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Identification and Characterization of Epitopes on the 120-Kilodalton Surface Protein Antigen of *Rickettsia prowazekii* with Synthetic Peptides

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The 120-kDa surface protein antigens (SPAs) of typhus rickettsiae are highly immunogenic and have been shown to be responsible for the species-specific serological reactions of the typhus group rickettsiae. To study the immunochemistry of these proteins, overlapping decapeptides encompassing the whole protein were synthesized on derivatized polyethylene pins. A modified enzyme-linked immunosorbent assay was used to identify epitopes recognized by rabbit hyperimmune antisera to *Rickettsia prowazekii* SPA. Eight distinct epitopes were mapped by this method in three regions. Four of the epitopes, which were located in the carboxy terminus of mature processed SPA, were strongly competitively inhibited by native folded SPA but not by intact rickettsiae, suggesting that they were on the SPA surface but not exposed on the rickettsial surface. Three of these epitopes were present on both *R. prowazekii* and *Rickettsia typhi* SPAs. The immunoreactivities of five epitopes were further characterized by synthesizing modified peptides. Glycine substitution experiments determined the critical residues involved in antibody recognition, while peptide truncation experiments defined the minimum number of residues in the epitopes. The dependence of binding of the peptide epitopes to the polyclonal antisera was mapped to single residues. The limited number and weak reactivity of linear peptide epitopes observed with human and rabbit sera, possibly due to a lack of the methylated amino acids which are present in rickettsia-derived SPA, suggest that the present approach will not provide useful synthetic antigens for diagnosis of typhus infections.

Rickettsiae are gram-negative, obligately intracellular bacteria which are responsible for a large number of arthropod-transmitted diseases in humans. *Rickettsia prowazekii* is the etiologic agent of epidemic typhus transmitted by the human body louse in areas of Africa, South America, the Middle East, and sub-Saharan Africa where the disease is endemic and by fleas of the southern flying squirrel in North America (23). *R. prowazekii* has a monomolecular layer of protein arranged in a periodic tetragonal array on its surface (32). This crystalline layer, representing 10 to 15% of the total protein mass of the rickettsiae, was identified as the immunodominant species-specific surface protein antigen (SPA). It has been isolated, purified, and biochemically characterized (8, 10, 19, 22). The earliest and dominant immunological antiprotein responses of mice, guinea pigs, rabbits, and humans following infection with *R. prowazekii* and *Rickettsia typhi* are directed against the SPAs (3, 4, 20, 24). We have shown that purified native typhus SPAs induce strong humoral and cell-mediated immune responses. Protective immunity was elicited by typhus SPAs in guinea pig and mouse protection models (3, 20, 21). S-layer surface proteins have also been shown to be the major virulence determinants in other gram-negative organisms, such as *Aeromonas salmonicida* and *Campylobacter fetus* (2, 31). These observations suggest that SPA is an appropriate target for developing diagnostic reagents and subunit vaccines.

We have studied the immunochemistry of the typhus SPAs very extensively. Both humoral and cellular responses to protein fragments and synthetic peptides of *R. prowazekii* and *R. typhi* have been investigated (9, 17, 18), and monoclonal antibody binding sites on CNBr fragments of the SPA S-layer proteins have been mapped by Western blot (immunoblot) analyses (8). Here, mapping with synthetic peptides (PEPSCAN), using overlapping decapeptides encompassing the whole open reading frame of the *spaP* (*rompB*) gene, was used to more definitively identify the major linear antigenic regions (5, 27). These epitopes were further evaluated for their topological locations on the purified native SPAs as well as on the tetragonal array layer located on the surfaces of intact rickettsiae. The critical amino acid residues involved in the epitope-antibody interaction, the minimum peptide length required for antibody recognition, and the importance of the chirality of the peptides were also determined (7, 15, 35, 36).

