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# Isolation and Detection of Human IgA Using a Streptococcal IgA-Binding Peptide<sup>1</sup>

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Bacterial proteins that bind to the Fc part of IgG have found widespread use in immunology. A similar protein suitable for the isolation and detection of human IgA has not been described. Here, we show that a 50-residue synthetic peptide, designated streptococcal IgA-binding peptide (Sap) and derived from a streptococcal M protein, can be used for single-step affinity purification of human IgA. High affinity binding of IgA required the presence in Sap of a C-terminal cysteine residue, not present in the intact M protein. Passage of human serum through a Sap column caused depletion of >99% of the IgA, and elution of the column allowed quantitative recovery of highly purified IgA, for which the proportions of the IgA1 and IgA2 subclasses were the same as in whole serum. Moreover, immobilized Sap could be used for single-step purification of secretory IgA of both subclasses from human saliva, with a recovery of ~45%. The Sap peptide could also be used to specifically detect IgA bound to Ag. Together, these data indicate that Sap is a versatile Fc-binding reagent that may open new possibilities for the characterization of human IgA. *The Journal of Immunology*, 2002, 169: 1357–1364.

The daily production of IgA in humans, ~66 mg/kg/day, exceeds that of all other Ig classes combined (1, 2). Most of this IgA is secreted on mucous membranes as dimeric secretory IgA (S-IgA),<sup>3</sup> which is believed to provide an important first line of defense against infections. Moreover, evidence is accumulating that IgA in serum contributes to a second line of defense by promoting phagocytosis (3–6). These important functions of IgA have focused interest on the development of mucosal vaccines (7) and on the possible therapeutic use of IgA for treatment of infections (8–13) and for killing of tumor cells (14, 15).

Studies of IgA would be much facilitated by the availability of a simple method to isolate and detect IgA. Indeed, purification of IgA is notoriously laborious and time consuming (16), and current methods to detect IgA are all based on the use of secondary Abs. In contrast, IgG can be easily purified and identified by means of the well-known bacterial proteins staphylococcal protein A (17) and streptococcal protein G (18, 19). This situation has raised the question whether a bacterial IgA-binding protein can be identified. However, extensive efforts to identify a bacterial protein suitable

for the isolation and detection of human IgA have thus far failed, although bacterial IgA-binding proteins are known.

Bacterial surface proteins that bind human IgA-Fc have been described in both group A *Streptococcus* (*Streptococcus pyogenes*) and group B *Streptococcus* (*Streptococcus agalactiae*), two important human pathogens (20–24), but attempts to use these proteins as IgA-binding reagents have been hampered by unsuitable binding properties (24) and/or affinity for human plasma proteins others than IgA (25–27). However, studies of an IgA-binding protein expressed by *S. pyogenes* have suggested that a domain in this protein might be developed into an IgA-binding reagent (28).

The IgA-binding proteins of *S. pyogenes* are members of the M protein family (29), a heterogeneous family of dimeric coiled-coil proteins that are important virulence factors (30–32). All M proteins bind one or more human plasma proteins, and ~50% of all *S. pyogenes* strains express an M protein that binds IgA-Fc (33). Studies of such IgA-binding M proteins have indicated that a sequence of 29 aa residues is sufficient for IgA-binding (34, 35). Based on this finding, we devised a 50-residue synthetic peptide including the 29-residue region (Fig. 1A) and showed that this peptide retained affinity for IgA (28). Thus, this peptide has the properties of an isolated IgA-binding domain.

The IgA-binding synthetic peptide represents a valuable model system for studies of human IgA-FcR (28, 36), but the specificity with which this peptide binds IgA has remained unclear, and it has not been known whether it can be used for the isolation of IgA and for detection of Ag-bound IgA. Here, we show that the peptide, which is designated Sap (for streptococcal IgA-binding peptide), binds human IgA with high specificity and can be used for single-step affinity purification of IgA and for specific detection of IgA bound to Ag.

## Materials and Methods

### Proteins and synthetic peptides

The Sap peptide corresponds to aa 35–83 of protein Sir22 (M22) (25) with addition of a C-terminal cysteine residue not present in Sir22 (28). The same peptide without the C-terminal cysteine residue is referred to as Sap(no Cys). These peptides were purchased from the Department of Clinical Chemistry, Malmö General Hospital (Malmö, Sweden). Analysis by

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<sup>3</sup> Abbreviations used in this paper: S-IgA, secretory IgA; C4BP, C4b-binding protein; mIgA, monomeric IgA; NIP, 3-nitro-4-hydroxy-5-iodophenylacetate; Sap, streptococcal IgA-binding peptide; Sap(no Cys), the Sap peptide lacking the C-terminal cysteine residue; SC, secretory component; SPR, surface plasmon resonance; CD, circular dichroism.

SDS-PAGE of the Sap peptide dissolved in PBS showed that most of the peptide occurred as a disulfide-bonded dimer, and such spontaneously dimerized preparations were used for most of the experiments reported here. However, dimerization may be enhanced by incubation in the presence of  $\text{CuCl}_2$ , as described (37), and peptide dimerized in this way was used for the experiments involving circular dichroism (CD) spectroscopy and surface plasmon resonance (SPR) studies. Before Sap was used in the latter experiments, small amounts of higher molecular mass forms of the peptide were eliminated by size exclusion chromatography on a Superdex 75 column (Amersham Pharmacia Biotech, Uppsala, Sweden), with a separation range of 7.5–60 kDa.

Streptococcal protein Sir22 (M22) was purified as described (25). Purified human serum IgA was from Cappel Organon-Teknika (Turnhout, Belgium) or Pierce (Rockford, IL), purified S-IgA was from Cappel or Sigma (Stockholm, Sweden), and purified IgG was from Sigma. Protein G was from Amersham Pharmacia Biotech. Recombinant anti-3-nitro-4-hydroxy-5-iodophenylacetate (NIP) IgA1, IgA2, and IgG1 Abs, purified from supernatants of CHO-K1 transfectants, have been described (38, 39); these Abs were chimeric (H chains composed of human constant domains and murine variable domain; the  $\lambda$  L chains were murine).

