Serum amyloid P component (SAP) forms a calcium-dependent complex with C4b-binding protein (C4BP) in human serum. This study demonstrated that heparin interacted with SAP in a calcium-dependent manner and prevented formation of the SAP•C4BP complex. Furthermore, the SAP-heparin interaction interfered with SAP binding to membranes. Therefore, all three of these interactions involved similar sites on SAP, or each interaction sterically obstructed the other binding sites. In addition to heparin, SAP bound to heparan sulfate and chondroitin sulfate. In each case, a distinct multimeric species was generated. Gel filtration and sucrose density gradient ultracentrifugation suggested that heparin and heparan sulfate produced a dimer of SAP. The dimer appeared to be the most stable structure since it was not dissociated by excess heparin. While low molecular weight heparin interacted with SAP and inhibited SAP association with membranes, the SAP dimer was not detected in sucrose density gradient ultracentrifugation studies. Polybrene prevented the interaction between SAP and heparin in both a purified system and in human serum that was enriched in SAP and heparin. In contrast, Polybrene did not seem to alter the SAP•C4BP complex. While the function of the SAP•C4BP complex is unknown, it may be important for regulation of complement and/or transport of SAP to sites in the body. Dissociation of the SAP•C4BP complex by sulfated polysaccharides such as heparin may be a physiological response that could be important during tissue damage or complement activation.

C4BP, functions as an inhibitory protein of the complement cascade. It is a large protein which consists of seven long, tentacle-like α-subunits and a shorter tentacle referred to as the β-subunit. The large subunits function by binding C4b (Dahlback et al., 1983) and promoting its activation by factor I (Scharfstein et al., 1978; Nagasawa and Stroud, 1977). C4BP binds about 60% of the vitamin K-dependent protein S in serum (Dahlback and Stenflo, 1981; Dahlback, 1983) through its small subunit (Dahlback et al., 1983; Hillarp and Dahlback, 1988, 1990). The protein S-C4BP interaction is calcium-dependent and of high affinity (Schwalbe et al., 1990a; Dahlback et al., 1990). The role of free protein S is to inhibit blood clot formation by serving as a cofactor for activated protein C (Walker, 1980; Walker et al., 1987). Protein S that is complexed to C4BP cannot function as an anticoagulant (Comp et al., 1984; Bertina et al., 1985; Dahlback, 1986). The role of protein S in the protein S-C4BP complex may be to localize C4BP at certain membrane sites, thereby protecting certain tissues or cells from complement activity. The protein S-C4BP complex interacts with specific membranes through the protein S component (Schwalbe et al., 1990a). Recently, we have reported that C4BP also forms a calcium-dependent complex with serum amyloid P component (SAP) in serum (Schwalbe et al., 1990b).

SAP is a member of the pentraxin family of proteins (for reviews see Pepys and Baltz, 1983; Skinner and Cohen, 1988). These proteins are characterized by five identical subunits which are noncovalently associated in a disc-like structure. SAP is a dimer with two pentameric discs noncovalently bound face to face (Painter et al., 1982; Skinner and Cohen, 1988). SAP is a normal constituent of serum and is a normal tissue protein (Dyck et al., 1980; Breathnach et al., 1981). While the biological function of SAP is not known, the fact that no deficiency or alteration in the amino acid sequence has been reported suggests an important function (Pepys and Baltz, 1983). It was suggested that SAP may act as an important regulatory protein in the complement and/or coagulation systems (Schwalbe et al., 1990b).

SAP has also been reported to interact with immobilized single-stranded and double-stranded DNA in a calcium-dependent manner (Pepys and Butcher, 1987). Furthermore, SAP was able to displace H-1 and interact with chromatin (Butler et al., 1990). It was proposed that SAP may solubilize extracellular DNA and help clear it from the circulatory system. In addition, SAP undergoes self-aggregation in the presence of calcium (Baltz et al., 1982; Schwalbe et al., 1990b). Heparin and other glycosaminoglycans have been shown to inhibit this polymerization (Hamazaki, 1987, 1989). This latter property raised the question of whether heparin might also influence the SAP-C4BP interaction, thereby altering the distribution of SAP and C4BP in serum.

