Chromatin-Independent Binding of Serum Amyloid P Component to Apoptotic Cells

Atoosa Familian, Bas Zwart, Han G. Huisman, Irma Rensink, Dorina Roem, Peter L. Hordijk, Lucien A. Aarden and C. Erik Hack

*J Immunol* 2001; 167:647-654; doi: 10.4049/jimmunol.167.2.647

http://www.jimmunol.org/content/167/2/647

**References**  This article cites 44 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/167/2/647.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Chromatin-Independent Binding of Serum Amyloid P Component to Apoptotic Cells

Atoosa Familian, Bas Zwart, Han G. Huisman, Irma Rensink, Dorina Roem, Peter L. Hordijk, Lucien A. Aarden, and C. Erik Hack

Human serum amyloid P component (SAP) is a glycoprotein structurally belonging to the pentraxin family of proteins, which has a characteristic pentameric organization. Mice with a targeted deletion of the SAP gene develop antinuclear Abs, which was interpreted as evidence for a role of SAP in controlling the degradation of chromatin. However, in vitro SAP also can bind to phosphatidylethanolamine, a phospholipid which in normal cells is located mainly in the inner leaflet of the cell membrane, to be translocated to the outer leaflet of the cell membrane during a membrane flip-flop. We hypothesized that SAP, because of its specificity for phosphatidylethanolamine, may bind to apoptotic cells independent of its nuclear binding. Calcium-dependent binding of SAP to early, nonpermeable apoptotic Jurkat, SKW, and Raji cells was indeed observed. Experiments with flip-flopped erythrocytes confirmed that SAP bound to early apoptotic cells via exposed phosphatidylethanolamine. Binding of SAP was stronger to late, permeable apoptotic cells. Experiments with enucleated neutrophils, with DNase/RNase treatment of late apoptotic Jurkat cells, and competition experiments with histones suggested that binding of SAP to late apoptotic cells was largely independent of chromatin. Confocal laser microscopic studies indeed suggested that SAP bound to these apoptotic cells mainly via the blebs. Thus, this study shows that SAP binds to apoptotic cells already at an early stage, which raises the possibility that SAP is involved in dealing with apoptotic cells in vivo. The Journal of Immunology, 2001, 167: 647–654.
Materials and Methods

Materials
Phosphorylcholine (PE) and phosphorylethanolamine (PC) were obtained from Sigma (St. Louis, MO). Phycocerythrin-labeled streptavidin (streptavidin-phycocerythrin) was obtained from Becton Dickinson Immunocytometry System (Mountain View, CA). Propidium iodide (PI) was purchased from Calbiochem (La Jolla, CA).

Recalculated plasma
Blood was collected from healthy volunteers in siliconized tubes containing sodium citrate at a final concentration of 10 mM. Plasma was separated by centrifugation at 1300 × g for 10 min at 4°C, supplemented with CaCl₂ to yield a final concentration of 10 mM, and incubated for 10 min at 37°C followed by 30 min at 4°C. Hereafter, a clot had formed that was removed by centrifugation at 1300 × g for 10 min at 4°C. The supernatant (recalculated plasma) was stored in aliquots at −70°C until use.

Purification of SAP
Human SAP was purified from normal human plasma by affinity column chromatography as described by Ying et al. (33). In brief, recalculated plasma was applied to a Bio-gel A 0.5-m column (Bio-Rad Laboratories, Hercules, CA) equilibrated with veronal-buffered saline, pH 7.4, containing 10 mM CaCl₂ and 2 mM MgCl₂ (VB). The column was washed extensively with the same buffer, and SAP was eluted with veronal-buffered saline containing 10 mM EDTA (VB/EDTA). Purity of SAP was determined by SDS-PAGE (12.5%, w/v). The purified protein gave a single band of Mr ∼ 25,000 under reducing conditions. Human SAP also was purchased from Calbiochem (La Jolla, CA).

Isolation of human neutrophils and preparation of cytoplasts
Human neutrophils were purified from heparinized peripheral blood as follows: mononuclear cells and platelets were separated by density-gradient centrifugation on isotonic Percoll with a specific gravity of 1.078 mg/ml at room temperature. Contaminating erythrocytes were lysed by ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.4). After centrifugation, the pellet was washed twice and resuspended in IMDM containing 5% human SAP-depleted plasma instead of FCS to prevent binding of bovine SAP to the cells. Neutrophil cytoplasts were prepared from isolated neutrophils of healthy persons as described by Roos et al. (35).