NIP-BSA was prepared by mixing a solution of BSA (10 mg/ml in PBS, pH 8.5) with 0.05 volume of a solution of NIP0-succinimide (Sigma-Genosys, Cambridge, U.K.; 100 mg/ml in dimethylformamide), with stirring. After incubation at room temperature for 2 h on a rotating mixer, the solution was dialyzed against PBS and stored at  $-20^\circ\text{C}$  in small aliquots.

### Antisera

Rabbit anti-human secretory component (SC) antiserum, rabbit anti-L chain antiserum, and the IgG fraction of rabbit anti-IgA, anti-IgG, and anti-IgM antiserum were from DAKO (Glostrup, Denmark), as were peroxidase- or alkaline phosphatase-conjugated rabbit Abs specific for human IgA, IgG, IgM, or SC. Rabbit antisera to human J chain were kindly provided by Dr. J. Mestecky (University of Alabama, Birmingham, AL) and by Dr. P. Brandtzaeg (University of Oslo, Oslo, Norway). Mouse mAbs specific for human IgA1 or IgA2 were from Nordic (Tilburg, The Netherlands). Rabbit anti-mouse IgG F(ab)<sub>2</sub> were from DAKO.

### CD spectroscopy

CD spectra were recorded on a Jasco J-720 spectropolarimeter supplied with a Peltier temperature control unit. Quartz cuvettes with 0.1-cm path length were used. The peptides were used in 10 mM potassium phosphate, pH 7.4. The exact concentrations of the peptides were determined by amino acid analysis after acid hydrolysis (Biomedical Center, Uppsala, Sweden). All spectra were recorded at  $25^\circ\text{C}$  from 260 to 185 nm with a scan speed of 5 nm/min, a response time of 8 s, and a step resolution of 1 nm. All spectra were accumulated 20 times to improve the signal-to-noise ratio. SELCON3 software (<http://lamar.colostate.edu/~sreeram/CDPro/>) was used for analysis of secondary structure. SELCON3 analyzes the experimental CD spectra using a database of 30 reference CD spectra of proteins with the amounts of secondary structure deduced from 3D structures (40).

### SPR studies

The interaction of IgA with synthetic peptides and with the intact Sir22 protein was studied using SPR technology on a BIAcore biosensor system. As described above, small amounts of higher molecular forms of the peptide had been eliminated by size exclusion chromatography. The flow buffer used for the SPR analysis contained 10 mM HEPES-KOH (pH 7.4), 0.15 M NaCl, and 0.005% Tween 20. Immobilization of monoclonal IgA2 or S-IgA to the sensor chip using amine coupling was performed as described (41), with 60  $\mu\text{l}$  IgA2 or S-IgA at 50  $\mu\text{g}/\text{ml}$  in 10 mM sodium acetate, pH 4.5. The association and dissociation of Sap, Sap(no Cys), and Sir22 to and from chip-immobilized IgA2 or S-IgA were studied at protein concentrations ranging from 0.004 to 0.08  $\mu\text{M}$  for the Sap peptide, from 0.5 to 2  $\mu\text{M}$  for the Sap(no Cys) peptide, and from 0.002 to 0.06  $\mu\text{M}$  for Sir22 using a flow rate of 20  $\mu\text{l}/\text{min}$ . The association was followed for 6 min, and the dissociation was followed for 2 h. The association and the dissociation were recorded separately. The surface was regenerated between binding experiments by injection of 0.1 M HCl. Control experiments were performed using a flow cell that had been prepared using the immobilization protocol above, but without coupling of IgA. No binding to this control surface was observed for any of the peptides or Sir22. The data were evaluated by nonlinear least squares analysis using the software KaleidaGraph, as described (41). The dissociation data could be well fitted with a single exponential decay. The values obtained for  $k_{\text{off}}$  were used in the subsequent analysis of the association data, which were also possible to fit with a single component.

### Radioimmunoassay

A modified solid phase RIA was used to analyze the binding of the synthetic peptides Sap and Sap(no Cys) to IgA (23). The wells of microtiter plates (Falcon 3912; BD Biosciences, San Diego, CA) were coated with serum IgA, using coating solutions containing different concentrations of IgA dissolved in PBS. The wells were washed and blocked with PBS with 0.02%  $\text{NaN}_3$  and 0.05% Tween 20 (PBSAT). Radiolabeled peptide ( $\sim 15,000$  cpm in 50  $\mu\text{l}$ ) was added to each well, followed by incubation for 2 h at room temperature. The wells were then washed three times with PBSAT, and the radioactivity associated with each well was determined in a gamma counter. The test was done twice with triplicate samples.

### Purification of IgA on immobilized Sap

Sap peptide (5 mg) was immobilized in a 1-ml HiTrap column (Amersham Pharmacia Biotech), according to the instructions of the manufacturer. The gel in the HiTrap column consists of highly cross-linked agarose beads, activated by *N*-hydroxysuccinimide to allow coupling of ligands that contain primary amino groups. The samples analyzed on the Sap column were human serum or whole saliva. Before use, the samples were centrifuged at 18,000 rpm for 30 min at  $4^\circ\text{C}$  and filtered through a 0.45- $\mu\text{m}$  pore size filter. In a typical experiment with serum, 1 ml of serum (diluted 3-fold in PBS) was applied to the column, which was then washed with PBS (5 ml). For work with saliva, a 4-ml sample of undiluted saliva, centrifuged and filtered as described above, was applied to the column, which was then washed with 2 ml of PBS. Bound proteins were eluted with 0.1 M sodium acetate buffer, pH 4.0, in fractions of 0.32 ml. The pH of the fractions was immediately adjusted by adding 0.32 ml of 1 M Tris, pH 8.3. The flow rate used for elution of serum IgA was 1.0 ml/min, and for sIgA it was 0.5 ml/min. After use, columns were regenerated with 3 M KSCN, washed with PBS, and kept at  $4^\circ\text{C}$ .

