FIBRONECTIN AND C4-BINDING PROTEIN ARE SELECTIVELY BOUND BY AGGREGATED AMYLOID P COMPONENT*

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Amyloid P component (AP)[1] is a glycoprotein found in all forms of amyloid deposit regardless of the chemical nature of the fibril protein (1–6). It is indistinguishable from a normal plasma protein, serum amyloid P component (SAP), in terms of antigenicity, molecular weight, polypeptide subunit composition, appearance in the electron microscope, and the partial amino acid sequences available hitherto (7–11). SAP/AP is also a normal tissue protein in man; it is an integral constituent of vascular basement membranes, where it is covalently linked to collagen and/or other matrix glycoproteins (12), and is located on the peripheral microfibrillar mantle of elastic fibres throughout the body (5).[2] SAP is very closely related to C-reactive protein (CRP), the classical acute-phase reactant (13), and there is ~60% homology of amino acid sequence between them in man (14). CRP and SAP are both composed of noncovalently associated subunits arranged with cyclic pentameric symmetry in an annular disclike configuration, and both proteins have the capacity for calcium-dependent ligand binding (10, 14, 15). The ligands for SAP identified so far include amyloid fibrils (16), agarose (10), and some other polysaccharides (17), and fixed complement (18), but the physiological role and possible pathogenetic significance of SAP/AP are not known.

We have previously reported that proteins that very closely resemble human SAP in terms of molecular structure and binding specificity for agarose are present in mammals and in lower vertebrates, including fish (19). There is ~50% homology of amino acid sequence in the N-terminal region between SAP of plaice (*Pleuronectes*

* Supported by Medical Research Council Programme grant 979/51 and by the Wellcome Trust.

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[1] *Abbreviations used in this paper:* AP, amyloid P component; BSA, bovine serum albumin; C4bp, C4-binding protein; CRP, C-reactive protein; EAC, sheep erythrocytes sensitized with rabbit IgM anti-erythrocyte antibody and coated with human complement; Fn, fibronectin; NHS, normal human serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.2; PMSF, phenylmethylsulphonyl fluoride; SAP, serum AP; SDS, sodium dodecyl sulphate; Tris-saline-Ca, 0.01 M Tris-buffered 0.14 M sodium chloride, pH 8.0, containing 0.002 M calcium chloride; Tris-saline-EDTA, 0.01 M Tris-buffered 0.14 M sodium chloride, pH 8.0, containing 0.01 M EDTA.

[2] Breathnach, S., S. Melrose, B. Bhogal, R. F. Dyck, F. C. de Beer, G. Tennent, M. M. Black, and M. B. Pepys. Amyloid P component is located on elastic fibre microfibrils of normal human tissues. Manuscript submitted for publication.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/10/1134/15 $1.00
Volume 154 October 1981 1134–1149

platessa L.) and man (20). The stable conservation of structure and binding reactivity of this protein throughout vertebrate evolution suggests that SAP has important functions and that these may relate to its ligand-binding properties. We have therefore investigated the binding specificity of human SAP for normal serum proteins and report here that, under suitable conditions, SAP, in the presence of calcium ions, selectively binds two distinct proteins from whole human serum. We have identified these as fibronectin (Fn) and C4-binding protein (C4bp) and have shown that pairing of SAP molecules is required for them to bind Fn.

Materials and Methods

Proteins. Isolated pure Fn (21) and SAP (10) were prepared from citrated human plasma and normal human serum (NHS), respectively, according to established methods. To ensure as far as possible the complete purity of the SAP used here, the protein, isolated as previously described (10), was passed over a column of Sepharose to which polyvalent anti-NHS antibodies were coupled, and it was then gel filtered on Sephacryl S-300 (Pharmacia G. B. Ltd., Hounslow, Middlesex, England). No contaminants were detectable by immunoelectrophoresis and crossed immunoelectrophoresis against polyvalent anti-NHS, by gradient polyacrylamide gel electrophoresis (PAGE) of the native protein or by sodium dodecyl sulphate (SDS)-PAGE (see below) with a 200 μg sample. Human IgM was isolated from a macroglobulinemia serum by repeated euglobulin precipitation, followed by gel filtration on Sephadex G-200 (Pharmacia). Isolated purified C4bp (22) was kindly provided by Dr. Victor Nussenzweig, New York University, and isolated human α_2-macroglobulin (23) was provided by Dr. A. J. Barrett, Strangeways Laboratory, Cambridge, England. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. Lt., Poole, Dorset, England.

Antisera. Monospecific antisera to SAP, Fn, and C3 (24) were raised by immunization of rabbits and sheep with the isolated proteins. Polyvalent anti-NHS was raised by repeated immunization of sheep with NHS. Serum proteins that were bound by SAP-Sepharose (see below) were used to immunize a rabbit. Monospecific rabbit anti-C4bp serum was kindly provided by Dr. Victor Nussenzweig. The IgG1 fractions of normal sheep serum and of sheep antisera to SAP and C3 were isolated by salt fractionation and DEAE cellulose chromatography. The IgG fraction of rabbit anti-Fn serum was isolated using protein A-Sepharose (Pharmacia G. B. Ltd.) according to the manufacturer's instructions. Antisera to fibrinogen, Factor XIII, Factor VIII, transferrin, α_2-macroglobulin, α and β lipoproteins, C1s, C1q, C4, IgM, IgA, and IgG were obtained from Behring Diagnostic Reagents, Hoechst Pharmaceuticals, Hounslow, Middlesex, England.