Antibodies
Rabbit antisera against human SAP was made by repeated i.m. injection with SAP in rabbits. Polyclonal anti-human SAP Abs (PaSAP) were purified from the serum by immunoaffinity column chromatography; SAP was covalently coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden); rabbit serum was passed through the column and recycled three times; the column then was washed with PBS, pH 7.4, until A₄₅₀ nm was <0.01. Finally anti-SAP Abs were eluted with 0.1 M glycine-HCl, pH 2.5, and dialyzed against PBS. Rabbit polyclonal anti-SAP also was purchased from Dako (Carpinteria, CA).

Mouse mAbs against human SAP were prepared by fusing spleen cells from mice immunized with purified SAP and SEAP2/Ag according to established procedures. Culture supernatants were screened by ELISA with a rat mAb against mouse κ-L chain as catching Ab, in combination with biotinylated SAP followed by streptavidin-HRP. Positive clones were cloned by repeated limiting dilutions. All mAbs were purified from culture supernatant by affinity chromatography on protein-A Sepharose (Pharmacia).

Biotinylation of anti-SAP and purified SAP
PaSAP either prepared in our laboratory or obtained commercially, mAb aSAP-14 or purified SAP were biotinylated with LC-biotin-cinimide (Pierce, Rockford, IL) according to instruction of manufacturer.

Depletion of SAP from plasma
SAP-depleted plasma was prepared by passage of recalculated plasma over a Bio-gel A 0.5-m column equilibrated with VB at 4°C. To ensure that trace amounts of SAP also were removed, this procedure was repeated once. By a quantitative ELISA performed with monoclonal anti-human SAP (aSAP-14) as a coating Ab and biotinylated PaSAP as a detecting Ab, it was established that this depleted plasma contained less than 1% of the normal plasma level of SAP.

Induction of membrane flip-flop in erythrocytes
Human erythrocytes were isolated from fresh heparinized whole blood by centrifugation at 1300 × g for 10 min, cleared from buffy coat, and washed carefully with sterile isotonic saline to remove plasma and contaminating white blood cells. On OCS-PAGE gels, these then were treated with ionomycin (Sigma) to induce exposure of phosphatidylserine in the outer leaflet of the membrane as described by Test and Mitsuyoshi (34). In brief, erythrocytes were incubated in TBS supplemented with 0.2% w/v glucose and 1 mM CaCl₂ for 5 min at 37°C. Then ionomycin was added at a final concentration of 4 μM and the cells were further incubated for 1 h at 37°C. Thereafter, an equal volume of TBS containing 1 mM CaCl₂ and 2%, w/v, BSA was added to the mixture, which then was incubated for 15 min at 37°C. The cells then were pelleted, washed once with the same buffer, and finally washed and resuspended with TBS containing 1 mM CaCl₂ without BSA.

Cell lines and cell culture
Jurkat (a T lymphocytic leukemia cell line), SKW, and Raji cells (both Burkitt’s lymphoma B cell lines) were cultured in IMDM containing 5% v/v FCS (Life Technologies, Grand Island, NY), penicillin-streptomycin (Life Technologies), 20 μg/ml transferrine (Sigma), and 50 μM 2-ME. Cultures were conducted at 37°C in a humidified 5% CO₂ atmosphere.

Isolation of human neutrophils and preparation of cytoplasts
Human neutrophils were purified from heparinized peripheral blood as follows: mononuclear cells and platelets were separated by density-gradient centrifugation on isotonic Percoll with a specific gravity of 1.078 mg/ml at room temperature. Contaminating erythrocytes were lysed by ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.4). After centrifugation, the pellet was washed twice and resuspended in IMDM containing 5% human SAP-depleted plasma instead of FCS to prevent binding of bovine SAP to the cells. Neutrophil cytoplasts were prepared from isolated neutrophils of healthy persons as described by Roos et al. (35).