### Quantification of total IgA and IgA subclasses

The levels of total IgA and of IgA subclasses in serum and saliva samples were estimated by titration in calibrated ELISA, the protocols of which shared several essential points: 1) Nunc polystyrene microplates (code 269620; Nunc, Roskilde, Denmark) were used as solid phase throughout, and solutions for coating were used at a protein concentration of 5  $\mu\text{g}/\text{ml}$  in PBS, using volumes of 100  $\mu\text{l}$  for incubation at room temperature overnight. Contrary to certain plates with high protein-binding capacity (42), these Nunc plates are readily blocked towards Igs, present in subsequently added serum or secretion samples, by using Tween 20 at low concentrations; 2) PBS with 0.15% Tween 20 served as combined washing and blocking solution and as diluent for test samples, standards, and Ab reagents, all of which were incubated at room temperature for 4 h. For dilution of test samples, the diluent was supplemented with 10 mM sodium azide; 3) optimal dilutions of commercial Abs including peroxidase conjugates were determined in pilot experiments; 4) test samples (appropriately prediluted) were analyzed at six serial 3-fold dilutions in duplicate wells, and a similar dilution series (eight steps) of the relevant standard was included in each plate; 5) after development of plates with chromogenic substrate ( $\sigma$ -phenylenediamine,  $\text{H}_2\text{O}_2$ ),  $\text{OD}_{492}$  was read with a PC-operated Multiscan RC ELISA Reader (Labsystems, Helsinki, Finland). Titration curves for standard and test samples were fitted from a four-parameter logistic model using the Genesis Lite software package (Labsystems), and concentrations for test samples were calculated as the mean of three, occasionally two, determinations based on optical densities corresponding to the log-linear part of the standard curve. Generally, the coefficient of variation for the three determinations was  $<10\%$ , testifying to the validity of the standards used.

The ELISA for quantification of total IgA in serum or saliva samples used rabbit Abs to human  $\alpha$ -chain (DAKO) for coating. After reaction with test samples and standard, wells were incubated with peroxidase-conjugated rabbit Abs specific for  $\alpha$ -chain (DAKO). Alternatively, to selectively quantify sIgA, conjugated Abs to SC (DAKO) were used for detection. In assays of serum IgA, purified human IgA (DAKO; code X594) served as standard. In assays of salivary IgA, a standard of human colostrum S-IgA purified as described (43) was used.

The ELISA for quantification of IgA subclasses used mouse mAbs, specific for either of the two subclasses (Nordic). Because these mAbs were available in the form of ascites, they were immobilized via a coating layer of rabbit Abs (F(ab)<sub>2</sub>) to mouse Igs (DAKO). After incubation with test samples and standard, plates were incubated with peroxidase-conjugated rabbit Abs to either human  $\alpha$ -chain or SC (DAKO). In subclass-specific assays of serum IgA, two human mAbs of the IgA1 and IgA2 subclass, respectively, served as standards. These had been produced, characterized (44), and donated by Dr. H. Baxendale (Institute of Child Health,

London, U.K.). Human colostral S-IgA1 and S-IgA2, purified by the use of jacalin agarose as described (43), was used for standardization of subclass-specific assays of salivary IgA.

#### Quantification of IgG and IgM

IgG and IgM contents were quantified by sandwich ELISA using rabbit Abs directed to human  $\gamma$ -chain and  $\mu$ -chain, respectively (DAKO). The wells of microtiter plates were coated overnight at 4°C with Abs, using 50  $\mu$ l of Ab solution (1  $\mu$ g total protein per ml) in PBS. All subsequent steps were performed at room temperature. The wells were washed five times with PBSAT and blocked for 1 h with PBS supplemented with 0.2% BSA. Serum and saliva samples (50  $\mu$ l) appropriately diluted in PBSAT were added to the wells and incubated for 1 h. The wells were then washed five times with PBSAT, and bound Ig was detected with rabbit alkaline phosphatase-conjugated Ab to either  $\gamma$ -chain or  $\mu$ -chain (DAKO) diluted 1/2000 and 1/1000 in PBSAT (50  $\mu$ l), respectively. After incubation for 1 h, the wells were washed five times with PBSAT. After development with *p*-nitrophenylphosphate in 10% diethanolamine, pH 9.8 (50  $\mu$ l; 1 mg/ml), the microtiter plates were examined in a Multiscan RC ELISA reader (LabSystems) at 405 nm. Dilutions of a human serum protein calibrator (DAKO; X0908) were used as standards for IgG and IgM. The contents of IgG and IgM in the test samples, appropriately diluted, were determined from the optical densities corresponding to the log-linear part of the standard curve. Optimal concentrations of Abs used for coating and detection were determined in pilot experiments.

#### Use of the Sap peptide to detect IgA bound to Ag

The wells of microtiter plates (Falcon 3912; BD Biosciences) were coated overnight at 4°C with NIP-BSA (5  $\mu$ g/ml) in PBS (50  $\mu$ l) and then washed and blocked with five changes of PBSAT during 1 h. All steps after the coating step were performed at room temperature. Anti-NIP IgA1, anti-NIP IgA2, or anti-NIP IgG1 (50  $\mu$ l; 0.5  $\mu$ g/ml) were added to the coated wells, as indicated. After 2 h, the presence of bound Abs was analyzed by the addition of radiolabeled protein G or radiolabeled Sap (~15,000 cpm in 50  $\mu$ l). All reagents were diluted in PBSAT.

#### Other methods and reagents

Radiolabeling with carrier-free  $^{125}$ I (Amersham) was performed with the chloramine-T method. N-terminal sequence analysis was performed on proteins transferred to ProBlot membranes (Millipore, Bedford, MA). All chemicals were of highest grade commercially available.

