The Journal of Immunology

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J Immunol 1998; 161:3161-3168; ;
http://www.jimmunol.org/content/161/6/3161

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

High Resolution Mapping of the B Cell Epitopes of Staphylokinase in Humans Using Negative Selection of a Phage-Displayed Antigen Library[1]

Stéphane Jenné, Katleen Brepoels, Désiré Collen, and Laurent Jespers[2]

Staphylokinase (Sak), a 16-kDa protein secreted by *Staphylococcus aureus*, induces fibrin-specific thrombolysis in patients with thrombotic disorders. However, Sak also elicits high titers of neutralizing Abs that persist for several months and preclude its repeated use in humans. To identify the antigenic determinants of Sak recognized by humans, a phage-displayed library of Sak variants was selected for mutants that escape binding to an affinity matrix derivatized with patient-specific polyclonal anti-Sak Abs. Fifty-six escape Sak variants were identified after three selection cycles using human polyclonal anti-Sak IgGs obtained from four different patients. DNA sequencing revealed 213 amino acid substitutions, of which 73% were found at 25 positions clustered in eight discontinuous Sak antigenic segments. Although each antigenic segment was recognized to a variable extent by each patient antiserum, the main epitopes of Sak in all patients were roughly targeted to two large discontinuous areas covering 35% of the solvent-accessible surface of Sak. The antigenic area I comprises three segments centered on residues 66, 73, and 135, while the antigenic area II consists of four segments centered on positions 20, 95, 102, and 121. These results suggest that a secondary immune response against Sak can occur in patients, and confirm an initial site-directed mutagenesis study wherein amino acid Lys^{74} was shown to play a prominent antigenic role. Comprehensive mapping of the most relevant sites of Sak that are antigenic for humans will guide efforts to modulate the immunogenicity of this therapeutically important molecule. ***The Journal of Immunology,*** **1998, 161: 3161–3168.**

Staphylokinase (Sak),[3] a plasminogen activator produced by certain strains of *Staphylococcus aureus*, consists of 136 amino acids in a single polypeptide chain without disulfide bridges (reviewed in Ref. 1). In contrast to endogenous activators such as tissue-type plasminogen activator and urokinase, Sak lacks proteolytic activity, but induces plasminogen activation capability in human plasmin upon formation of a 1:1 stoichiometric complex with its serine proteinase domain. The thrombolytic potential of Sak has recently been investigated in vitro and in animal models, and subsequently in patients with thromboembolic disorders (2–6). When compared with tissue-type plasminogen activator, Sak emerges as a potent profibrinolytic with a unique mechanism of fibrin specificity (7, 8).

Like streptokinase, Sak is a bacterial protein, and therefore induces neutralizing Abs in animal models (5, 9) and in a majority of patients (7, 8, 10). Although Sak was initially found to elicit a significantly lower humoral response than streptokinase in dogs and baboons, i.v. infusion of Sak in patients with acute myocardial infarction consistently resulted in high levels of anti-Sak IgGs (7). The Sak-neutralizing activity was low at baseline (0.07 ± 0.01 μg/ml, mean ± SEM), but raised to 32 ± 7.1 μg/ml from the second week onward. Intraarterial administration of Sak in patients with peripheral arterial occlusion yielded comparable anti-Sak Ab titers, which remained elevated for more than 1 yr (8). Although Sak apparently did not produce allergic reactions, the neutralizing Ab titers argue against its repeated use for thrombolysis, in view of the risk of partial or complete therapeutic refractoriness.

The long initial lag phase (about 10–14 days) before appearance of anti-Sak IgGs (7, 8, 10) may suggest that Sak elicits a primary immune response in humans. However, the very low (or almost undetectable) level of anti-Sak IgM and the large increase (from 200- up to 1000-fold) and persistence of anti-Sak IgGs for several months after treatment (10, 11) are more consistent with a secondary response, implying that, as observed with streptokinase (12, 13), an immunologic memory to Sak has been acquired by patients, presumably via *S. aureus* infections. In line with this, secretion of Sak by *S. aureus* has been observed in 4 to 50% of the clinical isolates (11, 14).

In a previous study, the immunodominant antigenic regions of Sak were investigated by competitive Ab-binding study and clustered charge to alanine mutagenesis (15). A panel of 15 murine mAbs was initially generated, and their respective binding sites on Sak were grouped into three nonoverlapping immunodominant epitopes, two of which could be mapped at positions Lys^{35}, Glu^{38}, Asp^{80}, and Glu^{82} on the one hand, and positions Lys^{74}, Glu^{75}, and Arg^{77} on the other hand (epitope clusters 1 and 3, respectively). Further studies using plasma samples from immunized animals (rabbits and baboons) and patients with acute myocardial infarction revealed markedly different patterns of Ab recognition between species and among individuals within the same species (16). Only one residue, Lys^{74}, was critical for antigenic recognition of Sak in humans. However, the Lys^{74} → Ala Sak variant still adsorbed 62% of the anti-Sak Abs from a pool of 10 patients treated

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Received for publication February 26, 1998. Accepted for publication May 11, 1998.

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[1] This work was supported in part by a sponsored research agreement between Flemish Institute for the Advancement of Scientific and Technological Research in the Industry (IWT) and Thromb-X NV, a spinoff company of the University of Leuven, in which D.C. has an equity interest.

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[3] Abbreviations used in this paper: Sak, wild-type staphylokinase; CNBr, cyanogen bromide; TU, transducing unit.

0022-1767/98/$02.00

with Sak, indicating that residues other than Lys^{74} should contribute to the Sak antigenicity in humans. Indeed, it has been demonstrated that proteins contain a multitude of antigenic determinants that cover large surface-exposed regions, if not the entire protein surface (17).

