AMYLOID P-COMPONENT IS A CONSTITUENT OF NORMAL HUMAN GLOMERULAR BASEMENT MEMBRANE*

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Amyloid P-component (AP) is a glycoprotein found in all forms of amyloid in which it has been sought (2–6), including primary localized cutaneous amyloid deposits (S. Breathnach, M. M. Black, R. F. Dyck, and M. B. Pepys. Unpublished observations). It is probably derived from a normal plasma protein, serum amyloid P-component (SAP), from which it is indistinguishable immunochemically in terms of polypeptide subunit size and composition, in electron microscopic appearance, and in the partial amino acid sequence that is presently available (4, 5, 7–9). SAP has calcium-dependent binding affinity for a variety of ligands including isolated amyloid fibrils (10). This interaction may be involved in the deposition of AP in amyloid in vivo but neither the role of AP in amyloidosis nor any other biological function for SAP is known.

AP was not originally detected in normal, non-amyloid-containing tissues (11), but more recently, Schneider and Loos (12) observed that fluoresceinated anti-AP antibodies stained vascular and perivascular structures in sections of a number of normal tissues and suggested that AP may be a form of type IV, basement-membrane-type collagen. We have confirmed their immunohistochemical findings and extended them with ultrastructural studies and immunochemical and biochemical characterization of the AP-like molecule in glomerular basement membrane (GBM). This molecule is not immunochemically related to collagen but is apparently a normal matrix protein of GBM and possibly other vascular basement membranes. These observations may be significant in relation to both amyloidosis and other aspects of the pathophysiology of basement membranes.

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Abbreviations used in this paper: AP, amyloid P component; CRP, C-reactive protein; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane(s); HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; pH 7.4; pl, isoelectric point(s); SAP, serum amyloid P-component; SDS, sodium dodecyl sulfate.
Antisera. Monospecific antisera to human SAP and C-reactive protein (CRP) were raised by immunization of sheep and rabbits with the isolated pure proteins (8, 13). Monospecific rabbit anti-sheep IgG and sheep anti-human C3 were raised by immunization with sheep IgG isolated by salt fractionation and DEAE-cellulose chromatography and with human C3 isolated from fresh plasma (14), respectively. Fluoresceinated swine anti-rabbit IgG was obtained from Dakopatts A/S, Copenhagen, Denmark. The F(ab')2 fragments, prepared by pepsin digestion of the IgG fractions of rabbit anti-SAP and anti-CRP sera, were conjugated with fluorescein isothiocyanate (FITC; BDH Chemicals Ltd., Poole, England) and, after chromatography on DEAE-cellulose, fractions with OD 495/OD 280 <1.0 were used for immunofluorescence staining (15). The IgG1 fractions of sheep anti-SAP and anti-C3 were conjugated with horseradish peroxidase (HRP; type VI; Sigma Chemical Co. Ltd., Surrey, England) (16). Antisera to pepsin-solubilized collagens types I, III, IV, and V, isolated from human placenta, were raised in rabbits (17-19). The type IV collagen had been further purified before immunization (20) and the purity of all types was assessed in sodium dodecyl sulphate (SDS; Sigma Chemical Co. Ltd.)-polyacrylamide gel electrophoresis (PAGE) (21). Anti-human GBM antiserum was raised by immunization of a sheep with isolated lyophilized normal GBM (see below).

Immunohistochemical Studies. Cryostat sections were cut at 6-8 μm from snap-frozen blocks of normal human kidney, esophagus, lung, heart, and skin, and were washed in phosphate-buffered saline, pH 7.4 (PBS) for 15 min at 21°C. The sections were then stained with optimal dilutions of FITC-F(ab')2 anti-SAP for 30 min at 21°C, washed three times with PBS, and mounted in buffered glycerol for examination by incident light fluorescence in a Leitz Orthopahn microscope (E. Leitz Ltd., Luton, England) equipped with water immersion objectives. Control sections were stained with FITC anti-SAP that had previously been incubated with 10-100 μg of isolated pure SAP. A further control was provided by staining with FITC-F(ab')2 anti-CRP, because CRP is very closely related structurally to SAP but is antigenically distinct (13, 22). For indirect tests, sheep IgG anti-SAP or rabbit anti-type IV collagen were incubated on kidney sections for 30 min at 21°C, followed, after three washes in PBS, by the appropriate fluoresceinated anti-Ig reagents. Further incubation, washing, and mounting were then completed as described above. Controls included absorption of the anti-SAP reagent with isolated pure SAP before staining and substitution of normal sheep IgG or normal rabbit serum for the specific antibodies in the first layer of indirect tests. Kidney sections were also stained with HRP-anti-SAP or anti-C3 and were processed for light and electron microscopy (23).