## Results

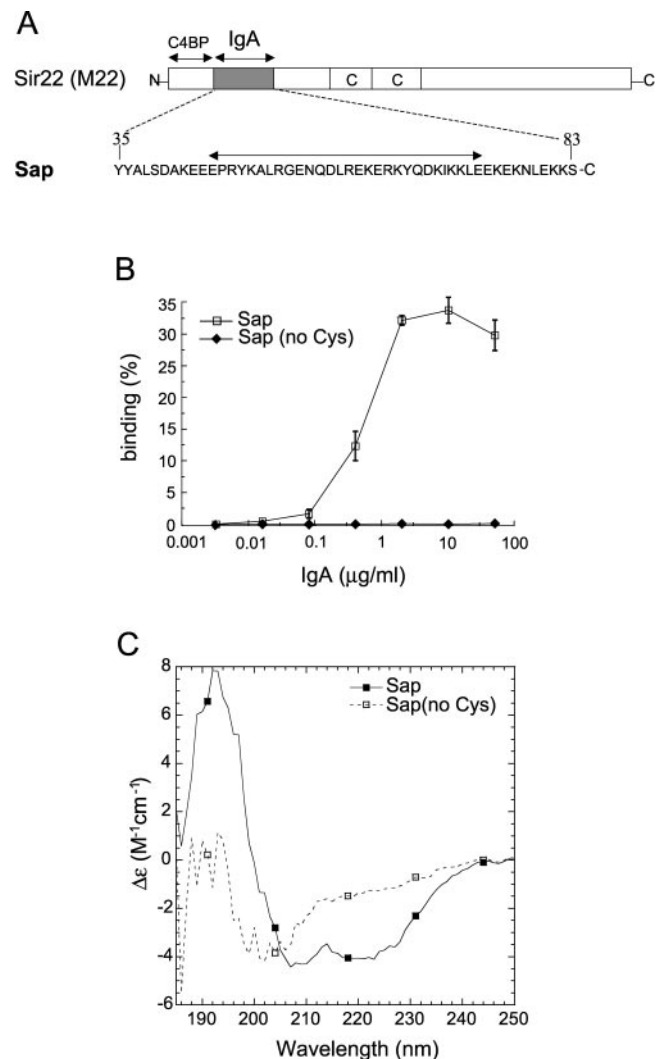
#### Dimeric Sap binds human IgA with high affinity

The Sap peptide (Fig. 1A) was derived from the streptococcal Sir22 (M22) protein. Like other members of the M protein family, much of the sequence of the Sir22 protein exhibits a heptad repeat pattern, causing the molecule to form a coiled-coil dimer (45–47), a feature that is important for the ability of M proteins to bind IgA (48). Although it seemed possible that the Sap peptide, which contains several heptad repeats, might tend to dimerize by itself, a cysteine residue, not present in the intact M protein, was added at the C-terminal end of the peptide, to initiate dimerization via a disulfide bond (28). The addition of this cysteine residue had the additional advantage of preventing dissociation of the two chains present in a putative coiled-coil structure. Analysis by SDS-PAGE under nonreducing conditions showed that Sap migrated as a dimer, whereas a derivative lacking the cysteine residue, designated Sap(no Cys), migrated as a monomer (data not shown).

To analyze whether the C-terminal cysteine residue in Sap is necessary for the peptide to bind IgA, we compared the binding properties of Sap with those of Sap(no Cys). Radiolabeled forms of the two peptides were analyzed for binding to microtiter wells that had been coated with solutions containing different concentrations of IgA (Fig. 1B). The Sap peptide showed significant binding to immobilized IgA, whereas no binding was observed for the peptide without cysteine. Similar data were obtained when the two peptides were immobilized in microtiter wells and analyzed for ability to capture radiolabeled IgA (data not shown). These data show that the cysteine residue is an important feature of the Sap peptide,

suggesting that the peptide lacking this residue may not form IgA-binding dimers, in spite of the presence of several heptad repeats.

The secondary structure of the peptides was studied using CD spectroscopy (Fig. 1C). The dimerized Sap peptide was estimated to be 43%  $\alpha$ -helical, with a CD spectrum showing typical minima around 208 and 222 nm and a maximum below 200 nm. The intensities at 222 and 208 nm are similar, whereas coiled-coil structures commonly give a ratio of the ellipticities at 222 and 208 of >1 (49, 50). This may represent spectral contributions from aromatic side chains or a deviation from coiled-coil structure. The CD



**FIGURE 1.** Characterization of the IgA-binding Sap peptide. *A*, Schematic representation of the streptococcal Sir22 (M22) protein, and sequence of the Sap peptide derived from this protein. The Sap peptide includes the 29-residue IgA-binding region (horizontal arrow) and 10 aa on either side of this region. In addition, Sap includes a C-terminal cysteine residue, not present in Sir22, to promote dimerization (28). The most N-terminal part of Sir22 is the hypervariable domain that binds the human complement regulator C4BP (26, 71). The position of the conserved C repeat region is indicated. The C-terminal end of Sir22 is covalently bound to peptidoglycan in the bacterial cell wall (32). *B*, Binding of Sap and its derivative Sap(no Cys), which lacks the C-terminal cysteine residue, to IgA immobilized in microtiter wells. The wells were coated with solutions containing different concentrations of serum IgA, as indicated, and analyzed for ability to bind radiolabeled peptides. The data are based on two different experiments with triplicate samples. *C*, CD spectra of the two peptides, Sap (0.10  $\mu$ M) and Sap(no Cys) (0.22  $\mu$ M), at 25°C.

analysis indicated that the Sap(no Cys) peptide assumed less secondary structure, and the  $\alpha$ -helical content was calculated to be only 8.4%. These data are in good agreement with the binding studies, and confirm that the cysteine residue has an important effect on the structure of the Sap peptide.