Apoptosis induction
Apoptosis was induced in Jurkat cells (5 × 10⁶ cells/ml) by incubation with 100 μM etoposide (Sigma) or 5 μg/ml anti-CD95 mAb for 5 h (early apoptotic cells) or 20 h (late apoptotic cells). SKW or Raji cells were treated with apoptosis by incubation with either 100 μM etoposide or 100 μM cyclohexamide (Sigma) for the same duration as Jurkat cells. Apoptosis induction always was done in the absence of FCS to prevent FCS-derived SAP from interfering. Therefore, cells were washed carefully with the culture medium without FCS before apoptosis induction and suspended in the same medium. Apoptosis in isolated neutrophils was induced by culturing the cells in IMDM containing 5% human SAP-depleted plasma for 24 h. Apoptosis of the cells was assessed by analysis of morphological changes under light microscope and by staining with annexin V and PI in cytometry. Prepared cytoplasts were cultured in IMDM overnight to induce “apoptosis” in these enucleated cells.

Binding of SAP to cells
Vital and apoptotic cells were washed with IMDM and distributed in 96-wells microtiter plates (2 × 10⁵ cells/well). The cells then were centrifuged at 1000 × g for 2 min, and the pellets were resuspended in 100 μl of IMDM containing varying amounts of plasma or purified SAP and incubated for 30 min at 37°C. Cells were washed four times with HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, pH 7.4 containing 0.5% BSA). Binding was detected by a subsequent incubation with biotinylated PaSAP or mAb aSAP-14 (5 μg/ml in HEPES buffer, final volume 50 μl) for 30 min at 4°C. The cells were washed three times with HEPES buffer and stained with Annexin V FITC (Boehringer Ingelheim, Ingelheim, Germany; final dilution 1/1500) and streptavidin-phycocerythrin (final dilution 1/150) in the same buffer, final volume 50 μl, for 20 min at 4°C in the dark. Binding assays also were performed with cells incubated with 5 μg/ml biotinylated SAP in HEPES buffer following the same procedure described above.

Binding of SAP cells also was tested by mixing vital and early and late apoptotic Jurkat cells. Thereafter, the mixture was washed and incubated for 30 min with 50% recalculated plasma or 20 μg/ml purified SAP in IMDM at 37°C. After extensive washing with HEPES buffer, cells were incubated for 30 min with biotinylated mAb aSAP-14 (5 μg/ml in HEPES buffer, final volume 50 μl) at 4°C. The cells were washed four times with HEPES and stained for 15 min at 4°C in the dark with streptavidin conjugated to allophycocyanin (final dilution 1/500; BD PharmMingen, San Diego, CA) and annexin V FITC (final dilution 1/150) in HEPES buffer, final volume 50 μl, followed by four washes and PI staining (final concentration 500 ng/ml) before analyzing of the cells by FACS.

Binding of SAP to neutrophils or cytoplasts was analyzed in similar experiments except that the Jurkat cells were replaced by the appropriate cells. In case cytoplasts were analyzed, permeability of the cells was assessed by staining with annexin V FITC first to determine the cytoplastic protein P67 as a indicator for loss of membrane integrity followed by a FITC-labeled second Ab (as an alternative for PI). As this prevented the use of FITC-labeled annexin V, merocyanin 540 (final concentration 20 μg/ml; Sigma) was applied as a
marker for membrane flip-flop. Binding of SAP was determined with biotinylated mAb and allophycocyanin-conjugated streptavidin as described above.

To confirm calcium dependency of the binding of SAP, recalcified plasma at the same indicated dilutions was preincubated either with or without 10 mM EDTA for 30 min at 37°C before incubation with vital or apoptotic cells. The experiments were completed as described above.

As other tests for specificity, recalcified plasma was preincubated for 30 min at 37°C with varying concentrations of PE (0 – 40 mg/ml), PC (0 – 40 mg/ml), or histones (H1, H2a, H2b, H3, or H4; 0 – 40 mg/ml) before incubation with cells.

Binding of SAP to apoptotic cells deprived of nucleic acids was investigated by incubating late apoptotic cells for 60 min at 37°C with different concentrations of DNase I (0 –250 μg/ml; Sigma), with or without RNase (0 –500 μg/ml; Sigma), before incubation with recalcified plasma. Complete digestion of DNA and RNA was assessed by PI staining of the cells with either fluorescence microscopy or FACS analysis.

**FACS analysis**

Binding of Annexin V-FITC or SAP was measured by flow cytometry. Leakage of the cells was assessed by staining with PI. After appropriate incubations, the cells were finally washed, and relative fluorescence intensity was measured with a flow cytometer (FACScan; Becton Dickinson). Experiments including streptavidin-allophycocyanin intensity were performed with a FACSCalibur (Becton Dickinson).

