. MOβDG inhibits binding of ^{125}I-SAP (0.55 μg) to AA fibrils (8.5 μg). Each point is the mean (±SD) of four replicates.

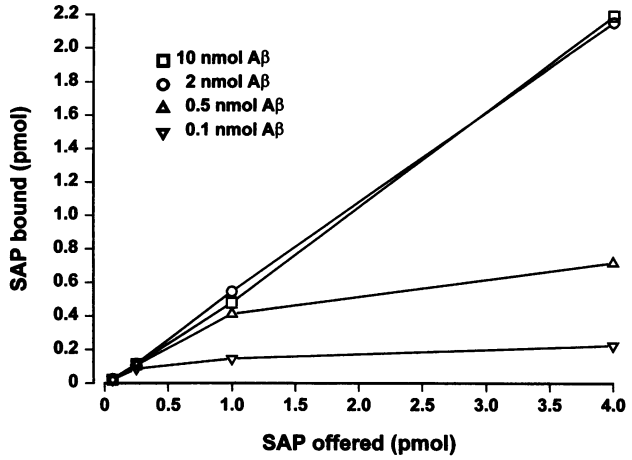


FIG. 4. Binding of SAP to synthetic Aβ fibrils. Each point is the mean of duplicate assays.

(26), are stabilized by the electrostatic and hydrogen bonds (27) involved in binding MOβDG.

DISCUSSION

Human neutrophil cathepsin G, and to a lesser extent elastase, as well as whole living phagocytic cells, were able to digest amyloid fibrils *in vitro* and this digestion was blocked by SAP. The pancreatic and microbial proteinases used in the experiments illustrated here also digested the various types of fibrils. Despite probably being much more aggressive than the enzymes responsible for degradation of amyloid fibrils *in vivo*, their action was inhibited by SAP, emphasizing how important the effect of SAP may be on persistence of amyloid fibrils *in situ*.

Although McAdam and colleagues have reported that SAP inhibited elastase (40) and reduced digestion of elastin and AA amyloid fibrils by elastase *in vitro* (41), we have not been able to detect any effect of SAP on cleavage of either synthetic substrates or soluble proteins by elastase or trypsin (21) (unpublished data). In the present experiments, SAP prevented digestion of fibrils only when bound to them. In the presence of the cyclic pyruvate acetal, MOβDG, which is the most specific carbohydrate ligand of SAP and inhibits its binding to amyloid fibrils (20, 28), SAP had no effect. SAP is thus not an enzyme inhibitor *per se*. CRP, another highly proteinase-resistant molecule (42) structurally very closely related to SAP (43), does not bind to amyloid fibrils (15) and neither it nor human serum albumin, an unrelated control protein, protected fibrils from proteolysis.

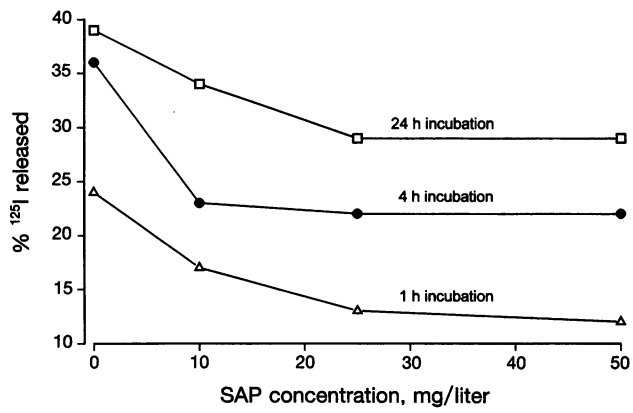


FIG. 5. Inhibition by SAP of digestion of ^{125}I-AA fibrils by Pronase, monitored by release of TCA-soluble radioactivity.