The affinity of Sap for monomeric IgA (mIgA) and dimeric S-IgA was studied using SPR technology (Fig. 2). In this analysis, the Sap peptide was compared with the intact Sir22 (M22) protein, from which Sap was derived, and with the Sap(no Cys) peptide. Experiments were performed five times for each peptide/protein, at five different concentrations. Association and dissociation rate constants are shown in Table I and sample sensorgrams are shown in Fig. 2. The sensorgrams obtained with intact Sir22 and with Sap indicated high affinity binding to IgA. For mIgA,  $K_D$  was calculated to be  $\sim 4$  nM for Sir22 and  $\sim 20$  nM for Sap. In this analysis, the Sap(no Cys) peptide also bound IgA, but with considerably lower affinity,  $K_D \sim 700$  nM. For S-IgA,  $K_D$  was  $\sim 3$  nM for Sir22,  $\sim 20$  nM for Sap, and  $\sim 1$   $\mu$ M for Sap(no Cys). These data indicate that Sap binds both mIgA and S-IgA with high affinity and confirm that the C-terminal cysteine residue is important for optimal binding. This cysteine-containing, dimeric, form of Sap was used for all experiments reported below.

#### Sap binds all IgA molecules in human serum

Previous analysis with a collection of monoclonal human IgA proteins indicated that a minority of these proteins did not bind Sap (28). However, it seemed possible that this result reflected aberrant behavior of certain purified mAbs rather than a low affinity of Sap for certain serum IgA molecules. To analyze this problem, human serum that had been passed through a Sap column was analyzed for remaining IgA. This single-step procedure caused  $>99\%$  depletion of IgA but did not have any apparent effect on the concentration of IgG or IgM (Fig. 3A). Thus, Sap binds to all, or almost all, IgA molecules in human serum.

#### Single-step affinity purification of serum IgA on immobilized Sap

To analyze whether a Sap column retains serum proteins other than IgA, a column that had been exposed to serum was eluted with 0.1

M acetate buffer, pH 4.0. Preliminary experiments indicated that this procedure caused elution of all proteins that had bound to the column (data not shown). The eluted material was analyzed by SDS-PAGE under nonreducing and reducing conditions and compared with two commercial preparations of serum IgA (Fig. 3B). In the nonreducing gel, a major protein species was seen in all three preparations (Fig. 3B, left), but all three samples contained less abundant protein species that migrated faster or slower than the major species. A similar size heterogeneity of IgA analyzed in nonreducing SDS-PAGE has been observed in another system (51). Analysis under reducing conditions showed the pattern expected for pure IgA, with a major H chain band at  $\sim 60$  kDa and a  $\sim 31$ -kDa doublet band typical for L chains (Fig. 3B, right). Thus, this analysis suggested that the different protein species observed in the nonreducing gel all represent forms of IgA with different migration behavior in SDS-PAGE. The nonreducing gel contained distinct protein species that migrated more slowly than the major form (Fig. 3B, left) and may represent the known polymeric forms of serum IgA (52).

The N-terminal sequence of the  $\sim 60$ -kDa polypeptide in the reducing gel (Fig. 3B, right) was EVQLVESG, which corresponds to the N-terminal sequence of the  $V_H$  domain of the IgA1 H chain (53). Thus, the major protein species eluted from the Sap column was indeed IgA.

In a typical experiment, 1 ml of serum was applied to a column that had been prepared with 5 mg of Sap, and the eluate contained the  $\sim 2$  mg of IgA present in the sample. The total capacity of such a column was  $\sim 8$  mg of serum IgA (data not shown). Analysis by ELISA of eluates obtained with serum from three donors showed the presence of  $\sim 0.45\%$  IgG and  $\sim 0.33\%$  IgM. Together, these data indicate that highly purified human serum IgA can be isolated by single-step affinity chromatography on a Sap column. Such columns, kept at  $4^\circ\text{C}$ , could be used many times and did not show any apparent loss of activity during a 1-year period.

Because a Sap column appeared to bind all IgA in serum and because this IgA could be quantitatively eluted from the column, the purified material was expected to contain IgA of both subclasses. Analysis by ELISA showed that the proportions of IgA1

**FIGURE 2.** SPR analysis of the interactions between the Sap peptide, the Sap(no Cys) peptide, or intact Sir22 protein with mIgA and S-IgA. **A**, Association data for Sap (19 nM), Sap(no Cys) (1006 nM), and Sir22 (19 nM) for mIgA. Symbols are explained in **B**. **B**, Dissociation data for Sap (19 nM), Sap(no Cys) (2013 nM), and Sir22 (62 nM) for mIgA, presented as a normalized response. The intensity at the start of the dissociation was set to 1.0, and the baseline value was set to 0.0. **C**, Association data for Sap (80 nM), Sap(no Cys) (1006 nM), and Sir22 (15 nM) for S-IgA. Symbols are explained in **B**. **D**, Dissociation data for Sap (20 nM), Sap(no Cys) (2013 nM), and Sir22 (15 nM) for S-IgA, presented as normalized response. The intensity at the start of the dissociation was set to 1.0, and the baseline value was set to 0.0.