Recently, we have devised an epitope-mapping strategy, whereby Ag variants that escape binding to an immobilized mAb are selected from a library of randomized Ag mutants displayed on filamentous phage (18). This negative selection approach was applied successfully to the mapping of two discontinuous epitopes on Sak. For this purpose, the *sak* gene was randomized by error-prone PCR (19) to generate a phage library encoding all reachable Sak variants resulting from single, double, and most triple amino acid changes (789, 3×10^5 and 8×10^7 combinations, respectively). After two negative selection cycles, the most frequently mutated positions in the selected Sak variants were shown to correspond to antigenic residues contacting the mAb used for the negative selection.

In the present study, the scope of the negative selection strategy has been extended to the mapping of dominant antigenic epitopes of Sak in several patients after thrombolytic therapy. Negative selection was performed using polyclonal anti-Sak Abs purified from four plasma samples. The results indicate that the molecular diversity of polyclonal anti-Sak IgGs does not preclude successful selection of escape Sak variants from the Sak-phage library. Overall, two main antigenic areas covering 35% of the solvent-accessible surface of Sak were deduced from the statistical analysis of the mutation occurrences in all selected Sak variants.

Materials and Methods

Reagents

Protein A-Sepharose, CNBr-activated Sepharose, HR-5/10 columns (5 mm diameter, 10 cm length), M13K07 helper phage, and horseradish peroxidase-conjugated anti-M13 IgG were purchased from Pharmacia (Roosendaal, The Netherlands). Streptavidin-coated magnetic beads were obtained from Dynal (Oslo, Norway). The murine anti-human IgG mAb GG-7 (Fc specific) was purchased from Sigma (St. Louis, MO). Synthetic oligonucleotides for PCR and automated DNA sequencing were synthesized at Eurogentec (Liège, Belgium). Recombinant Sak was produced in-house and purified, as described (20). Murine anti-Sak mAbs 32B2, 14H5, and 7H11 were produced as previously described (21). Biotinylation of mAb 32B2 was conducted with the NHS-SS-biotin (sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate) reagent from Pierce (Rockford, IL), according to the manufacturer's instructions. Sak coupling to CNBr-activated Sepharose (10 mg Sak/ml gel) was performed as recommended by the manufacturer.

Purification and immobilization of polyclonal human Sak-specific Abs

Polyclonal human anti-Sak Abs were purified from serum samples (20–40 ml) obtained from nine patients (MEE, CLA, BER, DRI, TOR, DAN, MAN, STA, and GOD) 14 to 28 days after Sak administration. All patients had received thrombolytic therapy (20–30 mg Sak over a period of 30 min) to treat an acute myocardial infarction. A two-step procedure was followed to purify human anti-Sak IgG fractions from each individual patient. Human IgGs were first prepared by adsorption on a 5-ml protein A-Sepharose column following standard procedures (22). After dialysis against TBS, the IgG samples were loaded on a 5 ml Sak-Sepharose column at a flow rate of 0.5 ml/min. After washing the column with 10 column volumes of TBS and 10 column volumes of 0.2 M glycine-HCl buffer, pH 5, bound human anti-Sak IgGs were eluted with 5 column volumes of 0.2 M glycine-HCl buffer, pH 3 (flow rate of 1 ml/min), and collected as 0.5-ml fractions that were neutralized immediately with 100 μl 0.5 M Tris-HCl buffer, pH 8. Polyclonal anti-Sak IgG-containing fractions were pooled, and IgG concentration was determined by measuring absorbency at 280 nm (1 $OD_{280\ nm}$ = 0.7 mg/ml). The purity of the polyclonal Ab preparations was evaluated by SDS-PAGE using the Phast System (Pharmacia) with 10 to 15% acrylamide gradient gels.

To immobilize polyclonal human anti-Sak IgGs on Sepharose, 2 ml CNBr-activated Sepharose was derivatized according to the manufacturer's instruction with 7 to 9 mg of affinity-purified anti-Sak IgGs. Three derivatized Sepharose batches were obtained using polyclonal human anti-Sak IgGs from patients MEE, CLA, BER, and DRI (the latter two being mixed in equal proportions before coupling, hereby named BERDRI), packed into HR-5/10 columns, and stored at 4°C in TBS buffer containing 0.02% sodium azide.

Selection method with the Sak library

To perform a negative selection against patient-specific anti-Sak Abs, each polyclonal anti-Sak IgG affinity column (MEE, CLA, or BERDRI) was adapted to an automated fast protein liquid chromatography (FPLC) system (Pharmacia) and washed with 10 column volumes of PBS. Purified phagemid particles (10^{11} transducing units (TU)) displaying the randomized Sak library (library size 8.3×10^8 clones, of which 7.5×10^8 had a *sak* insert) (18) were rescued from TG1 cells, as previously described (23), and loaded onto the anti-Sak column via a 500-μl injection loop at a flow rate of 50 μl/min. Unbound phage fractions, as identified by absorbency at 280 nm, were pooled (final volume approximately 1 ml) and submitted to positive selection with biotinylated mAb 32B2, as follows: 400 μl of the pooled phage fractions were mixed for 2 h at room temperature with 100 μl TBS buffer containing 0.5% BSA and 500 nM mAb 32B2. The Ag-Ab complexes were then captured on streptavidin-coated magnetic beads (0.5 mg) that had been blocked with TBS containing 0.1% BSA. After 20-min incubation at room temperature with moderate shaking, the magnetic beads were washed 10 times with TBS, 0.1% Tween-20, and bound phages were eluted by incubation in 0.5 ml TBS, 50 mM DTT for 10 min at room temperature. Eluted phages were used to infect *Escherichia coli* TG1, as described (23). The second and third cycles of negative/positive selection were conducted according to the same protocol.