**Confocal laser scanning microscopy**

Early, late, or mixed apoptotic Jurkat cells were incubated with 50% v/v recalcified plasma in IMDM for 30 min at 37°C. After washing with washing buffer (HEPES buffer containing 2% BSA), the cells were incubated with 5 μg/ml mAb aSAP-14 in washing buffer for 30 min at 4°C, washed, and stained with annexin V coupled to FITC (diluted 1/1500) and 20 μg/ml Alexa 568-conjugated goat anti-mouse Ab (Molecular Probes, Eugene, Oregon) in the same buffer for 20 min at 4°C in the dark. At the end of incubation, the cells were transferred to poly-L-lysine-coated microscope slides (Sigma) and fixed with 1% paraformaldehyde for 10 min. Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) using appropriate filter settings.

**Results**

**Binding of SAP to apoptotic cells**

Based on binding of annexin V-FITC and PI, cells can be divided into three subpopulations, i.e., vital and early and late apoptotic cells (Fig. 1A). To analyze binding of SAP to vital, apoptotic, or late apoptotic cells within one experiment, populations of these cells were combined and incubated with recalcified plasma. Bound SAP was detected by incubation with biotinylated mAb aSAP-14 and streptavidin coupled to allophycocyanin. Based on annexin V and PI staining, the cells were divided to three different populations (A). Histograms show binding of SAP to each population in presence (B) or absence (C) of recalcified plasma.

**FIGURE 1.** Binding of SAP to vital, early, and late apoptotic Jurkat cells. Early and late apoptotic Jurkat cells were prepared as described in Material and Methods, mixed, and incubated in IMDM with or without 50% v/v recalcified plasma and subsequently with biotinylated mAb aSAP-14. Thereafter, cells were stained with Annexin V-FITC, PI, and streptavidin-allophycocyanin. Based on annexin V and PI staining, the cells were divided to three different populations (A). Histograms show binding of SAP to each population in presence (B) or absence (C) of recalcified plasma.

**FIGURE 2.** Dose-response curves of binding of SAP to cells. The combination of early and late apoptotic Jurkat cells was incubated with increasing amount of purified SAP followed by biotinylated mAb aSAP-14. Top, Binding to early apoptotic cells. Bottom, Binding to late apoptotic population. Binding to vital cells is indicated in each panel, and curves represent MFI (n = 4).
To show specificity of binding for apoptotic cells, we also tested other cell lines, i.e., SKW and Raji cells (both Burkitt’s lymphoma B cell lines) as well as isolated neutrophils. Apoptosis was induced by incubation with etoposide or cyclohexamide for cell lines and 24 h culture for neutrophils as described in Materials and Methods. Binding of SAP to early and late apoptotic SKW, Raji, and neutrophils was observed, similar to Jurkat cells (data not shown).

Specificity of the binding of SAP to apoptotic cells

Specificity of the binding of SAP to apoptotic cells (Jurkat as well as SKW and Raji) was demonstrated in several ways.

Binding of purified SAP.

Binding of SAP was tested by incubating apoptotic or vital cells with the purified protein instead of recalcified plasma. A dose-dependent binding of purified SAP was observed only to the apoptotic cells (Fig. 2). The same results were observed when biotinylated SAP was used instead of purified SAP and biotinylated Abs (data are not shown).

Incubation with SAP-depleted plasma.

Cells were incubated with 50% v/v recalcified plasma either depleted or not for SAP, as described in Materials and Methods. The results showed that early and late apoptotic cells did not bind SAP on incubation with SAP-depleted plasma. Supplementing the SAP-depleted plasma with purified SAP again resulted in fixation of this protein to apoptotic cells (Fig. 3). These results indicated that the observed SAP binding was not due to aspecific binding of the anti-SAP Abs to the cells.

Binding in presence of EDTA.