Immobilized Proteins. Sepharose 4B and CNBr-activated Sepharose 4B were obtained from Pharmacia, and various proteins were coupled to the latter generally according to the manufacturer's instructions. IgG fractions of different antisera were coupled in pH 7.0 phosphate buffer at between 1 and 10 mg/ml of beads to produce, respectively, anti-SAP-Sepharose, anti-C3-Sepharose, anti-Fn-Sepharose, and normal sheep IgG-Sepharose. SAP, BSA, and Fn were coupled in pH 9.0 carbonate buffer. After coupling SAP to CNBr-Sepharose at between 0.1 and 10 mg/ml of beads, the beads were washed with 0.1 M borate buffer, pH 8.0, containing 1.0 M NaCl. SAP-Sepharose was not washed at pH 4.0 because SAP is labile at low pH. Control beads were prepared by blocking the active groups on CNBr-Sepharose with ethanolamine without coupling any protein. Fn was coupled to Sepharose at 4 mg/ml of beads. Gelatin-Sepharose (4 mg gelatin/ml beads) was prepared as described elsewhere (21) and was loaded with Fn by passing 100 ml of fresh citrated normal plasma over a 15-ml column and then washing with phosphate-buffered saline (PBS), pH 7.2, containing 0.01 M citrate.

Human Serum. Fresh NHS was obtained by allowing venous blood from laboratory volunteers to clot at room temperature for 2–4 h before centrifugation. In some experiments, serum from a single donor was used, in others different sera were pooled. In some cases benzamidine (5 mM) and phenylmethylsulphonyl fluoride (PMSF) (10 mM) (both from Sigma Chemical Co.) were added to the serum as soon as it was separated. Fn-depleted NHS was prepared by passing fresh citrated plasma over gelatin-Sepharose and then over anti-Fn-Sepharose before recalci-

fying it and allowing it to clot. Absence of Fn from the serum was confirmed by electroimmunoassay. SAP-depleted NHS was prepared by passing NHS over plain unsubstituted Sepharose that had been equilibrated with Tris-saline-Ca (10). The absence of SAP from the serum was confirmed by electroimmunoassay.

Immunochemical Assays. Proteins were detected and identified by double immunodiffusion in 1% wt:vol agarose gel (Indubiose A37, IBF S.A., Clichy, France) in 0.075 M veronal buffer, pH 8.6, containing 0.01 M EDTA. Electroimmunoassays for SAP, C4bp, C3, and Fn were performed by established techniques using monospecific antisera (25). These assays were calibrated using standards of the isolated pure proteins. IgM, IgA, and IgG were quantitated by radial immunodiffusion with a standard serum (Behring Diagnostics) for calibration.

PAGE. Gradient PAGE analysis of native proteins was performed in 4–30% gradient gels (Pharmacia) run exactly according to the manufacturer's instructions. Standard globular proteins used as markers; thyroglobulin, 669,000 mol wt; ferritin, 440,000 mol wt; catalase, 232,000 mol wt; lactate dehydrogenase, 140,000 mol wt; and albumin, 67,000 mol wt, were obtained from Pharmacia. SDS-PAGE analysis was undertaken in slab gels using the method of Laemmli (26). Marker polypeptides were fibronectin, 220,000–225,000 mol wt, and α_2-macroglobulin, 181,000 mol wt, and the Pharmacia kit of phosphorylase b, 94,000 mol wt; albumin, 67,000 mol wt; ovalbumin, 43,000 mol wt; and carbonic anhydrase, 30,000 mol wt. Stained gels were examined in a Helena QuickScan scanning densitometer (T & J Crump Ltd., Rayleigh, Essex, England) to quantitate the distribution of protein among different bands. Two-dimensional SDS-PAGE was performed using 2.5-mm Diam 7.5% rod gels in the first dimension. Samples were run on these without reduction in the Pharmacia GE4 apparatus and the whole gels were then immersed and incubated in the Laemmli sample buffer (26), including 2-mercaptoethanol, for 1 h at 37°C before being applied horizontally at the top of a 7.5% slab gel. After electrophoresis into the second dimension, the rod was discarded and the slab gel was stained. Replicate rod gels were stained after the first electrophoresis.

Affinity Chromatography Experiments. Tests for the binding of serum proteins to affinity media were performed at 4°C using either column or batch procedures. With the exception of experiments involving uptake of Fn onto gelatin-Sepharose, the Sepharose beads were always pre-equilibrated in 0.01 M Tris-buffered 0.14 M saline, pH 8.0, containing 0.002 M calcium chloride (Tris-saline-Ca) and isolated proteins were dialyzed into this buffer before use. Serum or isolated proteins were passed over or incubated with the affinity media, which were then washed with Tris-saline-Ca until no further material absorbing at 280 nm was eluted. The beads were finally eluted with Tris-saline containing 0.01 M EDTA instead of calcium (Tris-saline-EDTA), and the eluate was monitored by absorbance at 280 nm assuming $E^{1\%}_{1cm} = 13$. In experiments with isolated Fn, the quantities bound and eluted were measured by specific electroimmunoassay. When required for PAGE analysis, the EDTA eluates from SAP-Sepharose and anti-SAP-Sepharose columns were concentrated on PM30 membranes in the Amicon Diaflo system (Amicon Corp., Lexington, Mass.). To determine whether Fn in the EDTA eluate obtained after NHS had been passed over SAP-Sepharose retained its affinity for gelatin, 5.5 ml of eluate ($A_{280} = 0.39$) was passed over a 2-ml column of gelatin-Sepharose. Unbound protein was pooled and concentrated to 4 ml with $A_{280} = 0.15$. The column was washed with Tris-saline-EDTA and then eluted with 1.0 M arginine, pH 7.2, to yield 5 ml with $A_{280} = 0.15$. Starting material, absorbed and eluted protein were compared in gradient PAGE.

Digestion with Trypsin. Samples of EDTA eluate off SAP-Sepharose were dialyzed into Tris-saline-Ca and then incubated at 37°C for 5 min with 4% wt:wt trypsin (Sigma type XI). The reaction mixture was then run immediately in gradient PAGE.