Production of selected Sak phage clones and screening by ELISA

Single ampicillin-resistant colonies recovered after the third selection cycle were inoculated into 100 μl Luria-Bertani medium supplemented with 100 μg/ml carbenicillin and 1% w/v glucose using 96-well microtiter plates (Corning, NY) and grown overnight at 37°C with shaking. The overnight cultures were used to inoculate a second 96-well microtiter plate containing 200 μl of the same culture medium. After 1.5-h incubation at 37°C, each microculture was infected with 10^{10} TU of M13K07 and further incubated for 1 h. Glucose was then removed by spinning down 20 μl of each microculture (2000 rpm at room temperature). Each cell pellet was resuspended in 200 μl prewarmed LB medium containing 100 μg/ml carbenicillin and 50 μg/ml kanamycin, and grown at 37°C for 20 h. The culture supernatants were then mixed with 40 μl of a solution containing 20% polyethylene glycol 6000 and 2.5 M NaCl to precipitate phage particles (1 h at 4°C) that were then resuspended in 250 μl TBS after centrifugation (30 min at 2000 rpm).

For phage-ELISA with polyclonal human anti-Sak IgGs, 96-well microtiter plates were coated by overnight incubation at 4°C with murine anti-human IgG mAb GG-7 (1/1250 dilution in TBS), blocked with TBS containing 2% skim milk (37°C, 1 h), and then washed four times with TBS, 0.1% Tween-20. Human anti-Sak Abs (100 μl of 20 ng/ml in TBS, 2% skim milk) were then incubated in the wells for 1 h. After washing, phage samples (50 μl supplemented with 50 μl TBS containing 4% skim milk) were incubated in the wells for 2 h, and bound phage were detected by incubation with horseradish peroxidase-conjugated anti-M13 antiserum (1/1250 dilution in TBS for 1 h) and *o*-phenylene diamine dihydrochloride as substrate (0.5 mg/ml in 0.1 M citrate/0.2 M sodium phosphate buffer, pH 5). After 10 to 15 min, the 492-nm absorbency was measured. Control phage-ELISA was performed similarly with plates coated with an anti-Sak mAb (mAb 32B2) (15).

DNA sequencing and statistical analysis of selected Sak phage clones

The DNA sequences of selected clones were sequenced by the dideoxy method (24) using dsDNA (prepared with the Qiafilter Plasmid Maxi kit; Qiagen, Chatsworth, CA), the Cy5 AutoRead Sequencing kit (Pharmacia), Cy5-labeled primers 5′-CTA TGC GGC CCC ATT CAG ATC CTC (forward) or 5′-TCA TAA TGA AAT ACC TAT TGC (backward), and the automated ALF*express* DNA sequencing apparatus (Pharmacia).

To plot a patient-specific antigenic profile of Sak, a smoothing window of four amino acids length was run along the primary sequence of Sak (136 amino acids). For each window, the amino acid substitutions determined by DNA sequencing of *N* escape variants selected from the randomized Sak-phage library were summed, and the value (*i*) was plotted on the graph at the second amino acid position of the window. At maximal positions, the

probability (p) whereby the total number of selected amino acid mutations could have arisen by chance had been calculated to assess their statistical significance for occurrence. The p values were calculated for i amino acid mutations assuming a Poisson distribution of mutations, $p_{(i)} = e^{-\lambda}. \lambda^i/i!$, wherein λ is the average number of amino acid mutations in a four-amino acid window of N randomly mutated Sak sequences. Since each *sak* gene in the library encodes on average 2.5 amino acid mutations (18), λ is calculated as follows: $2.5 \times (4/136) \times N$ (where N is 14, 16, and 26 for escape Sak variants selected against MEE, CLA, and BERDRI polyclonal anti-Sak Abs, respectively). To assign the statistical significance of a single amino acid position, the probability p was calculated using $(2.5/136) \times N$ for λ.

Specificity patterns of patient-specific antisera against Sak

The reactivity profiles of purified polyclonal anti-Sak IgGs from patients MEE, CLA, and BERDRI, and of five additional patients (TOR, DAN, MAN, STA, and GOD) toward phage-displayed Sak variants were assessed by phage-ELISA, as described above. Seven variants were chosen from the pool of 56 selected Sak variants against MEE (clones 500 and 502), CLA (clones 604 and 605), or BERDRI (clones 704, 715, and 723) Abs. Briefly, microtiter wells coated with either 20 ng/ml patient-specific anti-Sak IgG, as described above, murine anti-c-*myc* tag mAb 9E10 (10 μg/ml), or an equimolar mixture of 15 murine anti-Sak mAbs (10 μg/ml) (15) were incubated with twofold serial dilutions of each phage-displayed Sak variant (from 5×10^{10} down to 2.4×10^7 TU/ml). mAb 9E10 and the mixture of 15 anti-Sak mAbs were used as controls to compensate for differences in the display efficiency of Sak variants on filamentous phage. Each experiment was made in duplicate using phage-displayed wild-type Sak as internal control, and Ab recognition for each Sak variant toward each patient anti-Sak Abs was ranked between 1% (>100-fold reduction in binding affinity) and 100% (unaffected binding affinity as compared with wild-type Sak).

Results

Selection of escape variants of Sak to polyclonal human anti-Sak IgGs

A phage-displayed library of randomized Sak, wherein each Sak molecule on average contains 2.5 amino acid substitutions (18), was used to map the dominant antigenic sites of Sak in humans. Each selection cycle comprised 1) a negative selection for escape Sak-phage to immobilized polyclonal human anti-Sak IgGs, 2) a positive selection for proper display and folding of Sak by capture on immobilized mAb 32B2 (15), and 3) a phage amplification by *E. coli* infection.

Three separate selection experiments (each comprising three negative/positive selection rounds) were performed against polyclonal anti-Sak IgGs of four patients (defining group I patients: MEE, CLA, BER, and DRI). An equimolar mixture of the latter two IgG samples (hereby named BERDRI) was used for the third experiment. After the third selection round, screening of 380 selected clones by phage-ELISA revealed that 4.1, 4.4, and 8.6% of the selected Sak clones against MEE, CLA, and BERDRI Abs, respectively, were not recognized by human anti-Sak IgGs. As previously observed with mAbs (18), the lack of phage binding to the polyclonal anti-Sak IgGs was not associated with display of unfolded Sak mutants or no Sak at all, since all selected clones still bound mAb 32B2 in a control phage-ELISA assay.