Binding of SAP to various ligands is calcium-dependent (3, 16). Incubation of apoptotic cells with recalcified plasma in the presence of 10 mM EDTA resulted in a decreased binding of SAP to apoptotic cells, i.e., from a median fluorescence intensity (MFI) of 4530–1950 for late apoptotic cells.
indicated histones. The experiments in was incubated with plasma preincubated with or without increas-

SAP on apoptotic cells, the mixture of early and late apoptotic cells was inhibited by EDTA or PE. Additional experiments revealed that the binding of SAP to the flip-flopped erythrocytes was considerably less than that observed with late apoptotic cells and comparable to erythrocytes (Fig. 4). The intensity of staining was considerably weaker but twice as much as that to the non-flip-flopped cytoplasts (Fig. 7).

The experiments with purified histones and DNase/RNase treatment, as well as the experiments with neutrophilic cytoplasts did not show any inhibitory effect on binding to early and late apoptotic cells (Fig. 6B).

Neutrophilic cytoplasts are enucleated cells consisting of a plasma membrane, cytoplasm, and some endoplasmic reticulum. Enucleation is achieved by separation of karyoplasts from neutrophils via specific gravity centrifugation (35). The composition of surface Ags of neutrophil cytoplasts is the same as intact neutrophils. Because cytoplasts have a similar cell membrane as nucleated neutrophils but are devoid of nuclear material, we examined binding of SAP to cytoplasts to establish the contribution of chromatin to the observed binding of SAP to apoptotic cells. Staining of the cytoplasts with mercocyanin 540 and anti-p67 to assess membrane flip-flop and leakiness revealed three populations of cells, i.e., non-flip-flopped (P1), flip-flopped but not leaky (P2), and flip-flopped/leaky cells (P3), corresponding to vital, early, and late apoptotic cells. SAP appeared to bind strongly to the flip-flopped/leaky population, whereas the binding to the flip-flopped/nonleaky cells was much weaker but twice as much as that to the non-flip-flopped cytoplasts (Fig. 7).

The experiments with purified histones and DNase/RNase treatment, as well as the experiments with neutrophilic cytoplasts did not show any inhibitory effect on binding to early and late apoptotic cells (Fig. 6B).

Ligands for SAP on apoptotic cells

Because of its specificity for phosphoethanolamine, SAP may bind to the flip-flopped membrane of apoptotic cells, but because of its specificity for nuclear materials, such as histones and DNA, it also may bind intracellularly to nucleosomes and other chromatin material. To determine whether SAP binding to apoptotic cells was attributable to interaction with flip-flopped membrane or with other (intracellular) structures, we did several experiments. First, we studied binding of SAP to erythrocytes that had been induced to undergo a membrane flip-flop by incubation with ionomycin. We presumed that binding to these cells would occur via the membrane only, because these cells lack a nucleus. Indeed, SAP, either in plasma or purified, appeared to bind to annexin V-positive erythrocytes (Fig. 4). The intensity of staining was considerably less than that observed with late apoptotic cells and comparable to that observed with early apoptotic cells. Additional experiments revealed that the binding of SAP to the flip-flopped erythrocytes was inhibited by EDTA or PE.

To expand the role of phosphatidylethanolamine as a ligand for SAP on apoptotic cells, the mixture of early and late apoptotic cells was incubated with plasma preincubated with or without increas-

FIGURE 6. Neither DNase/RNase treatment of cells nor preincubation of plasma with histones abolishes binding of SAP to apoptotic cells. A, Late apoptotic Jurkat cells were treated with 250 μg/ml DNase and 500 μg/ml RNase as described in Materials and Methods before incubation with plasma. SAP binding (A, right) was assessed with biotinylated mAb as described in Fig. 1. Hatched bars present MFI of untreated cells, dark bars show MFI after DNase/RNase treatment. PI staining is shown in A, left. B, Effect of soluble histones on binding of SAP to apoptotic cells. Recalculated plasma was preincubated with different amounts of histones (H1, H2a, H2b, H3, and H4), and then incubated with cells. The graph shows MFI of binding of SAP to vital (left), early (middle), and late apoptotic cells (right) after preincubation with or without 40 μg/ml of the indicated histones. The experiments in A and B were repeated twice with comparable results.

and from 140 to 28 for early apoptotic cells (MFI for vital cells was 27; n = 3). Furthermore, SAP bound to apoptotic cells could be dissociated by incubation with EDTA (results not shown).