MATERIALS AND METHODS

Synthesis of overlapping peptides. The synthesis of overlapping decapeptides was carried out as follows. Decamers were synthesized on derivatized polyethylene pins, arranged in a 96-well microtiter plate format, by using the solid-phase pin technology developed by Geysen et al. (14, 25, 26). Each sequential peptide overlapped with six amino acids of the preceding peptide. The number designating a decamer is the sequence position of the first amino acid of that peptide. A total of 403 peptides were synthesized in order to span the whole open reading frame of *spaP* from amino acid 1 to amino acid 1612 (excluding the signal pepitde) (5, 30). These peptides were tested for the presence of epitopes in a modified enzyme-linked immunosorbent assay (ELISA) (see below). The quality of the synthesis was assessed by synthesizing control pins with the sequences PLAQ and GLAQ, which were subsequently hydrolyzed for amino acid analysis after each step of coupling. Duplicates of the PLAQ control pin, which is reactive with a specific monoclonal antibody supplied by the manufacturer (Cambridge Research Biochemicals Wilmington, Del.), and GLAQ, which is nonreactive with the specific antibody, were tested.

Detection of antibody interactions with synthetic peptides. A modified ELISA was performed as described previously (26). Peptides attached to the polyethylene pin were precoated with 0.5% (wt/vol) casein and 0.5% (wt/vol) bovine serum albumin in 0.1 M phosphate-buffered saline (PBS), pH 7.2, for one h to prevent nonspecific binding of antibody. The pin-attached peptides were incubated with 100 μl of antibody solution overnight at 4°C. The next day the pins were washed four times for 10 min each in PBS containing 0.05% Triton X-100.

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The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy- and light-chain reactive) (Bio-Rad Laboratories, Richmond, Calif.). The A_{405} (against an air blank) was measured with a Molecular Devices plate reader. Pins with attached peptides were reused after the bound antibodies were removed by sonication for 1 h at 55°C in 0.1 M sodium phosphate, pH 7.2, containing 1% sodium dodecyl sulfate (SDS) and 0.1% β-mercaptoethanol. Sonication was followed by two washes of 2 min each in distilled water heated to 60°C and two washes of 2 min each in gently boiling methanol. The pin blocks were then air dried for 1 h before use. Amino acid analyses of the washed pins confirmed that only the amino acids of the synthesized peptide were present. In the competition experiments, native SPA proteins, intact rickettsial whole cells, and French pressure cell-disrupted rickettsiae were added to the rabbit antibody solution during the overnight incubation. All experiments were repeated twice.

Characterization of epitopes by synthesis of modified peptides. After the reactive peptides were identified, a series of modified peptides were synthesized. For each reactive peptide, glycine was substituted individually for each amino acid one at a time along the reactive peptide sequence, and glycine in the original sequence was replaced by alanine. Shorter peptides, ranging from pentamer to nanomer, were also synthesized by truncation of the decapeptide from the N and C termini. To test the requirement for amino acid chirality for antibody binding, peptides were also synthesized in the reverse direction or with all D-amino acids.

Preparation of rabbit antisera. Experiments were conducted according to the principles set forth in reference 31a. New Zealand White rabbits were immunized (half intramuscularly and half subcutaneously) four times at 2- to 3-week intervals with 10 μg of SDS-polyacrylamide gel electrophoresis (SDS-PAGE)-purified denatured SPA from *R. prowazekii* emulsified with Freund's incomplete adjuvant, boosted two times with 2 mg of adjuvantized French pressure cell-disrupted whole cell lysate of purified rickettsiae, and bled 2 weeks later (rabbit no. 51). Alternately, a rabbit (no. 64) was immunized four times monthly with 1.0 mg of an adjuvantized French pressure cell extract of purified rickettsiae whose soluble SPA had been removed by hypotonic shock with H_2O and centrifugation. The rabbit was then boosted biweekly with 1.0 mg of the extract without adjuvant and bled 3 weeks later. Pre- and postimmunization sera were diluted 1:500 for use in ELISAs.

Purification of rickettsiae and preparation of extracts. *R. prowazekii* Breinl, *R. typhi* Wilmington, and *Rickettsia canada* McKiel were grown in the yolk sacs of embryonated chicken eggs and purified from the host cell contaminants by differential centrifugation and isopycnic banding in Renografin density gradients. Whole-cell preparations were preserved in K36 containing 0.04% formaldehyde. Total rickettsial extracts were prepared by French pressure cell disruption at 22,000 lb/in^2 of cells suspended in H_2O, centrifugation at 17,400 × *g* for 15 min to remove intact cells, and addition of formaldehyde to 0.08% (22). A panel of monoclonal antibodies with different reactivities toward intact cells and disrupted cells was used to characterize the antigenicity and integrity of each whole-cell preparation (8, 33).