Amino acid mutations in escape Sak variants identified by DNA sequencing

Fifty-six escape clones were further analyzed by DNA sequencing of their *sak* genes (14 clones selected with MEE, 16 clones selected with CLA, and 26 clones selected with BERDRI). In total, 213 amino acid substitutions were detected and all 56 *sak* genes were genetically distinct. The average number of amino acid changes per Sak molecule (3.8 substitutions) is slightly higher than that observed in the unselected Sak library (2.5 amino acid substitutions), suggesting that selection of escape mutants to polyclonal Abs is more stringent for the phage library than previously observed with mAbs (18). In line with this, escape Sak mutants to polyclonal anti-Sak Abs were only detected after the third selection cycle, whereas two selection rounds are largely sufficient to isolate escape Sak variants with mAbs (18).

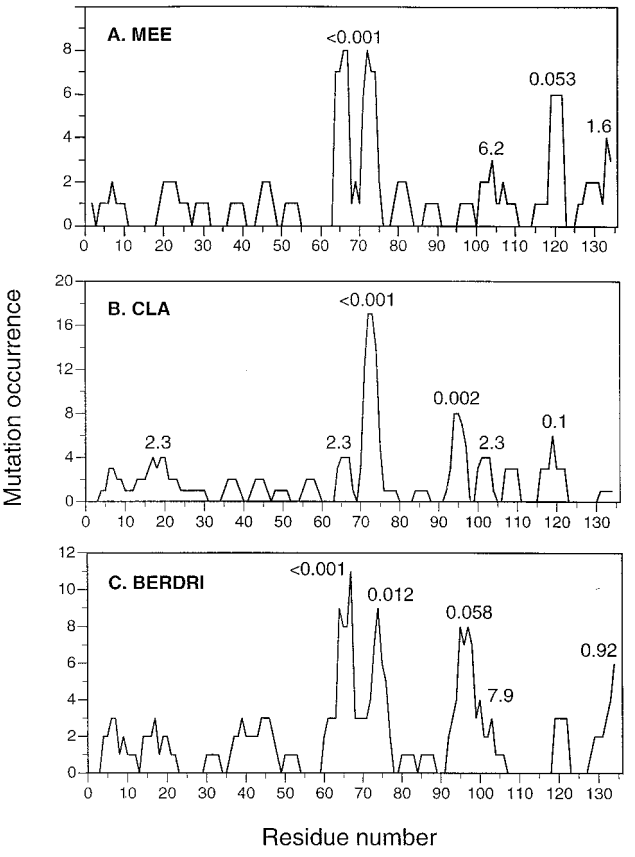

FIGURE 1. Smoothed antigenic profile of Sak in patients MEE (*A*), CLA (*B*), and BERDRI (*C*), as deduced from the amino acid sequences of negatively selected phage clones from a randomized Sak library. At each position (*i*) across the primary sequence, the mutation occurrence is calculated by adding the amino acid mutations from (i − 1) to (i + 2) in N selected sequences (where N is 14, 16, and 26 for the selection against MEE, CLA, and BERDRI, respectively). At peak values, the statistical significance is assessed by p, the probability whereby the number of selected amino acid substitutions in the four-amino-acid window could have arisen by chance.

Antigenicity profiles of Sak in patients using a four-amino-acid window

For each patient, the distribution and the frequencies of the amino acid substitutions were plotted across the primary sequence of Sak using a smoothing window of four amino acids length. The segments of highest mutation frequencies correspond to the major antigenic epitopes of Sak in a given patient.

The main epitopes of anti-Sak IgGs developed in patient MEE are targeted to amino acid positions 66, 73, and 121 in Sak (Fig. 1*A*). The statistical significance of these positions in targeting the humoral response in patient MEE can be assessed by calculating the probability (p) whereby the total number of observed mutations in a four-amino-acid window around these three positions could simply have arisen by chance. As shown in Figure 1*A*, the p values at positions 66, 73, and 121 are below 1%, and therefore highly significant with respect to their recognition by most anti-Sak Abs of patient MEE. Two other antigenic segments centered around positions 103 (p = 6.2%) and 135 (p = 1.6%) are less well

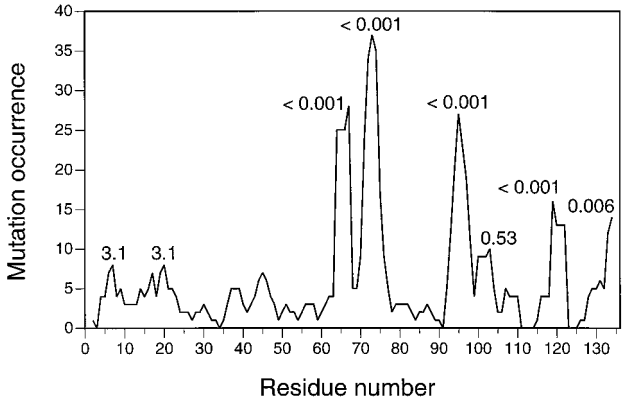

FIGURE 2. Smoothed antigenic profile of Sak in humans produced by adding all data from Figure 1 and using a four-amino-acid window across the primary sequence of Sak. The *p* values are calculated according to a total number of 56 selected Sak variants.

defined, and could therefore constitute minor antigenic determinants of Sak in this patient.

The antigenic profile of Sak in patient CLA (Fig. 1*B*) shows interesting features when compared with that of patient MEE. While the major antigenic segments centered on positions 73 and 121 are also targeted ($p < 1\%$) in patient CLA, amino acid position 66 in Sak is apparently less critical in patient CLA ($p = 2.3\%$) than in patient MEE ($p < 0.001\%$). Interestingly, a novel major antigenic determinant around position 95 ($p < 1\%$) of Sak is clearly identified in patient CLA. Finally, the minor antigenic epitope centered on position 102 ($p = 2.3\%$) of Sak is common to patients MEE and CLA, but the minor antigenic site on position 19 of Sak is patient CLA specific.