To elute the AP-like material from GBM, separate cryostat sections of normal kidney were incubated for 30 min at 21°C in the following reagents: PBS (control); 0.01 M EDTA-0.01 M Tris-0.14 M NaCl, pH 8.0; 2.0 M NaCl; 0.1 M diithiothreitol (DTT; Sigma Chemical Co. Ltd.)-0.01 M EDTA-0.01 M Tris-0.14 M NaCl, pH 8.0; 0.1 M DTT-0.01 M EDTA-2.0 M NaCl; 1% (vol:vol) Triton X-100 (BDH) in PBS; 1% (vol:vol) Triton X-100-0.1 M DTT-2.0 M NaCl-0.1 M EDTA; 0.1% (wt:vol) SDS-0.1 M DTT-PBS; 2.0 M hydroxylammonium chloride, pH 10.5. Other sections were incubated for 16 h at 37°C in 0.1 M DTT-2.0 M NaCl-0.01 M EDTA and for 5 h at 37°C in 6.0 M guanidine hydrochloride (Sigma Chemical Co. Ltd.)-0.5 M Tris-0.1 M EDTA-0.001 M EDTA, pH 8.5. After these various incubations, the sections were all washed three times in PBS and then stained with FITC-F(ab')2 anti-SAP.

Preparation of Human GBM. GBM was isolated (24) from normal kidneys removed at autopsy and stored at -20°C. 90-95% of the final product had the appearance of GBM by light microscopy. Lyophilized GBM was suspended at 25 mg/ml in 0.1 M Tris-0.005 M calcium acetate, pH 7.4, and stirred continuously during incubation at 37°C with 0.7% wt:wt bacterial collagenase. In pilot experiments, samples of the mixture were measured and centrifuged after 30, 60, 90, 120, 180, and 360 min, and after 19 h incubation. Digestion of collagen was assessed by assay of hydroxyproline released into the supernate (25), and release of AP-like material was measured by electrophoresis assay (see below). Collagenase was obtained from Sigma Chemical Co. Ltd.: type I (crude) and type VI (purified, free of nonspecific proteinase, clostripain, and trypsin activity), and type I was also used after purification. The isolation of collagenase (26).
**Amyloid P-Component in Basement Membrane**

**Immunochemical Studies.** The collagenase-solubilized GBM was tested by double immunodiffusion in gel against sheep anti-SAP and anti-GBM sera, in comparison with isolated pure SAP and normal human serum. It was also run in electroimmunoassay (27) against anti-SAP and polyvalent anti-normal human serum, and in crossed immunoelectrophoresis (28) against anti-SAP. 1% wt:vol agarose gel (Indubiose A37; L'Industrie Biologique Francaise S. A., Clichy, France) in 0.07 M barbitone-0.01 M EDTA, pH 8.6 was used in all these tests. Isolated lyophilized GBM and the insoluble residue after the 19-h digestion with collagenase were washed with PBS and then incubated for 30 min at 21°C with FITC-F(ab')2 anti-SAP. Control samples were treated with FITC-anti-SAP that had previously been absorbed with isolated SAP. The insoluble GBM was then washed three times in PBS and mounted in buffered glycerol for fluorescence microscopy. Cross-reactivity between anti-SAP serum and anti-collagen serum was sought by passive hemagglutination testing (29). Tanned sheep erythrocytes were coated with isolated collagen types I, III, IV, and V, and their agglutination by anti-SAP was compared with that produced by a mixture of antiserum specific for those collagen types. Rabbit anti-type IV collagen was also tested by gel immunodiffusion for reactivity with normal human serum and isolated SAP.