Immunoprecipitation Experiments. 40 μg of the mixture of serum proteins that underwent calcium-dependent binding by SAP-Sepharose were radiolabeled to a specific activity of 7.15 mCi/mg using $Na^{125}I$ (IMS30; Radiochemical Centre, Amersham, Bucks, England) in the chloramine T method (27). Separate 20-μl vol, containing 1.6 μg of the labeled material, were mixed with 20-μl vol of monospecific antisera to Fn and to C4bp and normal rabbit serum as a control. After incubation together on ice for 10 min, 100 μl volumes of a 40% suspension of fixed *Staphylococcus aureus* particles in PBS with 0.01 M EDTA were added. After mixing and a further incubation for 10 min the suspensions were centrifuged and washed twice in PBS/EDTA containing 0.05% desoxycholate. The sedimented *S. aureus* were then eluted by boiling

with the SDS-PAGE sample buffer containing mercaptoethanol (26), and the eluates were run in SDS-PAGE. The slab was stained, dried, and subjected to autoradiography using LKB ^{3}H-Ultrofilm (LKB Instruments Ltd., London, England). In other experiments, monospecific anti-Fn and anti-C4bp antisera were used in optimal proportions titrations against a pool of fresh NHS. The optimal amount of each antiserum, which precipitated all the respective specific antigen, was then added to aliquots of NHS in the presence and absence of 0.01 M EDTA. After incubation at 21°C for 1 h and overnight at 4°C, the concentration of SAP in the starting material and in the treated samples was measured. Complete depletion of Fn and C4bp, respectively, was confirmed by specific electroimmunoassay.

Results

Calcium-dependent Binding of Serum Proteins by SAP-Sepharose. The capacity of SAP to bind serum proteins was tested by passing fresh NHS over a column of SAP-Sepharose prepared by covalent conjugation of isolated pure SAP to CNBr-Sepharose. After washing with Tris-saline-Ca until the effluent had zero absorbance at 280 nm, the column was eluted with Tris-saline-EDTA and the recovered proteins were quantitated and analyzed. The same procedure was adopted with control columns of BSA-Sepharose and ethanolamine-Sepharose. In many separate experiments, columns of SAP-Sepharose always yielded more protein in the EDTA eluate than either of the control columns and a representative result is shown in Table I.

Characterization by PAGE of the Serum Proteins Bound by SAP-Sepharose. PAGE analysis of the serum proteins that were bound by SAP-Sepharose in the presence of calcium ions and eluted with EDTA revealed two major constituents that were the same in all the different preparations obtained by this procedure. In contrast, EDTA eluates from the control columns produced only a single faint band of SAP, which is known to have calcium-dependent binding affinity for agarose (10). In gradient PAGE run under nondenaturing conditions (Fig. 1), there was: (*a*) material of very high apparent molecular weight, which penetrated poorly into the 4% polyacrylamide at the top of the gel and did not form distinct bands, and (*b*) material forming a closely spaced set of two or three distinct, sharply focused bands with apparent molecular weights around 730,000 mol wt by comparison with the migration of standard globular protein markers. In three separate preparations, the distribution of the total protein between a and b was 42.3 ± 5.7% (mean ± SD) in the very high molecular weight material and 41.5 ± 4.2% in the 730,000 mol wt bands. In addition, there was a

TABLE I

Calcium-dependent Binding of Serum Proteins by SAP-Sepharose

Volume of serum offered	Total protein eluted with EDTA		
	SAP-Sepharose*	BSA-Sepharose‡	Ethanolamine-Sepharose§
ml		*mg*	
5	1.5	ND‖	ND
20	2.6	ND	ND
60	2.6	0.3	0.1

* 13.2 mg SAP coupled to 3.5 ml of CNBr-Sepharose.

‡ 14 mg BSA coupled to 3.5 ml of CNBr-Sepharose.

§ 3.5 ml CNBr-Sepharose blocked with ethanolamine, no protein coupled.

‖ Not done.

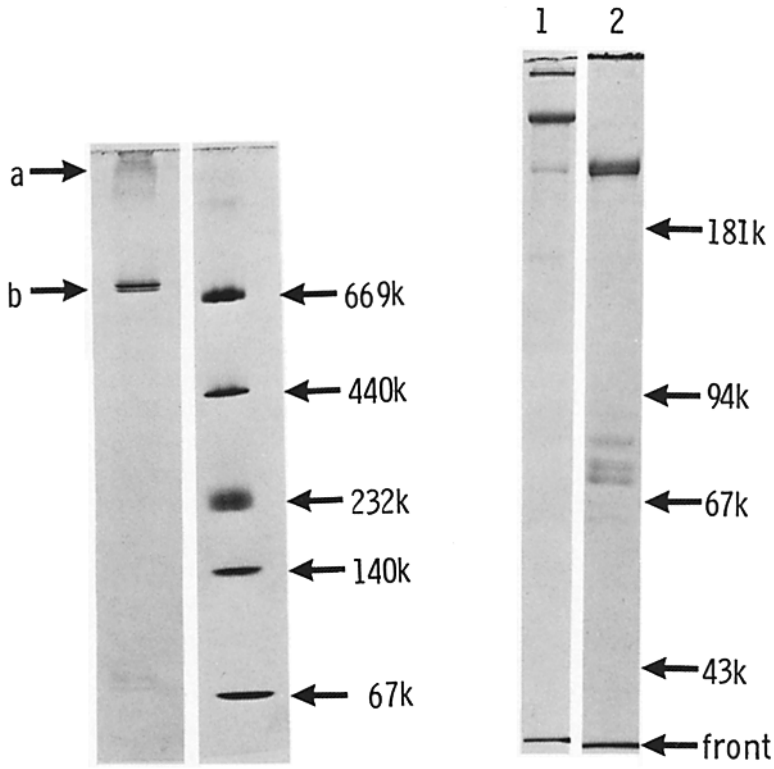

FIG. 1. 4–30% gradient PAGE of proteins eluted with EDTA after passage of NHS over SAP-Sepharose. The two major constituents are designated a and b. Positions of marker proteins of known molecular weight are indicated by arrows.