The combined antigenic profile of Sak in patients BER and DRI (Fig. 1*C*) reveals a similar trend for similarities and discrepancies as those observed with patients MEE and CLA. As previously observed, a major antigenic site is again detected around position 73 ($p < 1\%$), and can therefore be proposed as a dominant epitope of Sak in humans. A similar conclusion can be drawn for the minor antigenic site around position 103 of Sak (*p* values of 2.3, 6.2, and 7.9% with respect to MEE, CLA, and BERDRI Abs, respectively). In line with the antigenic profile of patient MEE, two major antigenic epitopes of Sak in patients BERDRI are detected at positions 66 and 135 ($p < 1\%$). Conversely, a common major antigenic epitope of Sak in patients CLA and BERDRI is identified around position 95 ($p < 1\%$).

Combined antigenicity profile of Sak using single- and four-residue windows

The mutation occurrences in all 56 Sak sequences were added to deduce a general antigenic profile of Sak in humans using a smoothing window of four amino acids length (Fig. 2). Altogether, Sak contains six major and two minor antigenic sites that are recognized to various extent by the purified anti-Sak IgGs from patients MEE, CLA, and BERDRI. All major antigenic sites (centered on positions 66, 73, 95, 103, 121, and 135, $p < 1\%$) are found within the second half of the Sak primary sequence. In contrast, the minor antigenic sites ($p = 3.1\%$) are located toward the NH_2-terminal end of Sak (positions 6 and 19). To further delineate the antigenic contribution of each Sak residue individually, the combined antigenic plot of Sak and the corresponding *p* values (up to a value of 6.2%) were recalculated for each amino acid residue using the data set of 56 escape Sak variants. As shown in Figure 3, a single amino acid residue (positions 66 and 121) defines two major antigenic segments in the primary sequence of Sak, while in two other antigenic segments, four succeeding residues are clearly

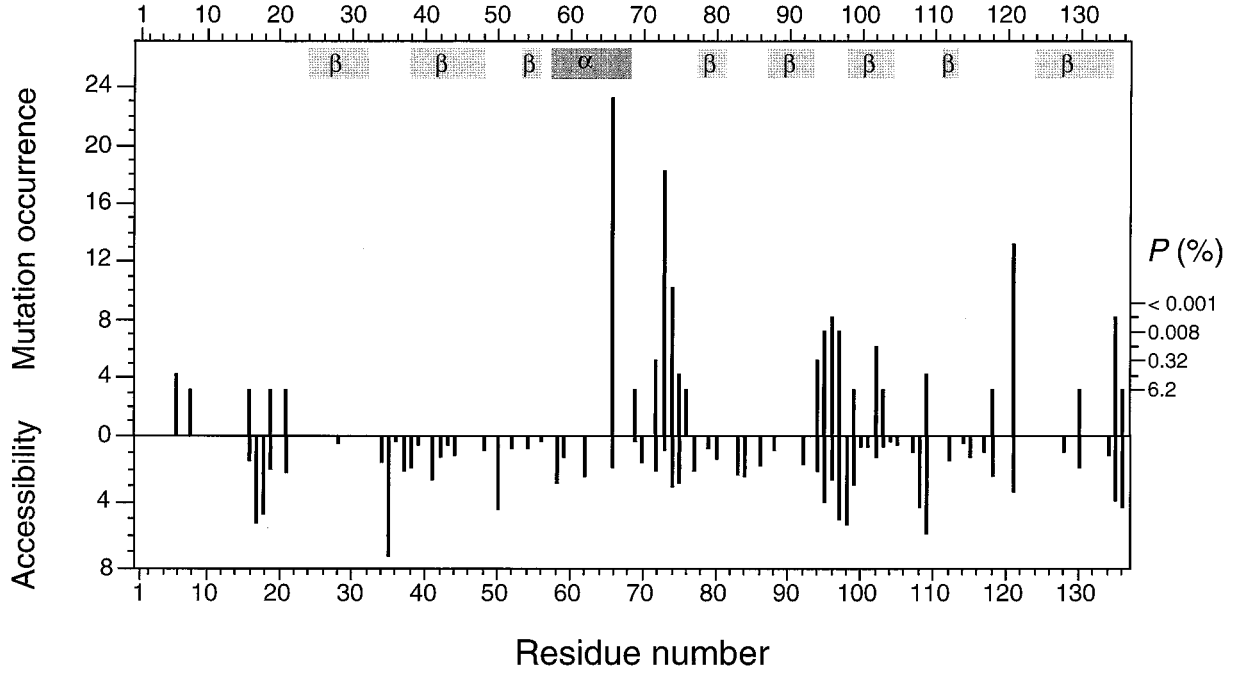

FIGURE 3. Single-residue antigenic profile (*upper part* of the figure) and predicted surface accessibility (*lower panel*) on Sak produced by summing all data from Figure 1. *Upper part*, At each position (*i*) across the primary sequence, the mutation occurrence was calculated by adding the amino acid mutations in 56 selected sequences. The probability (*p*) in percentage is calculated according to a total number of 56 selected Sak variants. Secondary structure elements (α-helix and β-sheets) are indicated according to the crystallographic coordinates of Sak (25). *Lower part*, Surface accessibility to a 9 Å radius probe (mimicking an Ab-combining site) (26) calculated at each position (*i*) across the primary sequence of Sak.