Heparin is a normal constituent of mast cells and is released by these cells when their Fc receptors are cross-linked (Kimball, 1986). Heparin is also used as a clinical anticoagulant (for review see Becker and Alpert, 1990). It functions by enhancing the activity of antithrombin III, which inactivates thrombin and other coagulation proteases (Mourey et al., 1990).

This study was initiated to examine the potential influence of heparin on the C4BP-SAP complex. We report that heparin did interact with SAP in the presence of calcium and did prevent formation of the SAP•C4BP complex. Thus, heparin released from mast cells may function as a regulator of the SAP-C4BP complex in serum.
**MATERIALS AND METHODS**

Human C4BP was purified as previously described (Dahlbäck, 1980) and was quantitated by its extinction coefficient of 14.1 (Perlman and Hymes, 1980). Human SAP used in this investigation was either prepared by published methods (Thompson and Enfeldt, 1978) or was purchased from Sigma. Gel electrophoresis under denaturing conditions showed essentially homogeneous protein preparations (Laemmli, 1970). In addition, there appeared to be little difference in the properties of the two preparations. The glycosaminoglycans used in this study were purchased from Sigma. Chondroitin sulfate and heparin sulfate were from a bovine source, and heparin (186.8 units/mg) was from a porcine source. Low molecular weight heparin was reported to have a molecular weight of 4,000–6,000. Polybrene was also purchased from Sigma.

Phospholipids were radiolabeled by reductive methylation using radioactive formaldehyde as described by Jentoft and Dearborn (1983). Briefly, the procedure consisted of adding 20 mM NaCNBH₃ (Sigma) followed by radioactive formaldehyde (50 μCi/mg of protein), to protein (0.5 to 0.99 mg/ml) in 50 mM phosphate buffer at pH 7.0 containing 0.1 mM NaCl. The specific activities for [H]formaldehyde and [14C]formaldehyde are 100 μCi/mmol and 56.4 mCi/mmol, respectively. The radioisotopes were purchased from Du Pont New England Nuclear. The specific activities of the final protein preparations were: [3H]C4BP, 9,870 cpm/μg; [14C]C4BP, 8,500 cpm/μg; and [14C]SAP, 10,900 cpm/μg.

Sucrose density gradient ultracentrifugation was carried out using isokinetic gradients as previously described (McCarty et al., 1974). The centrifuge tubes (14-x 95-mm polyallomer, Beckman) contained a 10-ml sucrose gradient. Samples (0.3 or 0.4 ml) were applied to the top of the centrifuge tube, and they were centrifuged at 40,000 rpm at 5°C for 22 hr in a Beckman model SW 40 rotor and Beckman model L5-50 preparative ultracentrifuge.

The gradients were fractionated by pumping a solution of 31% sucrose into the bottom of the tube so that fractions were collected starting from the top of the tube. Fractions were collected by drop counting, and fraction volume was determined. Ultracentrifugation was carried out in a 50 mM Tris buffer, pH 7.5, that contained 0.1 mM NaCl 0.1% bovine serum albumin, plus either 1 mM calcium or 1 mM EDTA as indicated (Schwalbe et al., 1990b). Some samples also contained 0.05 mM of serum. In this case, 0.05 ml of serum was diluted to 0.3 ml, or 0.1 ml of serum was diluted to 0.4 ml with buffer. When serum was used, the purified radiolabeled proteins were added to the samples and they were incubated for 30 min at 37°C prior to centrifugation. Buffer that did not contain bovine serum albumin was used for the standards that were to be detected by absorbance at 280 nm. Sedimentation positions for the samples were highly reproducible with a variation of ±0.3 ml for the sedimentation position for multiple runs of the similar samples.