FIG. 2. 7.5% SDS-PAGE of proteins eluted with EDTA after passage of NHS over SAP-Sepharose. Track 1, nonreduced proteins showing two main bands of high apparent molecular weight; track 2, reduced proteins showing pair of bands at 220,000–225,000 mol wt and poorly focused set of bands at about 70,000 mol wt. Positions of marker proteins of known molecular weight are indicated by arrows.

fainter band at an apparent molecular weight of 647,000, which comprised 9.7 ± 6.0% of the total protein.

Analysis of these preparations without reduction in SDS-PAGE again revealed two major components: a closely spaced pair of bands with apparent molecular weight greater than 500,000, and a single band at 450,000 (Fig. 2, track 1). After reduction, these proteins yielded a well defined pair of bands at 220,000–225,000, and a poorly focused set of bands at ~70,000 (Fig. 2, track 2). Two-dimensional SDS-PAGE (not shown) revealed that the 220,000–225,000 bands were derived from the 450,000 protein, and the 70,000 subunits were derived from the higher molecular weight material.

The sizes and polypeptide chain compositions of the two serum proteins bound by immobilized SAP corresponded with the known structures of Fn (28–31) and C4bp (22), respectively. Fn and C4bp, which had been isolated separately by established techniques (21, 22), were therefore run as standards for comparison in the various PAGE systems (Figs. 3 and 4) and they clearly identified precisely with the two

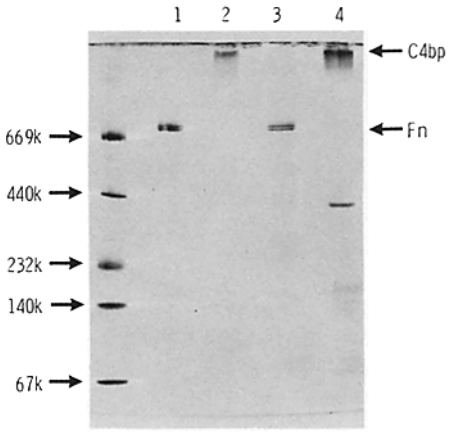

FIG. 3. 4–30% gradient PAGE. Track 1, isolated pure Fn; track 2, isolated pure C4bp; track 3, proteins eluted with EDTA after passage of NHS over SAP-Sepharose; track 4, proteins eluted with EDTA after passage of Fn-depleted NHS over SAP-Sepharose. Position of marker proteins of known molecular weight are indicated by arrows.

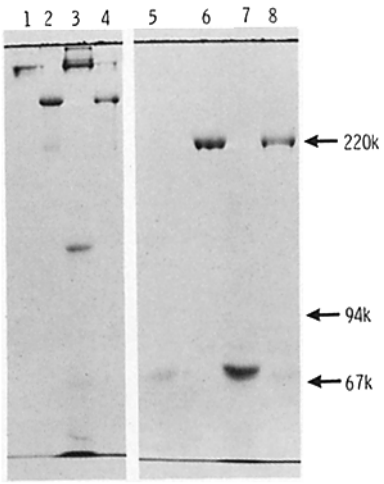

FIG. 4. 5.5% SDS-PAGE. Samples in tracks 1–4 were not reduced; tracks 5–8, reduced with mercaptoethanol. Tracks 1 and 5, isolated pure C4bp; tracks 2 and 6, isolated pure Fn; tracks 3 and 7, proteins eluted with EDTA after passage of Fn-depleted NHS over SAP-Sepharose; tracks 4 and 8, proteins eluted with EDTA after passage of NHS over SAP-Sepharose. Positions of marker proteins of known molecular weight are indicated by arrows.

proteins in question. It is evident that both Fn and C4bp in the native state run with anomalously high apparent molecular weights in gradient gels.

Immunochemical Identification of the Serum Proteins Bound by SAP-Sepharose. Immunoprecipitation in agarose gel with monospecific antisera to Fn and C4bp, respectively, confirmed the presence of these two proteins in the material eluted from SAP-Sepharose (Fig. 5). Furthermore, immunization with the eluted material induced the production of an antiserum giving two distinct precipitation lines against the eluate and these were identified as being anti-Fn and anti-C4bp, respectively (Fig. 5).

To correlate the immunochemical identity of the two proteins with their polypep-

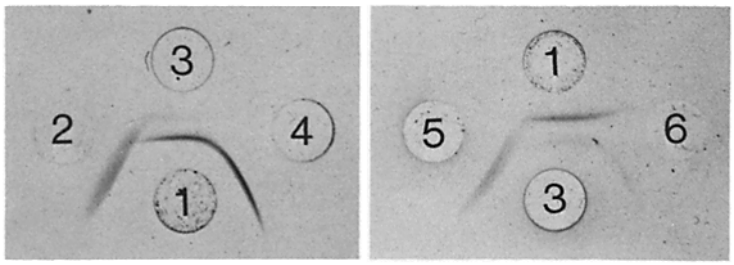

FIG. 5. Immunodiffusion in agarose gel between 1, proteins eluted with EDTA after passage of NHS over SAP-Sepharose; 2, monospecific rabbit anti-Fn serum; 3, rabbit antiserum raised against the proteins in 1; 4, monospecific rabbit anti-C4bp serum; 5, isolated pure C4bp; 6, isolated pure Fn.