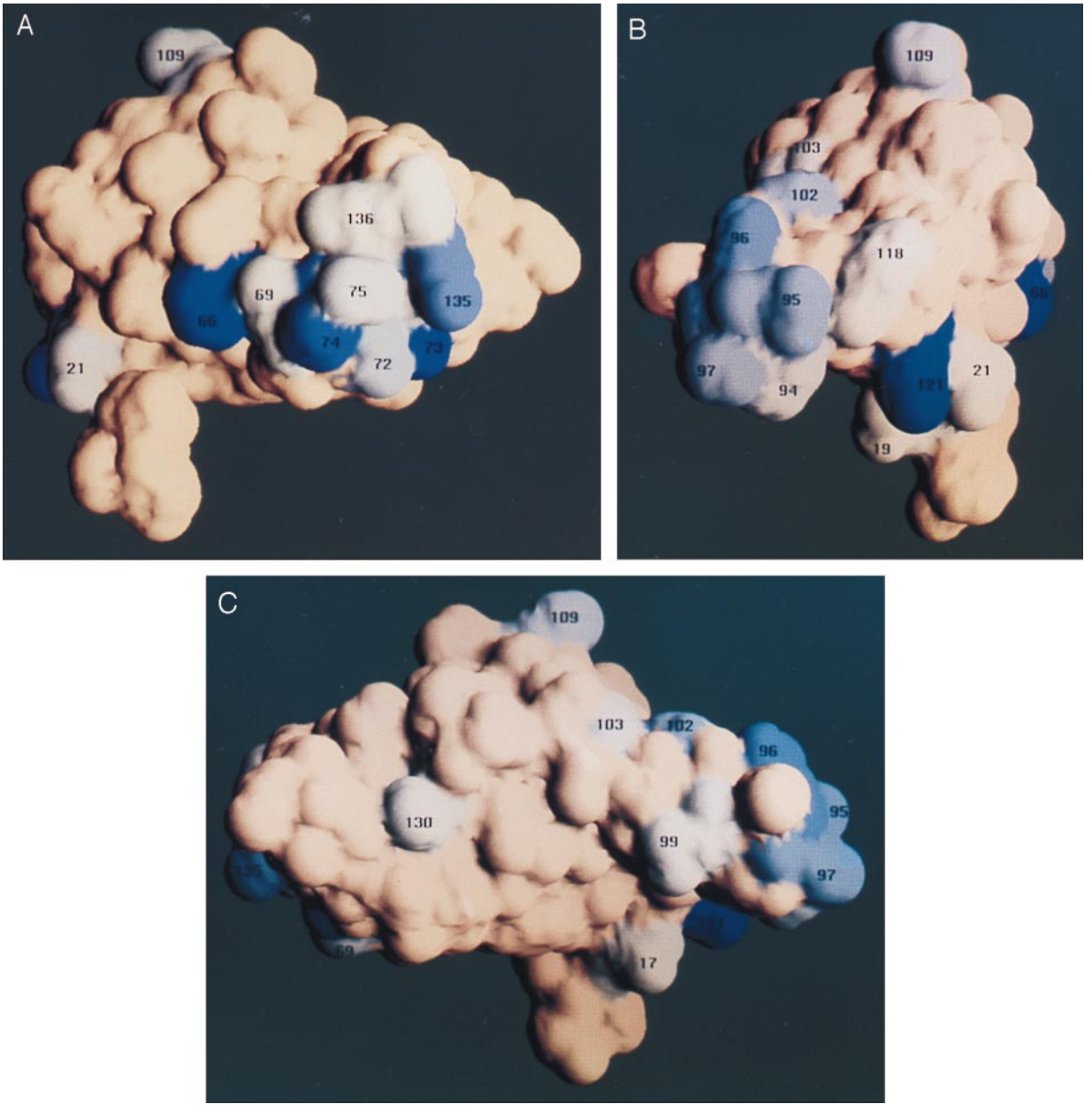

FIGURE 4. Spatial distribution of the antigenic residues on Sak according to the data of Figure 3. A surface-accessible area on Sak was calculated using a 1.4 Å radius probe and the crystallographic coordinates of Sak (25), and displayed using the software package GRASP (27). All 25 antigenic residues in humans are numbered and colored according to their antigenic importance (from white ($p = 6.2\%$) to dark blue ($p \leq 0.01\%$) surfaces). The three pictures (*A*, *B*, and *C*) represent successive views of Sak while rotating along the vertical axis.

critical (positions 72 to 76 and 94 to 97). Intermediate situations are observed elsewhere, depending on which threshold value is chosen for *p*. For example, the antigenic segment centered on position 135 of Sak comprises at least one residue (position 135, $p <$ 1%), but most probably two if position 136 is taken into account ($p = 6.2\%$). The same reasoning can be applied for three other antigenic segments (centered on positions 6, 19, and 102). In addition, by setting the threshold value of *p* to 6.2%, five additional residues located outside of the antigenic segments (positions 69, 99, 109, 118, and 130) can be identified in the single residue-based antigenic profile.

Spatial distribution of antigenic epitopes on the three-dimensional structure of Sak

Figure 4 shows the spatial distribution of all antigenic residues with *p* values $\leq$ 6.2% on the three-dimensional structure of Sak, according to the combined data of patients MEE, CLA, and BERDRI. The antigenic residues are found within each type of secondary structure elements, such as the α-helix (position 66), β-sheets (positions 102, 103, 135, and 136), or loops (residues 72 to 76 and 94 to 97) (Fig. 3). All 25 antigenic residues display a substantial solvent-exposed area (121 $\pm$ 40 Å^2, mean $\pm$ SD) that represents about 35% of the total water-accessible area. Except for a few residues (positions 16, 109, and 130), two major antigenic areas in Sak can roughly be deduced: the first major antigenic area (area I, 12% of the total water-accessible area) includes three discontinuous segments centered on positions 66, 73, and 135, the latter two contacting each other very closely, while the second major antigenic area (area II, 18% of the total water-accessible area) comprises four discontinuous segments centered on positions

Table I. *Amino acid substitutions of Sak variants selected for antigenicity fingerprints*

	Amino Acid Position																				
Sak Variant	G 8	A 12	H 43	E 46	F 47	W 66	D 69	A 72	Y 73	K 74	K 94	N 95	K 96	K 97	E 99	K 102	S 103	E 118	E 121	K 130	V 132
500	–	–	–	–	L	Q	–	–	–	R	–	–	–	–	–	–	–	–	E	–	Y
502	–	–	–	Q	–	–	–	–	–	E	–	–	–	–	–	–	T	–	I	–	–
604	G	A	–	–	–	–	–	G	–	N	–	–	–	–	–	–	–	K	–	–	–
605	S	–	–	–	–	–	–	S	C	E	–	–	–	–	–	Q	–	–	–	–	–
704	–	–	Y	–	–	R	–	–	–	–	–	–	R	–	–	–	–	–	–	–	–
715	–	–	–	–	–	Q	–	–	–	–	–	D	–	E	G	E	–	–	–	–	–
723	–	–	–	–	I	–	G	–	–	–	M	–	–	–	–	–	–	–	E	Q	–
	*[a]					*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