Each of the radiolabeled protein samples was sedimented in a sucrose density gradient to show protein and radiochemical purity. The sedimentation patterns were analyzed for absorbance at 280 nm and for radioactivity by scintillation counting. A Beckman SL 5000 spectrophotometer was used, the purified radiolabeled proteins were added to the samples and they were incubated for 30 min at 37°C prior to centrifugation. Buffer that did not contain bovine serum albumin was used for the standards that were to be detected by absorbance at 280 nm. Sedimentation positions for the samples were highly reproducible with a variation of ±0.3 ml for the sedimentation position for multiple runs of the similar samples.

**RESULTS**

**Binding of SAP to C4BP, Phospholipid Vesicles, and Various Glycosaminoglycans**—Earlier studies reported that C4BP, phospholipid vesicles, heparin, heparan sulfate, and chondroitin sulfate all prevented calcium-induced self-association of SAP (Schwalbe et al., 1990b; Hamazaki, 1987, 1989). In the case of the glycosaminoglycans, the nature of this inhibition and the physical nature of the products formed were not determined. Fig. 1 shows the sedimentation positions of radiolabeled SAP in a sucrose density gradient. The sedimentation coefficients were determined by comparison to protein standards (Schwalbe et al., 1990b). The sedimentation coefficients for SAP and for the SAP-C4BP complex were 7.5 and 11, respectively. The results also show the sedimentation position of radiolabeled SAP that was mixed with heparin. This mixture gave a single sedimentation peak and, therefore, a discrete complex. The sedimentation position of the SAP-heparin complex was identical with that of the SAP-C4BP complex.

The large heparin-induced shift in the sedimentation position of SAP could most easily be explained by formation of a dimer of the normal SAP species. Dimerization of spherical particles should cause a 1.6-fold increase in the sedimentation coefficient (van Holde, 1985) to an expected value of 12 for SAP. The observed sedimentation value of about 11 was slightly smaller but well within a reasonable range for dimerization of a nonspherical species. Thus, the results suggested that interaction of SAP with heparin generated a protein dimer. Furthermore, concentrations of heparin that corresponded to 115 times the amount needed to generate the maximum yield of SAP dimer (see below) did not shift or diminish the yield of this peak (data not shown). This sug-

![Fig. 1. Interaction of SAP with heparin and C4BP detected by sucrose density gradient ultracentrifugation. The sedimentation patterns are shown for samples consisting of [3H]SAP, 9.6 μg, 10,900 cpm/μg in buffer containing 1 mM EDTA; a mixture of [14C]SAP (9.6 μg) plus heparin (0.18 μg) in buffer containing 1 mM calcium; and [14C]SAP plus unlabeled C4BP (■) in buffer containing 1 mM calcium. All buffers contained 0.1% bovine serum albumin. When corrected for counting efficiency, the total recovery of radioactivity was ≥70% of the radioactivity applied to the tube.](image-url)
ggested that the SAP dimer represented a highly stable arrangement that included protein-protein interaction as well as protein-heparin interaction. For example, if SAP bound only to heparin, an excess of heparin should result in one SAP bound to each heparin molecule, and the amount of the $S = 11$ material should decrease.

The sedimentation positions of radiolabeled SAP in the presence of various glycosaminoglycans are shown in Fig. 2. In every case, the carbohydrates interacted with SAP and appeared to form a single multimeric form of SAP. Similar results were obtained with heparin or heparan sulfate (Fig. 2, A and B). The complex formed with chondroitin sulfate had a somewhat higher sedimentation coefficient (Fig. 2C). Other experiments indicated that chondroitin sulfate was also different in that a large amount was needed to generate the complex (data not shown). This suggested that chondroitin sulfate had a lower affinity for SAP than did heparin or heparan sulfate. Low molecular weight heparin did not produce the $S = 11$ complex (data not shown) and did not appear to be competent in forming the discrete SAP complex that is characterized by this sedimentation value.