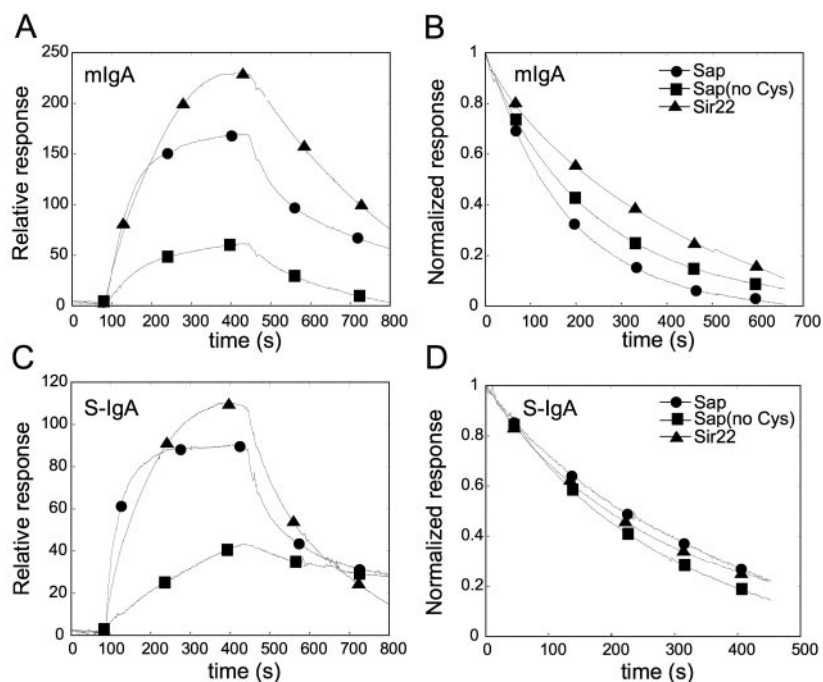


Table I. Quantitative analysis of the interaction of Sap, Sap(no Cys), and Sir22 with mIgA and S-IgA<sup>a</sup>

	Association Rate Constant (M <sup>-1</sup> s <sup>-1</sup> )	Dissociation Rate Constant (s <sup>-1</sup> )	Equilibrium Association Constant (M <sup>-1</sup> )	Equilibrium Dissociation Constant (M)
<b>mIgA</b>				
Sap	3 × 10 <sup>5</sup>	6 × 10 <sup>-3</sup>	6 × 10 <sup>7</sup>	2 × 10 <sup>-8</sup>
Sap(no Cys)	6 × 10 <sup>3</sup>	4 × 10 <sup>-3</sup>	1 × 10 <sup>6</sup>	7 × 10 <sup>-7</sup>
Sir22	8 × 10 <sup>5</sup>	3 × 10 <sup>-3</sup>	3 × 10 <sup>8</sup>	4 × 10 <sup>-9</sup>
<b>S-IgA</b>				
Sap	1 × 10 <sup>5</sup>	3 × 10 <sup>-3</sup>	4 × 10 <sup>7</sup>	2 × 10 <sup>-8</sup>
Sap(no Cys)	3 × 10 <sup>3</sup>	4 × 10 <sup>-3</sup>	7 × 10 <sup>5</sup>	1 × 10 <sup>-6</sup>
Sir22	1 × 10 <sup>6</sup>	3 × 10 <sup>-3</sup>	3 × 10 <sup>8</sup>	3 × 10 <sup>-9</sup>

<sup>a</sup> Association and dissociation rate constants were obtained by SPR analysis. Each peptide/protein was analyzed five times, at five different concentrations, and the values shown are the average of these five determinations. The equilibrium constants were calculated from the rate constants. Due to the error inherent in the SPR method, no decimals are shown.

and IgA2 were indeed the same in purified IgA as in whole serum (Fig. 3C).

#### Purification of S-IgA

To analyze whether immobilized Sap can be used for purification of S-IgA, human saliva was applied to a Sap column, which was eluted as described above. Analysis of eluted material by non-reducing SDS-PAGE demonstrated the presence of a doublet band of high molecular mass, present also in whole saliva and in two commercial preparations of S-IgA (Fig. 4A, left). Analysis under reducing conditions demonstrated the presence of three bands at ~80, ~60, and ~31 kDa in the material purified on the Sap column, and the same three bands were also seen in the commercial S-IgA preparations (Fig. 4A, right). Western blot analysis with specific antiserum identified the ~80-kDa band as SC (data not shown). The major band at ~60 kDa was similarly identified as the  $\alpha$ -chain. The identity of this band was confirmed by N-terminal sequencing. The doublet band at ~31 kDa corresponds to L chains and to the J chain, as shown with specific Abs (data not shown). Although the J chain has a calculated molecular mass of 15–16 kDa, it migrated more slowly than expected, as previously observed (51, 54, 55).

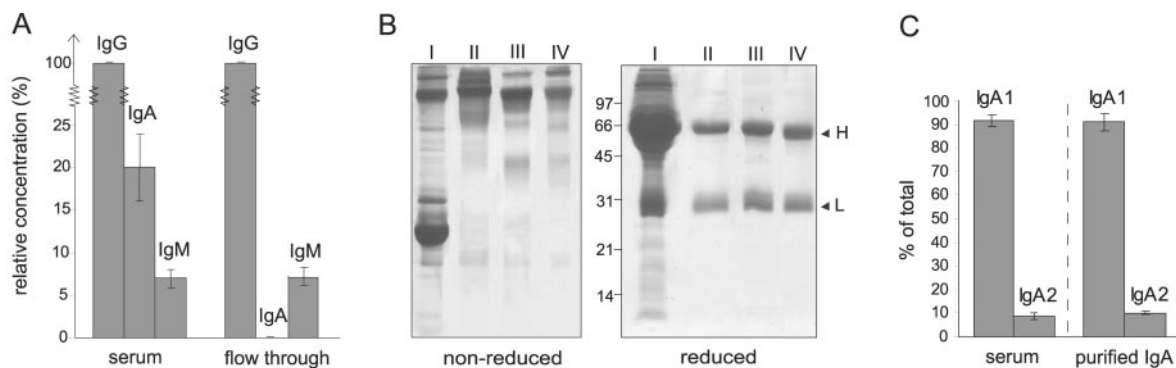
In general, the S-IgA eluted from the Sap column appeared to be at least as pure as the commercial preparations of S-IgA. For example, one of the commercial S-IgA preparations contained an

unidentified ~14-kDa polypeptide not present in S-IgA purified on a Sap column. The total capacity of a column prepared with 5 mg of Sap was ~3 mg of S-IgA (data not shown).