**Biochemical Studies.** GBM solubilized by collagenase was radiolabeled with carrier-free Na[125I] (IMS 30; Radiochemical Centre, Amersham, England) by the Iodogen (Pierce Chemical Co., Rockford, Ill.) method (30). Protein-bound 125I was separated from labeled small peptides and free 125I on Sephadex G-25 in PBS. The labeled protein had a 77.5 µCi/A280 sp act.

Normal human serum, isolated SAP, and 125I-GBM were subjected to isoelectric focusing in agarose gel over a pH 3.5–9.5 range with the reagents, Multiphor apparatus, and procedure provided by LKB Instruments Ltd., London, England. The gel was then immunofixed (31) with sheep anti-SAP serum, washed, dried, and subjected to radioautography with LKB 1H-Ultronfilm.

Some of the 125I-GBM was also coprecipitated with an optimal-proportions mixture, determined by previous titration, of isolated SAP and sheep anti-SAP serum. The mixture was incubated for 45 min at 37°C and then at 4°C for 16 h, after which, it was centrifuged at 10,000 g for 5 min. and the precipitate washed twice in PBS. A control coprecipitation was performed simultaneously under identical conditions with isolated CRP and sheep anti-CRP serum. The precipitates were dissolved by heating at 100°C for 5 min in 2% vol:vol mercaptoethanol (Sigma Chemical Co. Ltd.), 0.06 M Tris-HCl, pH 6.8, and run in a 12% slab SDS-PAGE (32). Samples of unlabeled collagenase-solubilized GBM, 125I-GBM, pure SAP, and pure CRP were run on the same gel under reducing conditions, as well as marker proteins of defined molecular weight (obtained from Pharmacia G. B. Ltd., London, England): rabbit muscle phosphorylase b, subunit 94,000 mol wt; bovine serum albumin, 67,000 mol wt; hen ovalbumin, 43,000 mol wt; bovine erythrocyte carboxyl anhydrase, 30,000 mol wt; soybean trypsin inhibitor, 20,100 mol wt; bovine α-lactalbumin, 14,000 mol wt. After electrophoresis, the gel was fixed, stained with 0.2% Coomassie blue (BDH), dried, and an autoradiograph prepared using LKB 3H-Ultronfilm. In a further experiment, 1.5 ml of 125I-GBM was mixed with 3.5 ml of 0.01 M Tris-0.14 M NaCl-0.01 M EDTA, pH 8.0 and chromatographed on a 2.6 × 90-cm column of Sephacryl S-300 (Pharmacia) equilibrated with the same buffer. The sample was eluted at 16 ml/h and fractions of 5.8 ml were collected; 100 µl of each was counted in a Packard 5210 γ-scintillation counter (Packard Instrument Co., Downers Grove, Ill.) and then coprecipitated with an optimal-proportions mixture of SAP and anti-SAP. After 1 h at 37°C and then 16 h at 4°C, these precipitates were washed with Tris-EDTA and counted again to determine the proportion of total radioactivity per fraction attributable to anti-SAP-reactive material. A sample of isolated SAP was mixed with a 1:5 dilution of normal human serum, heated at 60°C for 30 min to mimic the collagenase inactivation step in the GBM solubilization procedure, and was then run on the same Sephacryl column under identical conditions to the 125I-GBM. SAP was quantitated in the eluted fractions by electroimmunoassay. The column was calibrated using samples of dextran blue 2000 and marker proteins of known molecular weight.
Results

Immunochemical Localization of AP in Normal Human Tissues. With direct and indirect immunofluorescence and immunoperoxidase techniques on sections of normal kidney, rabbit and sheep anti-SAP antibodies produced linear staining of glomerular capillaries and structures in the walls of small arterioles (Fig. 1). The immunological specificity of this staining was established by its complete inhibition when the anti-SAP reagents were absorbed with microgram quantities of isolated pure SAP before application to the tissue sections (Fig. 1). Anti-CRP antibodies did not stain normal kidney tissue at all, nor was there staining in indirect tests in which normal rabbit or sheep sera were substituted for anti-SAP. Electron microscopy of glomeruli stained with HRP-sheet IgG anti-SAP revealed a homogeneous linear band of staining confined to the endothelial side, the lamina rara interna, of the GBM (Fig. 2). No staining was observed in control sections treated with HRP-sheet IgG anti-C3.