Gel filtration chromatography showed the elution positions of radiolabeled SAP by itself, in the presence of heparin, or in the presence of C4BP (Fig. 3). A problem with gel filtration of SAP was its apparent tendency to interact with the column matrix and generate a trailing edge of protein in the elution profile, despite the presence of bovine serum albumin. Nevertheless, the results showed that the primary heparin-SAP complex eluted at a position approximately midway between free SAP and the SAP-C4BP complex (Fig. 3). Thus, despite similar sedimentation coefficients, the SAP-C4BP complex had a substantially larger hydrodynamic radius than the SAP-heparin complex. This indicated that the SAP-C4BP complex, which has a larger molecular weight, also has a much larger frictional coefficient than the SAP-heparin complex. This would be expected for SAP-C4BP ($M_r = 235,000$ plus 570,000) versus SAP$_2$ ($M_r = 2 \times 235,000$). Heparin appeared to make a minor contribution to the mass of the SAP-heparin complex (see below).

SAP binds to negatively charged phospholipid vesicles in a calcium-dependent manner. Experiments were conducted to determine if this interaction competed with heparin binding. This approach was also used to determine if low molecular weight heparin interacted with SAP. Calcium addition to a solution of phospholipid vesicles (25 μg, phosphatidylcholine: phosphatidylserine, 75:25) and SAP (7 pg) in the absence of heparin (C), in the presence of heparin (●, 1.19 μg), and in the presence of various amounts of low molecular weight heparin (○, 0.6 μg; □, 3 μg). Protein-membrane binding was determined from the light scattering intensities. Equation 1 was used to determine $M_2/M_1$, the molecular weight ratio, of the protein-phospholipid complex to that of the phospholipid.

![Fig. 2. A distinct calcium-dependent complex of SAP with various glycosaminoglycans. The sedimentation patterns of a mixture of [14C]SAP (0.96 μg, 10,900 cpm/μg) plus heparin (1.86 μg, panel A), [14C]SAP plus heparan sulfate (10 μg, panel B), and [14C]SAP plus chondroitin sulfate (20 μg, panel C) are shown. All the protein samples were diluted to 0.3 ml with a buffer containing 1 mM calcium and 0.1% bovine serum albumin. Total recovery of radioactive protein was approximately 70% in all three cases.](image)

**Fig. 3.** Gel filtration of the SAP-C4BP and SAP-heparin complexes. A mixture of [14C]SAP (●, 2.4 μg, 10,900 cpm/μg) plus unlabeled SAP (20 μg) was chromatographed on a column of Sephacryl S-500 (1.8 × 29 cm) in buffer containing 1 mM calcium and 0.5% bovine serum albumin. Similar amounts of [14C]SAP were also gel-filtered in the presence of heparin (●, 4 μg) and in the presence of C4BP (■, 27 μg). The flow rate of the column was 1.9 ml/h, and the volume per fraction was 0.45 ml.

**Fig. 4.** Heparin or low molecular weight heparin inhibition of SAP with membrane association. Calcium was added to a solution (1.6 ml) of phospholipid vesicles (25 μg, phosphatidylcholine: phosphatidylserine, 75:25) and SAP (7 pg) in the absence of heparin (●, 1.19 μg), in the presence of heparin (■, 1.19 μg), and in the presence of various amounts of low molecular weight heparin (○, 0.6 μg; □, 3 μg). Protein-membrane binding was determined from the light scattering intensities. Equation 1 was used to determine $M_2/M_1$, the molecular weight ratio, of the protein-phospholipid complex to that of the phospholipid.
of heparin showed that both proteins sedimented primarily at the position of the SAP-C4BP complex (Fig. 5A). The SAP-C4BP interaction is of high affinity (Schwalbe et al., 1990b), and SAP which sedimented at the position of the SAP-C4BP complex. While this was indistinguishable from the position of the SAP-C4BP complex, it was possible to show that C4BP was no longer complexed with SAP. That is, the sedimentation of C4BP was slower when heparin was present (Fig. 5B). Thus, heparin prevented C4BP from interacting with SAP.