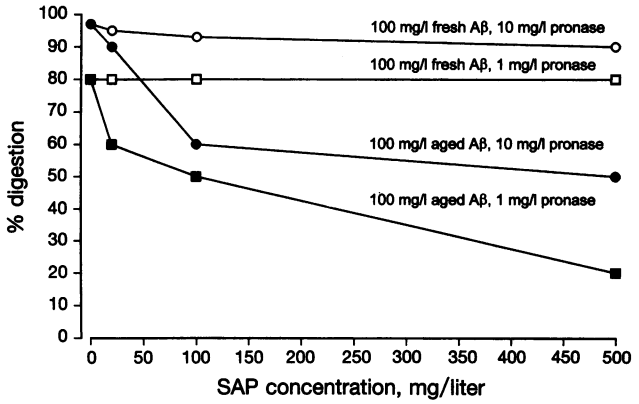
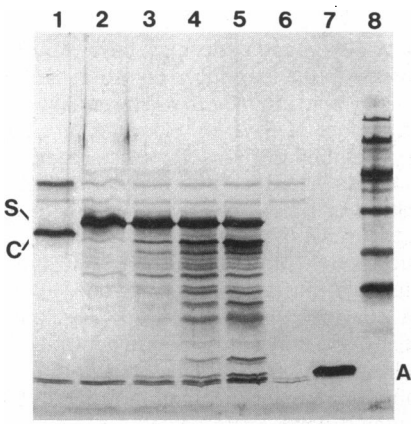


FIG. 6. Digestion of Aβ by Pronase monitored by densitometry of reduced SDS/PAGE. Only aged Aβ that had formed amyloid fibrils was significantly protected by SAP.

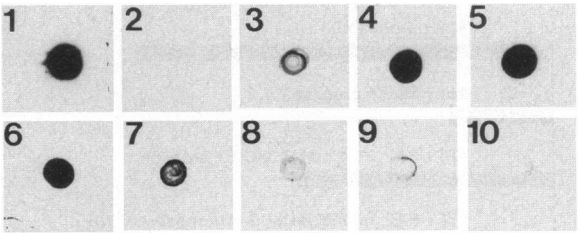
The calcium-dependent binding of SAP to fibrils formed from synthetic Aβ, which is demonstrated here, indicates that SAP can recognize a pure protein structure. Although glycosaminoglycans are universally associated with amyloid fibrils *in vivo* (32, 44), and SAP binds to glycosaminoglycans *in vitro* (45), SAP can evidently bind to amyloid fibrils in the absence of carbohydrate. Inhibition by MOβDG of binding of SAP to Aβ fibrils implicates the same site in the SAP molecule that recognizes naturally occuring amyloid fibrils, and since SAP did not prevent proteolysis of freshly dissolved Aβ, which had not formed fibrils (46), SAP apparently recognizes a protein conformation specific for fibrillar Aβ.

Our results identify SAP, an abundant universal constituent of all amyloid deposits, as potentially a key factor in persistence



1 aged Aβ 1-40 + 4.5 μg CRP + pronase
2 aged Aβ 1-40 + 10 μg SAP + 40 mM MOβDG + pronase
3 aged Aβ 1-40 + 10 μg SAP + 8 mM MOβDG + pronase
4 aged Aβ 1-40 + 10 μg SAP + 1.6 mM MOβDG + pronase
5 aged Aβ 1-40 + 10 μg SAP + pronase
6 aged Aβ 1-40 + pronase
7 aged Aβ 1-40
8 molecular weight markers

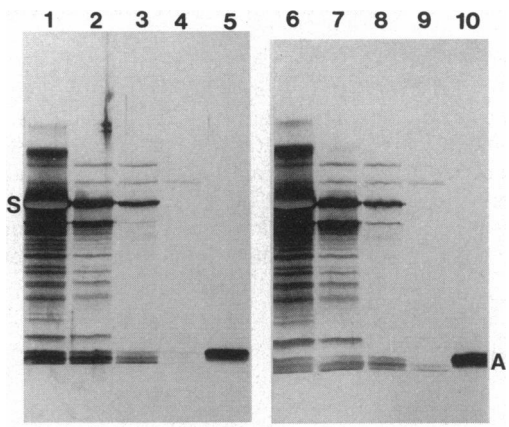
FIG. 7. Effects of SAP and MOβDG on digestion of Aβ fibrils by Pronase monitored by SDS/PAGE. Size markers: 94, 67, 43, 30, 20.1, and 14.4 kDa. Migration positions of intact undegraded proteins are indicated: S, SAP; C, CRP; A, Aβ. Note the dose-dependent protection against digestion conferred by MOβDG on SAP itself (lanes 2–4 compared to lane 5) and the failure of CRP to protect Aβ despite the intrinsic proteinase resistance of the CRP itself (lane 1).