[a] Antigenic amino acid of Sak in humans ($p \leq 6.2\%$)

19, 95, 102, and 121. The humoral response in patient MEE is mainly directed against amino acid residues of area I, while a large fraction of the B cell specificities in patient CLA is targeted to area II. Except for the isolated positions 16, 109, and 130, the residues with a borderline *p* value (6.2%) nicely fit into the antigenic map of areas I and II (e.g., residues 69 and 76 in area I; residues 19, 21, 99, and 118 in area II), thereby confirming their modest but significant contribution to the antigenicity of Sak.

Comparisons with structure-based prediction of antigenicity on Sak

Qualitatively, all 25 antigenic residues deduced from our experiments correspond to predicted antigenic amino acids of Sak based on a search algorithm for Ab-accessible side chains (calculated using a 9 Å radius probe mimicking an Ab-combining site) (26) (Fig. 3). Since the predicted antigenic area of a given residue does not strictly correlate with its mutation occurrence (e.g., positions 66 and 73), other parameters such as segmental mobility (reviewed in Ref. 28) could also be involved in determining a priori the antigenicity of a residue. Alternatively, this discrepancy could be related to the present epitope mapping approach wherein mutations introduced at preferred amino acid positions in escape variants may abolish Ab recognition either by the disruption of an interaction (electrostatic or hydrophobic), the modulation of the local flexibility, or the introduction of repulsive effects (by increasing the size of a side chain or by reverting its polarity). Finally, it should be pointed out that, as observed with the total water-accessible area, only a fraction (47%) of the predicted antigenic area using a 9 Å radius probe has been identified as such by the phage display approach. The discrepancy between prediction and observation is particularly strong within the first half of the Sak primary sequence (Fig. 3).

Antigenicity fingerprints of Sak among nine patients using escape Sak variants

Finally, we sought to evaluate whether the depicted antigenic map of Sak in patients MEE, CLA, and BERDRI (group I patients) could be extrapolated to other patients that were not included in the negative selection study (group II patients: MAN, DAN, TOR, STA, and GOD). Seven Sak variants selected with MEE (clones 500 and 502)-, CLA (clones 604 and 605)-, and BERDRI (clones 704, 715, and 723)-specific Abs were chosen according to their composite patterns of amino acid substitutions within the six major antigenic segments of Sak (Table I). By phage-ELISA, each phage-displayed Sak variant was tested for its recognition by groups I and II polyclonal Abs. After correction for the display efficiency of each variant on phage and normalization with respect to wild-type Sak, the data were used to plot antigenicity fingerprints according to the recognition efficiency (in percentage) of each Sak variant by the patient-specific antisera (Fig. 5). In line with the negative selection results, the specificities of MEE, CLA, BER, and DRI Abs are not exactly superimposable, although some Sak variants, such as clones 500 and 605, are consistently poorly recognized. As shown in Figure 5, with the exception of patients TOR and DAN, the antigenicity fingerprints of group II patients can be matched with corresponding patterns in the group I patients (e.g., GOD with DRI, STA with CLA, . . .). It should be noted that the antigenicity fingerprints of patients TOR and DAN are quite similar to that of patient DRI, except for a lower recognition of Sak variant 502.

Discussion

In the present study, a strategy has been devised to map in humans the dominant antigenic sites of a therapeutically promising bacterial protein, Sak. Traditionally, mapping of dominant epitopes with

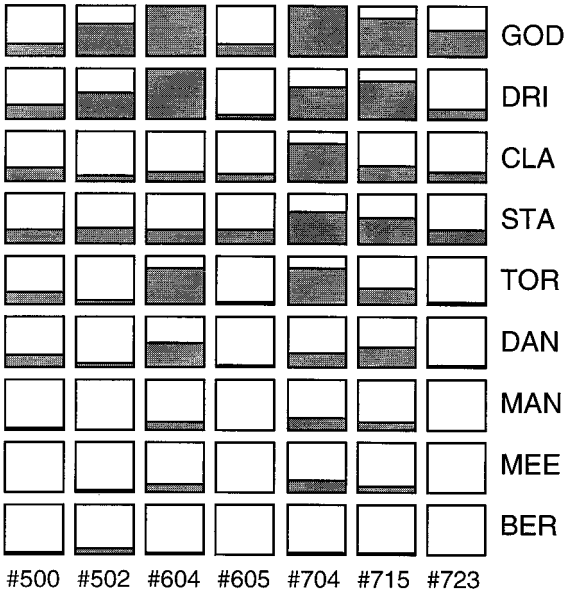

FIGURE 5. Antigenicity fingerprints of Sak in various patients. The ability of Sak variants selected against MEE (clones 500 and 502), CLA (clones 604 and 605), and BERDRI (clones 704, 715, and 723) to recognize nine patient-specific anti-Sak IgGs (GOD, DRI, CLA, STA, TOR, DAN, MAN, MEE, and BER) was determined by phage-ELISA and plotted according to their binding activity with respect to wild-type Sak. Gray squares represent full binding property, whereas white squares correspond to ≥100-fold reduced ELISA signals. The antigenicity fingerprints are grouped according to their similarities.

a polyclonal antiserum against a particular Ag is performed by ELISA or Western blots, using either truncated forms of the protein Ag or synthetic peptides overlapping the whole Ag sequence (reviewed in Ref. 29). Recently, phage display was also introduced as a tool to rapidly identify polyclonal serum specificities by positive selection from phage-displayed random peptide libraries (30, 31). However, since protein epitopes are essentially discontinuous and therefore highly dependent on the Ag conformation, it would be preferable to map these epitopes within the context of the native protein Ag. In a previous study, we have developed a phage display strategy, whereby discontinuous epitopes are rapidly and accurately delineated by negative selection of phage-displayed Ag variants against an immobilized mAb (18). In this study, we have further expanded the scope of the negative selection approach by using immobilized affinity-purified polyclonal IgGs obtained from four patients immunized against wild-type Sak following thrombolytic therapy.