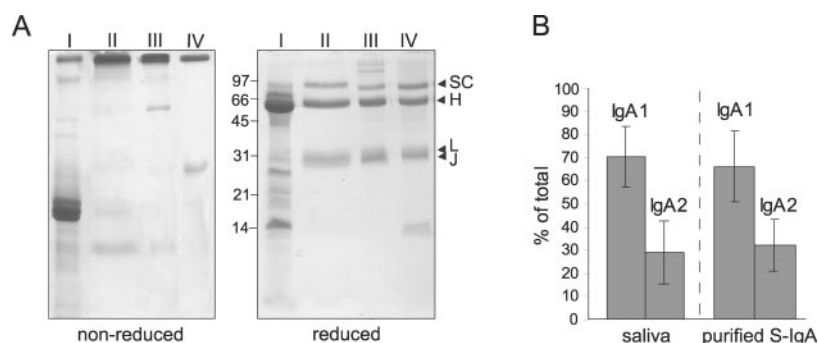
In all experiments with saliva, a considerable fraction of the S-IgA did not bind to the Sap column but was found in the flow through, and the recovery was ~45%. Attempts to improve the recovery by changes in pH, ionic strength, or washing procedures were unsuccessful. The incomplete recovery of S-IgA was not due to limited binding capacity of the column or to lack of elution of some S-IgA from the column (data not shown). Moreover, the incomplete recovery was not due to selective loss of one subclass, because the proportions of S-IgA1 and S-IgA2 were similar in whole saliva and in purified S-IgA (Fig. 4B). Thus, the reason for the incomplete recovery of S-IgA is not clear. Although these data indicate that S-IgA is not quantitatively recovered on a Sap column, they also indicate that S-IgA of both subclasses may be highly purified by single-step chromatography on a Sap column. Results similar to those obtained with saliva were obtained when a Sap column was used for the purification of S-IgA from human milk (data not shown).

#### Use of Sap to detect IgA bound to Ag

The binding of Sap to IgA with high specificity and affinity suggested that Sap might be used to specifically identify IgA bound to Ag. This possibility was evaluated using a set of three recombinant



**FIGURE 3.** Affinity chromatography of human serum on immobilized Sap and analysis of purified IgA. *A*, Depletion of IgA by chromatography of human serum on a Sap column. The concentration of IgG, IgA, and IgM was determined before (left) and after (right) passage of serum through the column. The concentration of IgG is set to 100%. These data are based on three experiments with serum from two different donors. Standard deviations are indicated. *B*, Analysis of serum proteins that bind to a Sap column. Bound proteins were eluted with 0.1 M sodium acetate buffer, pH 4.0, and subjected to SDS-PAGE under nonreducing (left) or reducing (right) conditions. Lane I, whole serum; lane II, material eluted from the Sap column; lanes III and IV, commercial serum IgA from Cappel and Pierce, respectively. The two arrowheads indicate the position of  $\alpha$  H and of L chains, respectively, in the reducing gel. Molecular mass markers are in kilodaltons. Because such markers are suitable for analysis only under reducing conditions, their positions are indicated only for the reducing gel. *C*, Analysis of subclass composition in serum IgA purified on a Sap column. The figure shows the percentage of IgA1 and IgA2 in whole human serum (left) and in IgA purified from the same serum (right). Data based on six experiments with serum from three donors.



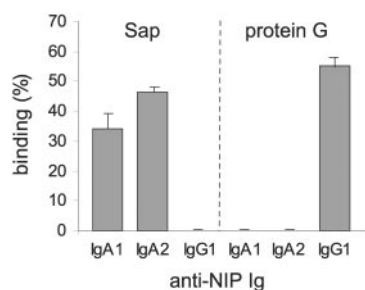
**FIGURE 4.** Analysis of human S-IgA purified by affinity chromatography of saliva on immobilized Sap. *A*, Analysis of salivary proteins that bind to a Sap column. Bound proteins were eluted with 0.1 M sodium acetate buffer, pH 4.0, and subjected to SDS-PAGE under nonreducing (*left*) or reducing (*right*) conditions. *Lane I*, whole saliva; *lane II*, material eluted from the Sap column; *lanes III and IV*, commercial S-IgA from Sigma and Cappel, respectively. Arrowheads, Positions of SC,  $\alpha$  H chain (H), L chains (L), and J chain (J). Similar results were obtained in several different experiments. *B*, Analysis of subclass composition in S-IgA purified on a Sap column. The figure shows the percentage of IgA1 and IgA2 in whole human saliva (*left*) and in S-IgA purified from this saliva. Data are based on three experiments with saliva from three different donors.

IgA1, IgA2, and IgG1 proteins that recognize the same antigen (38, 39). These Abs have identical variable regions that recognize NIP, but the H chain constant regions correspond to human IgA1, IgA2, or IgG1. The use of these Abs allowed direct comparison of Fc reactivity, without possible effects due to differences in the variable regions. The three Abs were bound to NIP-BSA immobilized in microtiter wells, and the presence of bound Ig was analyzed by the addition of radiolabeled Sap or protein G. The results (Fig. 5) show that Sap recognized Ag-bound IgA1 and IgA2, but not IgG1, whereas protein G gave the opposite result, as expected (56). Thus, Sap can be used to specifically detect IgA bound to Ag.

## Discussion

The data reported here show that the Sap peptide binds human IgA with high specificity and affinity and that immobilized Sap can be used for gentle purification of IgA by a single-step procedure. Moreover, Sap can be used to specifically detect IgA of both subclasses bound to Ag. Together, these findings indicate that Sap has the potential to become a valuable tool for the isolation and detection of IgA, thereby facilitating studies on the biochemical and biological properties of this Ig class. In this context, it is of interest that Sap is a stable molecule that retains its IgA-binding ability even after boiling (28).