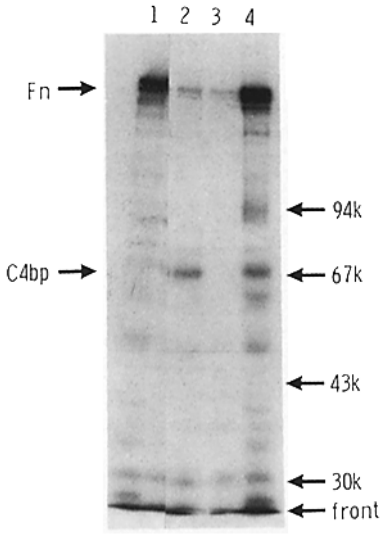

FIG. 6. Autoradiograph of reduced 7.5% SDS-PAGE analysis of proteins eluted with EDTA after passage of NHS over SAP-Sepharose. The eluate was radioiodinated and immunoprecipitated with 1, monospecific anti-Fn; 2, monospecific anti-C4bp; 3, normal rabbit serum control; track 4, whole nonprecipitated radiolabeled eluate. The positions of known marker proteins run in the same gel are indicated by arrows.

tide chain composition, a preparation of the eluate from SAP-Sepharose was radiolabeled and then immunoprecipitated in separate aliquots with anti-Fn and anti-C4bp antisera. After washing, denaturation, and reduction, the precipitates were run in an SDS-PAGE slab gel, which was then subjected to autoradiography (Fig. 6). This showed that anti-Fn antibodies had reacted selectively with the protein at 220,000–225,000 and that anti-C4bp antibodies had selectively complexed with the protein composed of 70,000 mol wt subunits. Traces of Fn in the anti-C4bp immunoprecipitate were also visible in the normal nonimmune rabbit serum control (Fig. 6) and are due to binding of Fn to the *S. aureus* particles (32) used to harvest the immune precipitates.

Trace Constituents of the Material Eluted from SAP-Sepharose. Although Fn and C4bp were always the major proteins eluted from SAP-Sepharose, other faint bands were seen in some preparations analyzed by PAGE techniques and in the autoradiograph

of the radiolabeled eluate. Many of these bands were derived by cleavage of Fn, which is known for its susceptibility to proteolytic degradation (30, 31). During storage of eluates at 4°C, there was progressive loss of staining from the band(s) corresponding to intact Fn and appearance of new bands at lower apparent molecular weights. This process was retarded by adding benzamidine and PMSF to the preparations. On the other hand, incubation with trypsin caused complete loss of the intact Fn band but had no effect on the C4bp.

In addition to the cleavage fragments of Fn, presumably generated by proteinases released or activated in serum, trace amounts of certain other serum proteins were detected immunochemically in the eluates. These proteins, which together never exceeded 10% of the total protein present, were IgM, IgG, IgA, C3, C1q, C1s, and SAP itself. With the exception of IgM, comparable amounts of these proteins were recovered in the EDTA eluates from the control, non-SAP-bearing Sepharose columns. More IgM was always eluted from SAP-Sepharose and, although it never comprised more than 10% of the total protein, it was sometimes identifiable in SDS-PAGE (Fig. 7). Traces of C4 were detected in the eluate from SAP-Sepharose only when aged serum instead of fresh NHS was used as the starting material. This was probably

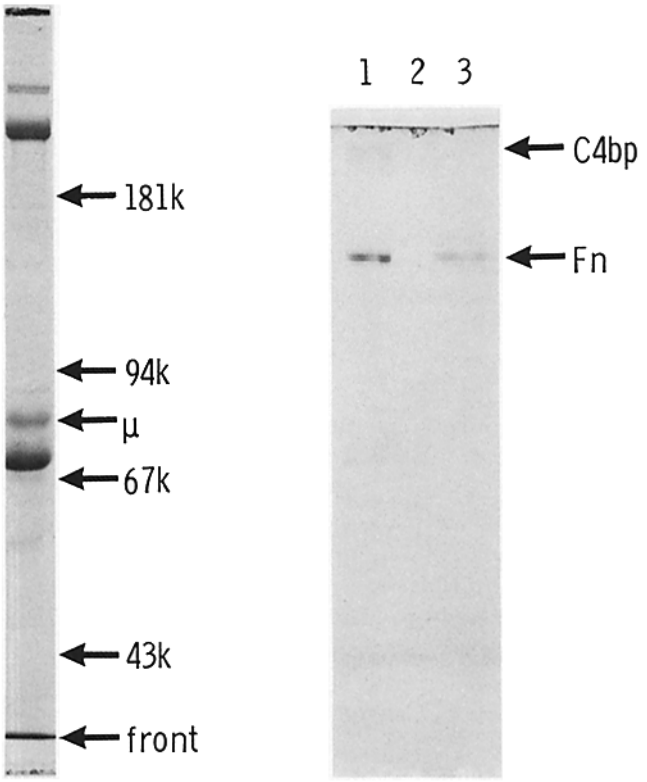

FIG. 7. 7.5% SDS-PAGE of reduced proteins eluted with EDTA after passage of NHS over SAP-Sepharose. Positions of marker proteins of known molecular weight, including IgM heavy chain (μ), are indicated by arrows.

FIG. 8. 4–30% gradient PAGE. Track 1, proteins eluted with EDTA after passage of NHS over SAP-Sepharose; track 2, same material as 1 after absorption with gelatin-Sepharose; track 3, protein eluted off gelatin-Sepharose with 1.0M arginine.

because some C4b that was generated during aging was bound by C4bp (22), which in turn was bound by the immobilized SAP.

Binding of Fn by SAP-Sepharose. The EDTA eluate obtained after NHS had been passed over SAP-Sepharose contained Fn, which retained its characteristic ability to bind to gelatin (33). Passage of the mixture of proteins eluted by EDTA (Fig. 8, track 1) over a column of gelatin-Sepharose selectively removed the Fn (Fig. 8, track 2), which was then recovered by elution with 1 M arginine (Fig. 8, track 3) (21).