FITC-F(ab')2 anti-SAP also stained alveolar capillary walls in sections of normal lung and linear intercellular structures in both cardiac and smooth muscle. In sections of skin, anti-SAP stained the capillary loops around the rete ridges but not the basement membrane at the dermo-epidermal junction.

To characterize the nature of the association of the AP-like material with GBM, attempts were made to elute it from sections of kidney. There was no change in uptake of anti-SAP after sections had been incubated in EDTA, 2.0 M NaCl, EDTA-2.0 M NaCl, DTT-EDTA, DTT-EDTA-2.0 M NaCl, SDS, Triton X-100, Nonidet P-40, Triton X 100-DTT-EDTA-2.0 M NaCl, or hydroxylamine. However, treatment with SDS-DTT or with 6.0 M guanidine-DTT-EDTA, both of which solubilized the glomeruli, abolished staining.

Lack of Immunological Cross-Reactivity between SAP and Collagen. Rabbit anti-type IV collagen antiserum stained both GBM and tubular basement membrane with equal intensity, producing a quite different pattern from that seen in kidney sections stained with anti-SAP. Furthermore, anti-SAP serum did not agglutinate sheep erythrocytes coated with isolated collagen types (Table I), nor did anti-type IV collagen precipitate in gel with SAP.

Digestion of Isolated GBM by Collagenase. Lyophilized GBM isolated from normal kidneys and suspended in PBS stained immunospecifically with FITC-anti-SAP. When GBM was digested with bacterial collagenase, part of it was solubilized, and the supernate contained an antigen that precipitated in gel with anti-SAP serum (Fig. 3). There was no difference in the rate at which GBM was digested by crude type I collagenase, purified type I collagenase, or pure type VI collagenase, as measured by release of hydroxyproline or by the appearance into solution of the AP-like antigen. The concentration of anti-SAP reactive material in the supernate reached a maximum after 30-60 min. digestion and in some experiments its concentration decreased after 2 h. Therefore, for further characterization of the AP-like material, GBM was incubated for 45 min with purified type I collagenase, after which, the supernate derived from 25 mg GBM/ml had an A280 of 10-15. Some insoluble residue always remained and this still stained specifically with FITC-anti-SAP.

Immunochemical Studies of the AP-like Material from GBM. After digestion of GBM with collagenase, as described above, the supernate contained an antigen that gave a precipitation line in gel of complete identity with that between anti-SAP and normal serum or isolated pure SAP (Fig. 3). Sheep anti-GBM serum reacted with normal
FIG. 1. Normal human kidney stained with FITC-anti-SAP. Linear staining of glomerular capillary walls (A) and specific inhibition of staining (B) when the fluoresceinated antibody was absorbed with pure SAP. × 400.
human serum and with pure SAP; in both cases, the precipitation line identified completely with that produced by sheep anti-SAP (Fig. 3). Although immunologically indistinguishable from SAP, the AP-like material in solubilized GBM was heterogeneous in electrophoretic mobility, a significant proportion of the molecules were more anodal than SAP in crossed immunoelectrophoresis (Fig. 4). This produced a differently shaped rocket of immunoprecipitation in the electroimmunoassay (Fig. 4) so that precise quantitation of the amount of antigen present by comparison with normal serum standards was not possible.
Approximate estimates of the concentration of AP-like antigen in different preparations of solubilized GBM lay between 40 and 90 µg/ml, whereas ~120 µg/ml of serum albumin was present. Polyvalent sheep anti-normal human serum also detected traces of two other serum proteins in the solubilized GBM.