1 aged Aβ 1-40
2 aged Aβ 1-40 + pronase
3 aged Aβ 1-40 + 0.25 μg SAP + pronase
4 aged Aβ 1-40 + 1.26 μg SAP + pronase
5 aged Aβ 1-40 + 3.15 μg SAP + pronase
6 aged Aβ 1-40 + 1.26 μg SAP + 0.2 mM MOβDG + pronase
7 aged Aβ 1-40 + 1.26 μg SAP + 1.0 mM MOβDG + pronase
8 aged Aβ 1-40 + 1.26 μg SAP + 5.0 mM MOβDG + pronase
9 aged Aβ 1-40 + 0.45 μg CRP + pronase
10 aged Aβ 1-40 + 0.26 μg HSA + pronase

FIG. 8. Effects of SAP and MOβDG on digestion of Aβ fibrils by Pronase monitored by dot-blot immunoassay. HSA, human serum albumin.

of amyloid *in vivo* and suggest another approach to treatment. Molecules active *in vivo*, that share with MOβDG the capacity to dissociate fibril-bound SAP and prevent attachment of free SAP (20, 28), should expose the abnormal fibrillar structure to endogenous protein clearance mechanisms, thereby preventing new amyloid deposition and accelerating regression of existing lesions. In many forms of amyloidosis, including Alzheimer disease and type II diabetes, it is not possible to reduce production of fibril precursor proteins. A strategy that enables the body's own protein clearance mechanisms to degrade amyloid fibrils is thus very attractive for both prevention and therapy. The high-resolution three-dimensional structure of SAP alone and complexed with MOβDG (27), and the recognition by SAP of amyloid fibrils formed from synthetic



1 aged Aβ 1-40 + 50 μg SAP + pronase
2 aged Aβ 1-40 + 10 μg SAP + pronase
3 aged Aβ 1-40 + 2 μg SAP + pronase
4 aged Aβ 1-40 + pronase
5 aged Aβ 1-40
6 fresh Aβ 1-40 + 50 μg SAP + pronase
7 fresh Aβ 1-40 + 10 μg SAP + pronase
8 fresh Aβ 1-40 + 2 μg SAP + pronase
9 fresh Aβ 1-40 + pronase
10 fresh Aβ 1-40

FIG. 9. Digestion of aged and fresh Aβ by Pronase monitored by reduced SDS/PAGE. Migration positions of intact undegraded proteins are indicated: S, SAP; A, Aβ. Slight protection by SAP of Aβ in the fresh preparation probably reflects the presence of traces of Aβ fibrils.

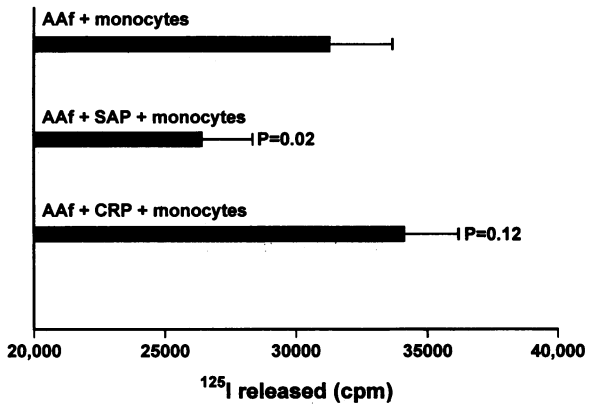


FIG. 10. Digestion of ^{125}I-AA amyloid fibrils (AAf) by activated monocytes, monitored by release of TCA-soluble radioactivity. Each bar is the mean (+SD) of four replicates; *P* values indicate differences (*t* test) from fibrils digested in the absence of added protein (top bar).

Aβ, provide valuable starting points for drug design (Patent Cooperation Treaty application no. PCT/GB94/01802).

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