Isolated pure Fn was also bound by SAP-Sepharose. An excess of purified Fn in Tris-saline-Ca was passed over 3.5 ml of Sepharose to which 13.2 mg of SAP had been coupled and 3.0 mg of Fn became bound. Elution with EDTA recovered all the bound Fn, whereas no Fn became bound to or was eluted from the control, BSA-Sepharose, or ethanolamine-Sepharose columns. There was therefore a specific interaction between Fn and SAP-Sepharose that was calcium dependent but independent of any other protein or serum constituent.

All the known ligand-binding reactivities of SAP are calcium dependent (10, 15, 16), whereas the binding of Fn to proteins such as collagen, gelatin, and fibrin (28–31) and hemagglutination by human plasma Fn (34) are independent of divalent cations. The present results therefore suggested that in these experiments SAP was the binding protein and Fn was the ligand.

Immobilized Fn Does Not Bind Soluble SAP. Fn was immobilized by directly coupling the isolated pure protein to CNBr-Sepharose or by passing an excess of citrated plasma over a column of gelatin-Sepharose. Both types of material were then equilibrated with Tris-saline-Ca, and fresh NHS, as a source of SAP, was passed over them. After further washing with Tris-saline-Ca, elution with Tris-saline-EDTA yielded no more SAP than was yielded by plain unsubstituted Sepharose beads that had been exposed to NHS in the same way. The interaction between Sepharose-SAP and Fn thus seems to depend on the molecular disposition of SAP when it is coupled to Sepharose.

SAP in Serum Is Not Complexed with Fn or C4bp. An alternative explanation for the failure of SAP in serum to become bound to immobilized Fn is that SAP, in its native state in the serum, may already be complexed with Fn and/or C4bp, or possibly some other ligand, and that it may not dissociate from such complexes and attach to the solid-phase Fn under the conditions tested. The existence of such complexes was therefore sought by adding optimal amounts of monospecific antisera to Fn and C4bp, respectively, to samples of normal serum. All the Fn and C4bp were precipitated, as confirmed immunochemically, but there was no depletion of SAP irrespective of whether the precipitations were conducted in the presence of calcium ions or EDTA. This is strong evidence against the existence of a significant proportion of native SAP in the form of calcium-dependent complexes with either Fn or C4bp.

Calcium-dependent Binding of Fn and C4bp from Serum Passed over Anti-SAP-Sepharose. The capacity of SAP to bind serum proteins was also investigated by passing normal serum over anti-SAP antibodies coupled to CNBr-Sepharose. SAP in the serum was absorbed by the anti-SAP antibodies, and after washing with Tris-saline-Ca the column was eluted with EDTA. A significant amount of protein was recovered (Table II) and consisted of a mixture of Fn and C4bp exactly as in the eluate from SAP-Sepharose. Control columns of normal IgG-Sepharose and of anti-C3-Sepharose yielded no Fn or C4bp whatsoever, only a small quantity of SAP itself,

TABLE II

Calcium-dependent Binding of Serum Proteins by Anti-SAP-Sepharose

Volume of serum offered	Total protein eluted with EDTA		
	Anti-SAP-Sepharose*	Normal IgG-Sepharose‡	Anti-C3-Sepharose§
ml		*mg*	
60	3.6	0.8	0.9

* 80 mg of IgG1 fraction of sheep anti-SAP coupled to 15 ml CNBr-Sepharose. This column removed all the SAP (2.9 mg) from the serum passed over it.

‡ 80 mg of IgG1 fraction of normal sheep serum coupled to 15 ml CNBr-Sepharose.

§ 80 mg of IgG1 fraction of sheep anti-C3 serum coupled to 15 ml CNBr-Sepharose.

TABLE III

*Calcium-dependent Binding of Serum Proteins to SAP-Sepharose**

Amount of SAP coupled (mg/ml Sepharose beads)	0	0.1	0.5	1.0	10.0
Total protein eluted with EDTA (mg)	0.05	0.07	0.08	0.14	0.61

* 10 ml vol of NHS were offered to separate 1.0 ml vol of Sepharose bearing the amounts of SAP shown.

and trace amounts of immunoglobulins, C1 and C3. The specific role of SAP in binding Fn and C4bp when NHS was passed over anti-SAP-Sepharose was confirmed by using SAP-depleted NHS. NHS that had been completely depleted of SAP by absorption with plain Sepharose (10) was passed over the anti-SAP-Sepharose column. After washing with Tris-saline-Ca, elution with EDTA yielded only traces of protein and no detectable Fn or C4bp. However, when this same serum was then passed over SAP-Sepharose and followed by washes of Tris-saline-Ca, elution with EDTA produced the mixture of Fn and C4bp exactly as before.

When identical aliquots of NHS were used as starting material, more Fn and C4bp were always recovered from anti-SAP-Sepharose than from SAP-Sepharose. In the representative experiments shown in Tables I and II, the yields of eluted protein from SAP complexed by anti-SAP-Sepharose and directly coupled to Sepharose were 1.24 mg/mg and 0.12 mg/mg, respectively.

In view of the failure of anti-Fn or anti-C4bp antibodies added to NHS to deplete any SAP at all, the results with the anti-SAP-Sepharose column cannot be adduced as evidence that SAP in serum is complexed with these proteins. Rather it seems that when SAP molecules are either immobilized or aggregated in some way, they acquire a calcium-dependent reactivity with Fn and C4bp. Binding of SAP by solid-phase anti-SAP antibodies appears to generate this reactivity much more efficiently than direct coupling of SAP to Sepharose.

Aggregation of SAP Molecules Is Required for Them to Bind Fn and C4bp. When serum was passed over plain unsubstituted Sepharose, the SAP was taken up, due to its calcium-dependent affinity for agarose (10), but no Fn or C4bp was yielded by subsequent elution with EDTA. The capacity of plain Sepharose beads for SAP is only ~60 μg/ml (16), and when serum was incubated with batches of SAP-Sepharose to which different amounts of SAP had been coupled, there was no significant yield of Fn and C4bp from beads bearing <1 mg SAP/ml (Table III). This suggests that

mere immobilization of SAP is not sufficient for it to express reactivity for Fn and C4bp and that there may be a need for close association of SAP molecules.