Panel C shows difference plots for the sedimentation of C4BP when complexed with SAP (Fig. 5A) versus when these proteins were diluted in the absence of excess heparin (Fig. 5B). The percent of radioactivity recovered in each fraction was determined, and the difference of values obtained in the absence of heparin minus those obtained in the presence of heparin is shown. The mass of heparin in the complex was approximately 0.08 pg, which is very close to the reported level of SAP in serum (Teng and Teller, 1981). Thus, the mass of heparin in the S = 11 complex was only about one-tenth the mass of SAP and probably corresponds to one or two molecules of heparin per SAP dimer.

Demonstration of Heparin Dissociation of the SAP-C4BP Complex in Serum—A mixture of serum containing radiolabeled SAP was prepared and was subjected to sucrose density gradient ultracentrifugation. The sedimentation patterns obtained for a mixture of [3H]C4BP and [14C]SAP (C) showed a broad double peak which was essentially unaffected by polybrene (Fig. 6A). The degree of dissociation of the SAP-C4BP complex was dependent on the amount of heparin added to the serum (Fig. 7C). The amount of heparin needed to initiate SAP-C4BP dissociation was low and corresponded to approximately therapeutic levels of heparin (0.4–1.0 unit/ml or 2.5–6.25 µg/ml (Holm et al., 1998; Boneu et al., 1989), Fig. 7C). These results suggested that physiological levels of heparin may be capable of altering the amount of the SAP-C4BP complex in serum and of generating a physiological effect that this might produce.

The effect of Polybrene on the SAP-heparin complex was examined. Polybrene is a cationic polymer that binds anionic polymers such as heparin. Once again, sufficient radiolabeled SAP was added to give approximately a 1:1 ratio of free SAP and SAP-C4BP. The sedimentation showed a broad double peak which was essentially unaffected by polybrene (Fig. 8A). When heparin was included in the mixture, the [14C]SAP peak became very narrow and sedimented at the position of the SAP-C4BP complex.
The purpose of this study was to determine how heparin or other glycosaminoglycans might influence the SAP-C4BP interaction in serum (Schwalbe et al., 1990b). Heparin has been shown to interact with SAP as evidenced by its ability to prevent SAP polymerization (Hamazaki, 1987, 1989). If heparin also influenced SAP-C4BP interaction, it may have a physiological role of altering the distribution of SAP and its many interactions. For instance, during tissue damage or complement activation, mast cells may be triggered to release heparin and other components (Kimball, 1986). Along with other effects, this may result in dissociation of the SAP-C4BP complex and formation of the SAP-heparin complex. Dissociation of SAP may influence, either positively or negatively, the action of C4BP, thereby influencing complement fixation. Alternatively, the SAP-heparin complex may trigger another reaction or serve as a regulatory signal. For instance, SAP interaction with heparin might help modulate the anticoagulant role of heparin. Otherwise, heparin released from mast cells might also displace SAP from other sites in the body, thereby producing alterations in tissue properties. Thus, while precise roles of these several interactions are not known, their existence suggests numerous possibilities which will require future examination.

In addition, the acute phase response is characterized by a modification of the concentration of several plasma proteins (Kushner, 1982; Pepys and Baltz, 1983). This response is stimulated by infection, tissue damage, and cell death. The serum levels of C4BP (Sacki et al., 1989) are reported to increase during the acute phase response. For this reason, the serum levels of free C4BP or C4BP complexed to protein S (Dahlbäck and Stenflo, 1981) might be elevated in the acute phase. This may constitute an important relationship as well. Thus, extensive future studies will be needed to examine these many possible roles for the influence of heparin and other factors on SAP and its many interactions.
Heparin Effect on SAP-C4b-binding Protein

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