Biochemical Characterization of the AP-like Material from GBM. After isoelectric focusing of 125I-GBM, immunofixation with anti-SAP revealed a broad series of bands over a pH 3.9–4.8 range that overlapped, but was more anodal, than the isoelectric points (pI) of pure SAP or SAP in serum. Radiiodinated protein that coprecipitated from 125I-GBM with complexes of SAP and anti-SAP was run on a 12% slab SDS-PAGE under reducing conditions. Some labeled material failed to enter the gel, indicating that it was of high molecular weight (>250,000), but two main bands and two fainter ones were seen (Fig. 5). One major band migrated to almost the same position as the subunit of isolated SAP. The other major band lay between ovalbumin and bovine serum albumin standards. The subunits of SAP and CRP are known to migrate with anomalously large apparent molecular weights in SDS-PAGE (33). When the calibra-
Fig. 5. Reduced 12% slab SDS-PAGE analysis of AP-like protein in GBM. Track 1, collagenase-solubilized GBM; 2, ^125I-labeled collagenase-solubilized GBM; 3, radioautograph of track 2; 4, coprecipitate of ^125I-GBM with SAP and anti-SAP; 5, radioautograph of track 4; 6, isolated SAP; 7, coprecipitate of ^125I-GBM with CRP and anti-CRP; 8, radioautograph of track 7; 9, isolated CRP; 10, standard molecular weight marker proteins, a, 94,000 mol wt; b, 67,000 mol wt; c, 43,000 mol wt; d, 30,000 mol wt; e, 21,100 mol wt; f, 14,000 mol wt.
tion curve for log molecular weight versus Rf, obtained with standard marker proteins, was corrected for the known molecular weights of CRP and SAP (33, 34), the apparent sizes of the two main labeled chains from the AP-like molecule in GBM were 24,000 and 46,000 mol wt, respectively. The control coprecipitate of 125I-GBM with complexes of CRP and anti-CRP did not contain any labeled material demonstrable by radioautography after they were run on the SDS-PAGE.

Most of the material with an A280 in the 125I-GBM preparation eluted from a Sephacryl S-300 column in the void volume, and most of the labeled material that was specifically precipitated by anti-SAP was also in this fraction, suggesting a >750,000 mol wt (Fig. 6). However, a second peak of AP-like protein eluted from the column in the same volume as SAP in normal human serum (235,000 mol wt) run as a standard (Fig. 6). Approximately 10% of the total activity in the 125I-GBM preparation was specifically immunoprecipitated with SAP-anti-SAP complexes.

Discussion

Our findings demonstrate that normal human GBM, and, probably, other vascular basement membranes, contain a protein that is immunochemically indistinguishable from AP. In electron microscopic studies of glomeruli, this material was confined to the lamina rara interna of the basement membrane. In collagenase-solubilized GBM, there were comparable amounts of the AP-like antigen and of serum albumin, which contrasts with their relative plasma concentrations of 1,000:1 in favor of albumin, and suggests that SAP is not just nonspecifically absorbed onto GBM. The AP in GBM was not held there via noncovalent intermolecular interactions or via the known calcium-dependent ligand binding of SAP and AP, because it could not be eluted by high-ionic-strength salt, EDTA, or either polar or nonpolar detergents. It was also not eluted by reduction of disulphide bridges alone (with DTT) or by treatment with hydroxylamine. These observations suggest that it may be covalently linked as one of the normal matrix glycoproteins of GBM.