This suggestion is supported by the high relative efficiency of binding by SAP complexed with anti-SAP-Sepharose (Tables I and II). The IgG fraction of anti-SAP serum that was used here contained only ~5% of specific anti-SAP antibodies and it was coupled at 5 mg/ml of beads, well below their capacity of 20 mg/ml or more. Anti-SAP molecules were therefore probably spaced out on the bead surfaces rather than being close to each other, and the greatest degree of aggregation of SAP that could have occurred would have been binding of pairs of SAP molecules by the adjacent combining sites of divalent anti-SAP IgG molecules. Alternatively, it was possible that the mere binding of individual SAP molecules by immobilized anti-SAP might be sufficient for the SAP, in turn, to express its binding reactivity for Fn and C4bp.

Pairs of SAP Molecules Bind Fn. Anti-SAP-Sepharose beads bearing 5 mg anti-SAP IgG fraction/ml of beads were divided into 1-ml and 10-ml portions, and the same volume of NHS was incubated with each portion in a batch procedure. Sufficient SAP was provided by the NHS to completely saturate the smaller portion. The larger one received only 10% of its capacity and the bound SAP molecules were therefore presumably widely scattered on the bead surfaces. After washing with EDTA and reequilibration with Tris-saline-Ca, only the beads that were saturated with SAP were capable of binding isolated pure Fn (Table IV), despite the fact that both sets of beads bore the same total amount of immobilized SAP.

In another experiment, two lots of 10 mg of the IgG fraction of anti-SAP were coupled, respectively, to 1 ml and 10 ml of CNBr-Sepharose. The spacing of anti-SAP molecules on these two lots of beads must have been widely different but after saturating them with an excess of isolated SAP, both had the same capacity for binding isolated Fn (Table V).

These results indicate that the capacity of SAP to bind Fn is expressed when two SAP molecules are near each other. This can be achieved either by directly coupling a high density of SAP to CNBr-Sepharose, or by providing sufficient SAP to anti-SAP-Sepharose for two SAP molecules to be taken up by each antibody, regardless of how widely spaced the anti-SAP molecules are. The quantitative data in Tables IV

TABLE IV

*Effect of Degree of Saturation of Anti-SAP-Sepharose with SAP on the Capacity of the SAP to Bind Fn**

Volume of beads	Anti-SAP IgG coupled (mg/ml of beads)	SAP	Percent capacity for SAP taken up	Amount of Fn bound	Molar ratio of SAP:Fn‡
ml		*μg*		*μg*	
1	5	130	100	153	2:1.3
10	5	130	10	0	—

* Anti-SAP-Sepharose beads were either fully or partially saturated with SAP and were then offered an excess of isolated pure Fn in Tris-saline-Ca for binding.

‡ Based on molecular weights of 450,000 for Fn and 250,000 for SAP.

TABLE V

*Effect of Amount of Anti-SAP Coupled to Sepharose on the Capacity of SAP to Bind Fn**

Volume of beads	Anti-SAP IgG coupled (mg/ml of beads)	SAP	Percent capacity for SAP taken up	Amount of Fn bound	Molar ratio of SAP:Fn‡
ml		*μg*		*μg*	
1	10	330	100	318	2:1
10	1	292	100	318	2:1.2

* Anti-SAP-Sepharose beads bearing either 10 or 1 mg anti-SAP IgG/ml were fully saturated with SAP and then offered an excess of isolated pure Fn in Tris-saline-Ca for binding.

‡ Based on molecular weights of 450,000 for Fn and 250,000 for SAP.

and V also indicate that each pair of SAP molecules usually binds a single Fn molecule.

Independent and Competitive Binding of Fn and C4bp by Aggregated SAP. When serum was depleted of Fn by passage over gelatin-Sepharose and then over anti-Fn-Sepharose before being offered to a column of SAP-Sepharose, the resultant EDTA eluate of the SAP column contained double the usual proportion of C4bp (82 vs. 42% of the total protein) (Figs. 3 and 4). The binding of C4bp was therefore independent of the presence of Fn and the two proteins seem to compete with each other for uptake by aggregated SAP. Presumably, each closely adjacent pair of SAP molecules is able to bind a molecule of either Fn or C4bp.

Discussion

We report here for the first time the specific calcium-dependent binding reactivity of SAP for two distinct normal serum proteins, Fn and C4bp. SAP in solution in the serum was not complexed with either of these proteins, but when SAP was directly coupled at a sufficient density to Sepharose beads, or was itself bound by immobilized anti-SAP antibodies, it became capable of selectively recognizing Fn and C4bp in whole undiluted normal serum. The capacity of SAP to bind Fn was found to depend on dimerization of SAP, such as was produced by allowing divalent anti-SAP molecules to bind pairs of SAP molecules. Uptake of Fn by SAP in this form required calcium ions but was independent of all other proteins or serum constituents. Similar considerations applied to the binding of C4bp and, although this was not tested with the isolated protein, it was shown to be independent of the presence of Fn.

Both Fn and C4bp are proteins with their own well-defined binding reactivities. Fn binds to collagen, gelatin, actin, fibrin, DNA, glycosaminoglycans, glycolipids, and cell surfaces of many cell types (28–31). C4bp binds very specifically to C4b, the major cleavage fragment of the complement component C4 (22, 35–37). However none of the reactions described hitherto of human plasma Fn with other proteins (28–31, 34) nor of C4bp with C4b requires calcium or any other divalent cations, even though C4bp does itself bind calcium ions (22). In contrast, all the binding properties of SAP reported previously are absolutely dependent on calcium ions (10, 15, 16). This, together with our present observation of the failure of immobilized Fn to bind soluble

SAP indicates that, in the associations we have described here, SAP is functioning as the binding protein and Fn and C4bp as the ligands.