With regard to serum IgA, our analysis indicates that virtually all IgA molecules in serum are retained on a Sap column and can be gently eluted. Indeed, the two subclasses of IgA are present in the purified material in the same proportions as in the starting material. A molecular basis for this result is provided by previous



**FIGURE 5.** Use of the Sap peptide to specifically detect IgA bound to Ag. Recombinant IgA1, IgA2, and IgG1 Ab with specificity for NIP were added to microtiter wells that had been coated with NIP-BSA. Bound Abs were detected by addition of radiolabeled Sap (*left*) or protein G (*right*). Data are based on three experiments with duplicate samples.

studies showing that Sap binds to a site in IgA-Fc conserved in both subclasses (36). This binding specificity can also explain why Sap recognizes IgA of both subclasses bound to Ag. The IgA purified on a Sap column probably contains all possible subpopulations of IgA present in human serum, including minute amounts of IgA covalently linked to other proteins (57–59). In particular, our data indicate that serum IgA purified on a Sap column contains polymeric serum IgA, a molecular form that has been suggested to play an important role in IgA-mediated defense against infection (4).

In contrast to serum IgA, the recovery of S-IgA after chromatography of human saliva on a Sap column was ~45%. The reason for this incomplete recovery of S-IgA is not clear, and it did not appear to be due to a reduced affinity of S-IgA for Sap, because S-IgA and serum IgA had similar affinities for Sap, as measured by SPR. In any case, it is important that the S-IgA purified on a Sap column contained IgA1 and IgA2 in the same proportions as in the starting material.

The Sap peptide not only shows specificity for IgA among human proteins but also shows species specificity. Our preliminary studies indicate that Sap does not bind mouse IgA but that it binds bovine IgA (J. M. Woof, R. J. Pleass, T. Areschoug, and G. Lindahl, unpublished data). This observation is in agreement with sequence analysis, which shows that amino acid residues in the C $\alpha$ 2-C $\alpha$ 3 interdomain region of IgA-Fc that are important for binding of Sap are conserved in human and bovine IgA, but not in mouse IgA (36, 39). Thus, it seems possible that Sap may become valuable also for studies of immunity mediated by bovine IgA (11).

Several proteins in addition to Sap have been reported to bind IgA, including the plant lectin jacalin (60) and some bacterial surface proteins. Jacalin is commonly used for the purification of human IgA, although the usefulness of this reagent is limited by its lack of reactivity with molecules of the IgA2 subclass (61), by its affinity for several plasma proteins besides IgA1 (62), and by its ability to cause complement activation due to binding of C1-inhibitor (63). However, the inability of jacalin to bind IgA2 molecules is also a valuable property. Indeed, a simple procedure to isolate and separate pure IgA1 and IgA2 might be to isolate total IgA by chromatography on a Sap column, followed by a second chromatography on a jacalin column.

Bacterial proteins that bind IgA have been studied most extensively in *S. pyogenes*, and the Sap peptide was derived from one of these proteins. However, many strains of group B *Streptococcus* also express an IgA-binding protein, designated  $\beta$  or Bac, that is

unrelated to M proteins (22, 24, 64, 65). Interestingly, these unrelated streptococcal IgA-binding proteins bind to the same region in IgA-Fc (36), but for unknown reasons  $\beta$  binds poorly to S-IgA, which limits its value as a possible IgA-binding reagent (24). Moreover,  $\beta$  has a separate binding site for the human complement regulator factor H (27). Another IgA-binding molecule of bacterial origin was derived from a region of staphylococcal protein A engineered to yield domains with novel binding properties (66). When expressed on the surface of a heterologous bacterial species, one of these domains allowed binding of a monoclonal IgA Ab, suggesting that it may be used as an IgA-binding reagent (66). Finally, some strains of *Streptococcus pneumoniae* express a SC-binding surface protein that is of potential interest for the isolation of S-IgA (67), although this protein probably also binds human factor H (68, 69).

Although this study was focused on the use of Sap as an immunological reagent, our findings are of relevance also to bacterial pathogenesis, because Sap was derived from the *S. pyogenes* Sir22 (M22) protein, which is a major virulence factor (30, 70). The Sir22 protein, like several other M proteins, has separate binding sites in the N-terminal part for two human plasma proteins, the complement regulator C4b-binding protein (C4BP) and IgA (34, 71, 72). We have previously shown that the N-terminal hypervariable C4BP-binding region of Sir22 (Fig. 1A) can be studied as a synthetic peptide that binds C4BP with high specificity (72). Similarly, the IgA-binding region of Sir22 retains its binding properties in isolated form and binds IgA-Fc with high specificity (Ref. 28 and this report). Thus, the N-terminal region of the Sir22 protein is composed of two distinct protein domains, each of which specifically binds a component of the human humoral immune system. These two domains correspond to a total sequence of ~80 aa residues, but in spite of this limited length the two large ligands C4BP and IgA, which have molecular masses of ~540 and ~160 kDa, respectively, bind independently to the two sites, i.e., binding of one ligand does not interfere with binding of the other one (26). The biological significance of these highly specific interactions is still not clear, but there is good evidence that the binding of C4BP to the N-terminal hypervariable region of the Sir22 protein contributes to phagocytosis resistance (73), a well-known property of M proteins (30).

Streptococcal M proteins are known to have dimeric coiled-coil structure, as determined by sequence analysis and CD spectroscopy (45–47, 74), but this situation does not exclude that certain regions of M proteins may have a different structure. With regard to the Sir22 protein, studies of the most N-terminal C4BP-binding region indicated that it is largely  $\alpha$ -helical (72), and the CD analysis reported here indicates that the IgA-binding region also is  $\alpha$ -helical, but it is not yet known whether these regions have a dimeric coiled-coil structure. However, our data indicate that dimerization of Sap via a C-terminal cysteine residue has an important effect on secondary structure and on affinity for IgA-Fc, suggesting that formation of the binding site requires interaction between two chains, as in a coiled-coil. More definite conclusions concerning the conformation of the IgA-binding domain must await structural analysis.

In summary, we have demonstrated that the synthetic peptide Sap, derived from the IgA-binding region of a streptococcal M protein, can be used for the purification and detection of human IgA of both subclasses. This peptide promises to become a valuable tool for structural and functional studies of IgA.

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