The AP-like antigen may be associated with the collagen moiety of GBM because

![Diagram](https://via.placeholder.com/150)

Fig. 6. Gel filtration of 125I-GBM on Sephacryl S-300. Vo, void volume estimated with dextran blue 2000; Vi, column volume; Ve, eluted volume; SAP designates the elution volume of SAP subsequently run on the same column.
it was released into solution when GBM was digested by pure collagenase free of neutral proteinase, clostripain, and trypsin activity. Although it gave precipitation lines of identity with SAP, the antigen from GBM was heterogeneous in pI and electrophoretic mobility and much of it was of high molecular weight (>750,000 mol wt). However some did have the same apparent molecular weight, 235,000, as SAP. Studies of the polypeptide chain structure in SDS-PAGE also suggested that there were some subunits marginally larger (24,000 mol wt) than those of SAP (23,500 mol wt), whereas others were approximately twofold larger (46,000 mol wt) and a major portion were >250,000 mol wt. Taken in conjunction, these observations indicate that the AP-like protein in GBM is very similar to SAP, but is covalently linked to other components of GBM. When released into solution by the action of collagenase, most, or even all, the AP subunits are still associated with other peptide chain fragments that are possibly derived from collagen or other matrix proteins. It is not possible now to estimate precisely the amount of AP in GBM, but it is probably more than a trace component, because ~10% of the radioactivity in the iodinated collagenase-solubilized GBM was specifically precipitated by anti-SAP.

The source of AP in basement membrane is not known, but it is of interest that such closely related proteins should be present both in the circulation and in basement membranes. One possibility is that the basement membrane AP derives from plasma SAP and is incorporated into nascent basement membrane as it is laid down or becomes covalently linked to already established basement membrane. Alternatively, the basement membrane AP may be produced locally by vascular endothelial cells or fibroblasts. It has been reported that human fibroblasts cultured in vitro in the absence of human serum can be stained with anti-AP antibodies (35), but this has not been confirmed, nor was it possible to demonstrate incorporation of radioactive amino acids into SAP in such cultures despite unequivocal demonstration of de novo synthesis of C1q at the same time (M. B. Pepys and K. Reid. Unpublished observations.). On the other hand, circulating SAP levels are low in patients with hepatocellular insufficiency (36) and the liver is the site of SAP synthesis in the mouse (37).

The presence of AP in normal basement membranes raises a number of intriguing possibilities. In the first place, vascular basement membranes are among the most important filters between the intra- and extravascular compartments both in the kidney and elsewhere. Although there is as yet no direct evidence regarding a physiological role for AP in this respect, it is known that the net negative charge on the glomerular capillary wall, derived in part from anionic matrix glycoproteins of the GBM, is necessary for integrity of the filtration barrier (38). AP could thus be a target for various pathological processes that affect basement membrane, and we have recently observed distinctive abnormal patterns in the disposition of AP in GBM of patients with membranous glomerulonephritis, acute post-infectious nephritis, and diabetic glomerulosclerosis. Secondly, GBM is one of the earliest sites at which amyloid fibrils are deposited (39). Because AP is present in amyloid deposits both in man and experimental animals (37, 40) and SAP binds to amyloid fibrils in vitro (10), it is conceivable that basement membrane AP contributes to localization of fibrils in this site. Thirdly, SAP has calcium-dependent binding affinity for as yet

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unidentified ligands in agarose (8, 13), other galactans (41), some microbial polysaccharides (10) and also a modified form of the complement component C3.3 If the AP within basement membranes expresses this binding capacity in vivo, it could provide a mechanism whereby circulating ligands could localize and focus immunological or other inflammatory processes onto basement membrane.

Summary

Glomerular and other vascular basement membranes were found to contain an antigen that was immunochemically indistinguishable from serum amyloid P-component. There was no immunological cross-reactivity between antisera to serum amyloid P-component and to collagen types I, III, IV, or V. The amyloid P-component antigen was confined to the endothelial aspect, the lamina rara interna, of glomerular basement membrane. It could not be eluted by high-ionic-strength saline, EDTA, dithiothreitol, or either polar or nonpolar detergents, but was released into solution when isolated glomerular basement membrane was digested by highly purified bacterial collagenase. Most of these P-component molecules and their constituent polypeptide chains were of higher molecular weight and lower isoelectric point than serum amyloid P-component. These findings indicate that, as well as being a normal plasma protein and a universal constituent of amyloid deposits, P-component is also a normal matrix glycoprotein of basement membrane in which it is covalently linked to collagen and/or other matrix proteins. This may be relevant both to the pathogenesis of amyloidosis and to other aspects of physiology and pathology of basement membranes.

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