Binding of SAP to neutrophilic cytoplasts

Cytoplasts were prepared from neutrophils as described in Materials and Methods and incubated for 24 h in IMDM to induce “apoptosis.” The cells then were incubated with 50% recalcified plasma (RP) or 20 μg/ml purified SAP followed by biotinylated mAb aSAP-14 as described. For evaluation of binding of SAP via flip-flopped membrane, cytoplasts were stained with mercocyanin 540 (as a marker for flip-flop of membrane), a rabbit Ab against cytotoxic protein p67 followed by a FITC-labeled goat anti-rabbit Ab (as an indicator for loss of membrane integrity), and allophycocyanin-conjugated streptavidin for detecting binding of SAP. Based on staining with mercocyanin 540 and anti-p67 Ab, cytoplasts were divided into three populations: P1, cytoplasts negative for either mercocyanin 540 or anti-P67, i.e., comparable with viable cells (left); P2, cytoplasts only positive for mercocyanin 540, i.e., comparable with early apoptotic cells (middle); and P3, cytoplasts positive for both dyes, i.e., comparable with late apoptotic cells (right). Bars represent MFI of binding of SAP to each population in presence of 50% recalcified plasma ( ), purified SAP ( ), and IMDM alone ( , n = 2).

FIGURE 7. Binding of SAP to neutrophilic cytoplasts. Cytoplasts were prepared from neutrophils as described in Materials and Methods and incubated for 24 h in IMDM to induce “apoptosis.” The cells then were incubated with 50% recalcified plasma (RP) or 20 μg/ml purified SAP followed by biotinylated mAb aSAP-14 as described. For evaluation of binding of SAP via flip-flopped membrane, cytoplasts were stained with mercocyanin 540 (as a marker for flip-flop of membrane), a rabbit Ab against cytotoxic protein p67 followed by a FITC-labeled goat anti-rabbit Ab (as an indicator for loss of membrane integrity), and allophycocyanin-conjugated streptavidin for detecting binding of SAP. Based on staining with mercocyanin 540 and anti-p67 Ab, cytoplasts were divided into three populations: P1, cytoplasts negative for either mercocyanin 540 or anti-P67, i.e., comparable with viable cells (left); P2, cytoplasts only positive for mercocyanin 540, i.e., comparable with early apoptotic cells (middle); and P3, cytoplasts positive for both dyes, i.e., comparable with late apoptotic cells (right). Bars represent MFI of binding of SAP to each population in presence of 50% recalcified plasma ( ), purified SAP ( ), and IMDM alone ( , n = 2).
not point to chromatin as the main ligand for SAP in late apoptotic cells. To further examine this, binding of SAP to apoptotic cells also was studied with a confocal laser microscope. A combination of the cells incubated with etoposide for 5 or 20 h were incubated with 50% recalcified plasma followed by incubation with monoclonal anti-SAP Abs. The cells were stained with Annexin V-FITC and Alexa 568-conjugated streptavidin. In control experiments, cells incubated without plasma were found to be negative for red staining (Fig. 8A). Subsequent to incubation with plasma, the majority of the annexin V-positive cells appeared to bind SAP (Fig. 8B). Each population was tested separately as well. Cells incubated for 5 h with etoposide predominantly showed a patchy binding of annexin V and SAP (Fig. 8, C–E). Fluorescent intensity predominantly occurred in clusters on the surface of the cells, some clusters being positive for both reagents. Cells incubated with etoposide for 20 h showed nuclear staining for SAP as well as membrane staining (Fig. 8, F–H). Vital cells showed neither staining for annexin V-FITC nor SAP (results not shown).

**Discussion**

The function of human SAP, a member of the pentraxin family of proteins, is unknown. In the present study, we provide evidence that SAP, either in recalcified plasma or purified, binds to apoptotic, but not to normal, Jurkat, Raji, or SKW cells. Moreover, binding of SAP was more pronounced to late than to early apoptotic cells. Experiments with purified SAP confirmed that binding of SAP in plasma to apoptotic cells did not occur via other plasma components.