Fifty-six genetically distinct escape Sak variants were recovered from a phage-displayed library of 7.5×10^8 randomized Sak variants using negative selection against polyclonal anti-Sak Abs from several patients. Most amino acid substitutions were shown to be clustered in eight discrete antigenic segments centered around positions 6, 19, 66, 73, 95, 102, 121, and 135, the latter six being assigned as major antigenic sites in Sak. Two types of antigenic segments were defined: those composed of several contiguous residues (e.g., positions 72–76 and 95–99) that are preferentially detected using a smoothed antigenic profile, and those almost exclusively associated with a single residue (e.g., positions 66 and 121) that are best characterized by a single-residue antigenic profile. In total, 25 amino acid positions were considered as critical for Sak recognition by human polyclonal anti-Sak IgGs. Of these, 68% are charged amino acid residues (11 Lys and 6 Glu), thereby confirming a previous mutagenesis study wherein a prominent role was attributed to charged side chains in mediating Ab-Ag complex formation (via hydrogen bonds and charge-charge interactions) (32). However, it should be noted that the negative selection approach (i.e., the search of escape mutants in a library of weakly mutated Sak variants) may overlook weaker operative forces such as van der Waals interactions.

The antigenic residues are not scattered on the Sak surface, but rather converge into two large antigenic areas encompassing several discontinuous sequences. These antigenic areas cover only 35% of the solvent-accessible surface of Sak. Furthermore, a prediction search on Sak for Ab-accessible amino acid residues (using a sphere of 9 Å radius probe) (26) indicates that only a fraction of the eligible amino acid residues (47%) have indeed been identified by the phage display approach. These results support the hypothesis that, although the whole surface of a protein Ag is potentially antigenic, the B cell response is preferentially directed toward a limited set of exposed regions (32), especially during a secondary immune response (33). In the present study, the polyclonal anti-Sak IgGs were purified from blood samples collected from strongly immunized patients (probably as a result of previous *S. aureus* infections). Indeed, the mean Sak-neutralizing activity in these four plasma samples was on average 5.3-fold higher than that previously reported for patients 2 wk or more after treatment with Sak (7).

The positive selection step using mAb 32B2 is critical for the isolation of properly folded escape Sak variants. Since the antigenic sites of Sak in humans may overlap with the mAb 32B2 epitope on Sak, the amino acid residues assembled in the latter epitope are not eligible for the negative selection step. A comprehensive alanine-scanning mutagenesis on Sak has revealed that only five amino acids at positions 91, 99, 101, 116, and 117 are critical for Sak recognition by mAb 32B2 (unpublished results, S. J. and L. J.). Moreover, these residues are confined to a single patch in close vicinity to the major antigenic area II. This indicates that most of the solvent-exposed residues in Sak can indeed be scanned for reactivity to human polyclonal anti-Sak IgGs. In theory, the use of an oligoclonal mix of nonoverlapping mAbs would be preferable than using a single one for the positive selection step.

This mapping study corroborates a previous study aiming at the identification of critical amino acid residues involved in the recognition of Sak by human polyclonal anti-Sak Abs (15, 16). In our analysis, the contribution of Lys^{74} to the antigenicity of Sak in humans is clearly confirmed. Since both studies were performed using different plasma samples, the same solvent-exposed motifs on Sak (areas I and II, as shown in Fig. 4) may therefore be targeted for immune reaction in the general population. Although this hypothesis should be confirmed by performing several new negative selection experiments, it is confirmed provisionally by the observation that the specificity patterns of five additional human anti-Sak antisera were very similar to the reactivity profiles of those used for the negative selection experiments.

High resolution mapping of the immunodominant antigenic epitopes of Sak in humans will help the present efforts to reduce Ab-mediated Sak neutralization in patients with thromboembolic disorders. A significant but incomplete reduction of the immunogenicity of Sak was previously reported by our group using site-directed mutagenesis of several residues within (Lys^{74}, Glu^{75}) or close (Lys^{35}, Glu^{38}, Arg^{77}, Glu^{80}, Asp^{82}) to the major antigenic area I (34). Further work aiming at the optimization of the ratio of antigenicity to sp. act. will depend on the extent to which the antigenic areas mapped in this study overlap with the binding site of plasmin on Sak. A preliminary structure-function study with recombinant Sak mutants revealed that three clusters of charged amino acids, Lys^{11}-Asp^{13}-Asp^{14}, Glu^{46}-Lys^{50}, and Glu^{65}-Asp^{69}, are critical to establish a functional Sak:plasmin activator complex (35). Except for the Glu^{65}-Asp^{69} cluster, which partially overlaps with the major antigenic area I, the other two appear to be antigenically silent in humans, thereby suggesting that reengineering of a large fraction of the antigenic residues identified in this study will be compatible with an intact profibrinolytic activity.

In summary, the scope of the negative selection by phage display has been extended to the detailed epitope mapping of polyclonal Ab response against Sak in humans. Provided that a given Ag can be functionally displayed on phage, this approach may surpass mapping techniques based on protein truncation (either enzymatically or genetically) and linear peptide scanning, which often overlook the conformational elements of antigenic epitopes. Even epitopes on a large protein such as streptokinase (44 kDa) have been mapped successfully with this negative selection strategy (unpublished results, S. J. and L. J.). Beyond the Sak study, this strategy may also have practical benefits in the context of vaccine development.

Acknowledgments

We are grateful to Dr. I. Lasters for helpful discussion and Dr. M. De Maeyer for assistance in computer modeling.

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