The basis for the specificity of the reaction of aggregated SAP with these two disparate serum proteins is not known. There is no known similarity between Fn and C4bp, although it is possible that their carbohydrate moieties may be related. SAP is clearly able to bind to polysaccharides, agarose, for example (10), a linear galactan hydrocolloid, and experiments are currently in progress to determine whether aggregated SAP binds the glycopeptides isolated after exhaustive proteolysis of purified Fn. In fact, the present observations provide the first opportunity to identify and characterize chemically potentially physiological ligand(s) for SAP.

Studies of the formation of complexes of isolated fluid-phase SAP with Fn and with C4bp were not possible because in the presence of sufficient free calcium ions for SAP to express its binding reactivity, the SAP molecules aggregate on themselves (16). However, density gradient ultracentrifugation studies using whole serum, the results of which are to be reported elsewhere,[3] failed to show any evidence of interaction between soluble SAP and either Fn or C4bp under physiological conditions.

C4bp is evidently an important regulatory protein of the classical pathway of complement activation (35–38). It is bound to antigen-antibody complexes in vitro and has been detected at sites of their in vivo deposition (39). Studies of the effects, if any, of SAP on the behavior and function of C4bp and of the presence of SAP in antigen-antibody-complement complexes will be of considerable interest.

We have recently observed that, in the presence of calcium ions, isolated SAP agglutinates complement-coated erythrocytes (EAC) (18). The physiological significance of the reaction is not clear because it is inhibited by the presence of serum or even by human serum albumin at concentrations of 4 mg/ml or more.[4] Furthermore, when EAC are generated from serum as the source of complement, SAP is not detectable on the cells.[4] In marked contrast, the reaction of aggregated SAP with soluble Fn and C4bp proceeds as well in serum as with the isolated proteins and is therefore more likely to relate to in vivo functions.

The one well-established in vivo reactivity of SAP is its binding to amyloid fibrils (16). AP is present in all forms of amyloid regardless of the chemical nature of the fibril proteins (1–6), and it can be eluted from the deposits by chelating agents (11). In preliminary immunohistochemical studies, we have not yet been able to identify either Fn or C4bp in amyloid deposits. Although this negative result requires confirmation it may indicate that the density of AP molecules in amyloid is insufficient for them to express their binding reactivity for Fn and C4bp, or that Fn and C4bp do not gain access to amyloid deposits in vivo.

Fibronectin is both a plasma protein and a constituent of the extracellular matrix of connective tissues, as well as being a major cell surface protein of many different cell types (28–31). It seems to have important functions in cell-cell and cell-substratum interactions and to participate as a nonspecific opsonin in the phagocytic function of the reticuloendothelial system (28–31). It is intriguing that SAP is the only other protein known so far that is both a plasma protein and a normal constituent of

[3] Baltz, M. L., F. C. de Beer, A. Feinstein, and M. B. Pepys. Calcium-dependent aggregation of human serum amyloid P component. Manuscript submitted for publication.

[4] Baltz, M. L., S. Holford, and M. B. Pepys. Studies of the interaction between serum amyloid P component and the complement system. Manuscript in preparation.

connective tissues. Immunohistochemical staining with anti-SAP at the ultrastructural level reveals an apparently continuous "layer" of SAP in the *lamina rara interna* of vascular basement membranes (12) and on the peripheral microfibrillar mantle of elastic fibres throughout the body.[3] In contrast to circulating SAP, SAP molecules in these tissue situations may be sufficiently closely aligned to express their reactivity for Fn. Although the distributions of Fn and of SAP within tissues are certainly not always coincident, it is possible that there may be a functionally significant interaction between cell surface Fn and tissue SAP at sites, such as the capillary wall, where they are in juxtaposition. Experiments are in progress to test this hypothesis by investigating a possible role for SAP in processes of cell adherence and growth in vitro.

Summary

Serum amyloid P component (SAP) is a normal plasma protein, closely related to C-reactive protein, which is deposited together with amyloid fibrils in all forms of amyloidosis. It is also a normal constituent of human tissues, where it is found in vascular basement membranes and in association with the peripheral microfibrillar mantle of elastic fibres throughout the body. Very similar, highly conserved, homologous proteins are present in the sera of all vertebrates in which they have been sought, and in all cases these proteins display calcium-dependent binding affinity for agarose. The physiological function or pathogenetic significance of this reactivity are not known but we report here for the first time that under appropriate conditions human SAP can also bind certain serum glycoproteins. SAP, which had been aggregated either by direct conjugation to CNBr-activated Sepharose beads, or by complexing with anti-SAP antibodies immobilized on such beads, selectively took up fibronectin and C4-binding protein from whole normal human serum. The reaction was calcium dependent and the two ligands were bound independently of each other or of other serum constituents. Experiments with isolated fibronectin and SAP complexed by anti-SAP-Sepharose indicated that close association of pairs of SAP molecules was required for fibronectin to be bound and that each SAP dimer was capable of taking up a single molecule of fibronectin. There was no evidence that SAP in its native state in the serum was complexed with either fibronectin or C4-binding protein. The present findings significantly extend knowledge of the properties of SAP and open the way to characterisation of its physiological ligand(s) and thence to elucidation of its function.

We thank Dr. R. C. Hughes and Dr. J. R. Couchman for valuable discussions and for provision of fibronectin and anti-fibronectin reagents used in preliminary experiments not included in the present paper, and Dr. A. J. Barrett for his suggestions and for providing isolated α_2-macroglobulin. We are particularly indebted to Dr. V. Nussenzweig for his generosity in making available isolated C4-binding protein and monospecific antiserum to it. The photographs of polyacrylamide gels were printed by Mr. W. Hinks.

Received for publication 9 June 1981.

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