A recent study has demonstrated binding of CRP, a homologue of SAP, to apoptotic tumor cell lines (36). Binding of CRP to apoptotic nontumor cells was not mentioned in that study. Here we demonstrate binding of SAP to flip-flopped erythrocytes as well as to flip-flopped cytoplasts (see Figs. 4 and 7). In addition, we have observed binding of SAP to flip-flopped neutrophils. Hence, these data rule out the possibility that SAP binds exclusively to tumor cell lines and not to normal cells.
To establish that SAP indeed binds to apoptotic cells via phosphatidylethanolamine exposed in the flip-flopped membrane, we also studied binding of SAP to erythrocytes. A membrane flip-flop was induced in these cells by increasing the intracellular calcium concentration (34). Indeed most erythrocytes incubated with calcium-ionophore stained positively for annexin V, a well-established marker for membrane flip-flop (32, 37). SAP was found to bind to flip-flopped, but not to normal, erythrocytes. This binding was specific, as it was observed with different SAP preparations, dependent on the presence of calcium, and inhibited by PE. The binding of SAP to flip-flopped erythrocytes much resembled that to early apoptotic cells. Altogether, these data led us to conclude that SAP binds to early apoptotic cells via phosphatidylethanolamine exposed in the outer leaflet of the membrane.

We consistently found that SAP bound to late apoptotic cells with a higher affinity compared with early apoptotic cells or flip-flopped erythrocytes. The difference in binding affinity between late and early apoptotic cells may be attributable to presence of additional ligands on late apoptotic cells like chromatin, or alternatively an enhanced exposure of phosphatidylethanolamine on these cells. On double-staining of late apoptotic cells with PI and SAP, it appeared that most cells that had taken up PI, indicating that these cells were leaky, strongly bound SAP. Considering the specificity of SAP for DNA, histones, and chromatin (18–22), it hence can be speculated that SAP bound to nucleosomes disposed in the blebs of apoptotic cells or pass through the cells and bind to nuclei. To investigate this, we used confocal laser scanning microscopy. This approach revealed that SAP predominantly bind to membranes of bleb-like structures on the apoptotic cells, although in some apoptotic cells nuclear staining was observed next to membrane clusters. Treatment of the late apoptotic cells with DNase and RNase to digest DNA and RNA or preincubation of plasma before incubating those cells with various histones did not decrease binding of SAP to late apoptotic cells, whereas preincubation with PE showed complete inhibition of the binding to early and a significant inhibition of that to late apoptotic cells. Experiments with neutrophil cytoplasts, which are devoid of nuclear material (35), also showed restricted binding of SAP to flip-flopped cytoplasts and strongly to cytoplasts staining with an Ab against the cytoplasmic protein P67, which reflects loss of membrane integrity. Hence, although we cannot exclude the possibility that SAP binds to nucleosomes as well, we suggest that the stronger binding of SAP to late apoptotic cells was, at least in part, attributable to binding to the flip-flopped membranes of apoptotic blebs. Notably, strong binding of CRP also has been postulated to occur mainly to blebs (36).

Previously, we have hypothesized that another pentraxin, CRP, binds to flip-flopped membranes in particular when these membranes contain phospholipids hydrolyzed by secretory phospholipase A2. The reason for this requirement for secretory phospholipase A2 is likely that is to be able to bind CRP, the tight package of phospholipids needs to be loosened to allow CRP to get access to the PC groups of phosphatidylycholine (38). A similar behavior of SAP would explain its preferential binding to blebs on apoptotic cells: because of the small diameter of these structures, phosphoethanolamine groups may be more easily accessible in the flip-flopped membrane of these structures than in the planar membranes of early apoptotic cells.

Several mechanisms have been suggested for the clearance of apoptotic cells by phagocytes including direct recognition via specific PS receptors, lectin-like receptors, and thrombospondin-dependent vitronectin receptors (39–42). Bound to a ligand, SAP, as well as its homologue CRP, can bind to C1q and activate the classical complement pathway (3, 10). Therefore, our results are consistent with a scenario that apoptotic cells either via direct interaction of the blebs with C1q (43) or indirectly via fixation of SAP and CRP can activate complement. Hence, we suggest that in addition to the mechanisms just mentioned, apoptotic cells may also be cleared via opsonization with complement. Recent studies in mice made deficient for SAP or C1q by targeted gene disruption (23, 44) support this idea: these mice have a high risk for developing systemic lupus erythematosus, a disease which likely results from impaired clearance of apoptotic cells and subsequent exposure of the immune system to nucleosomes (45). Currently, we are investigating whether SAP bound to apoptotic cells indeed can activate complement.

In conclusion, our study demonstrates that SAP can bind to cells in the early stage of apoptosis, presumably via phosphatidylethanolamine exposed in flip-flopped membranes. This data suggest that SAP may be involved in the clearance of these cells in vivo.

References