Preparation of native SPA protein. SPA was obtained from Renografin density gradient-purified rickettsiae by two or three successive extractions with distilled water at 4°C (19). The pooled extracts were ultracentrifuged at 200,000 × *g* for 2 h, the supernatants were sterilized by passage through a 0.45-μm-pore-size membrane filter, and the filtrate was stored in aliquots at 4 or −60°C. The SPA in the supernatant was then further purified by ion-exchange high-pressure liquid chromatography (10).

RESULTS

Pin mapping of epitopes. The close spacing of peptides on the surface of polyethylene pins allows for multivalent interactions with antibodies and probably results in a high sensitivity of antibody detection. Rabbit polyclonal antibodies (from rabbit 51) were tested against a set of peptides encompassing the complete open reading frame of the *R. prowazekii* SPA gene (excluding the signal peptide) (Fig. 1). Comparisons of the reactivities of each of the peptides with pre- and postimmunization sera were made. Those peptides which exhibited a stronger interaction with immune serum than with preimmune serum (a minimum increase of 0.5 optical density unit at 405 nm) were considered potential epitopes. Table 1 lists the sequences of eight strongly reactive *R. prowazekii* SPA peptide domains which exhibited increases of more than 1 absorbance unit. Seven of these domains were identified with rabbit 51 antiserum, and one (epitope VIII) was identified with rabbit 64 antiserum. In three cases (epitopes I, IV, and V) in which two successive reactive peptides were identified, the beginning of the first peptide to the end of the second peptide was designated the epitope. Clusters of reactive peptides were found in two regions: one cluster contained three epitopes (II, III, and IV; amino acids 647 to 784), and the other contained another four epitopes (V, VI, VII, and VIII; amino acids 1239 to 1300). While the highly reactive epitope I was in the amino terminus at amino acids 45 to 58, no dominant epitopes were found in the C-terminal region previously found to be absent in the truncated form of SPA extracted from the rickettsial surface (8).

Determination of critical epitope residues. The interaction of rabbit antibody with five of the identified epitopes (I and V to VIII) was further characterized. Antibody binding to peptides in which glycine was substituted individually at each position was determined (Fig. 2). For epitope I, peptide 45, the identity of residues 45 to 48 was not critical, while alteration of residues 49 to 54 reduced the binding activity to less than half (Fig. 2A). The replacement of T-48 by glycine enhanced reactivity with antibodies. Peptide truncation experiments which indicated that the binding affinity of the hexapeptide from position 49 to 54 was about the same as that of the parent decapeptide from position 45 to 54 (Table 2) confirmed the conclusions obtained in the glycine substitution experiments and the observation that the adjacent decapeptide from position 49 to 58, which contained the hexapeptide from position 49 to 54, exhibited a reactivity similar to that of the decapeptide from position 45 to 54 (Fig. 1).

For epitope V, peptide 1243, three amino acids, L-1243, T-1246, and N-1247, were critical for antibody recognition (Fig. 2B). Replacement of these residues by glycine reduced the binding activity to 20% or less, while replacement of the other seven residues did not substantially reduce the binding activity. Truncation experiments (Table 2) showed that the binding activity of the pentapeptide from position 1243 to 1247 was about the same as that of the parent decapeptide and the preceding overlapping peptide 1239 (Fig. 1), which contained residues 1243 to 1247.

For epitope VI, peptide 1259 (Fig. 2C), the most critical residues involved in antibody binding were T-1265 and V-1267. Replacement of residues D-1259 and P-1260 had no effect on the antibody recognition, while replacement of the rest of the six residues only slightly decreased the binding affinity. Truncation experiments (Table 2) showed that the heptapeptide from position 1261 to 1267 had the same reactivity as the parent decapeptide from position 1259 to 1268, while the pentapeptide from position 1263 to 1267 had very little binding activity (<20%). Only two residues, S-1261 and D-1262, differ between the heptapeptide and the pentapeptide. While replacement of S-1261 and D-1262 by glycine decreased the activity only 20 to 40%, the exclusion of these two residues led to nearly complete loss of binding activity. The preceding and the successive adjacent decapeptides, which did not contain all of the required residues, 1261 to 1267, had no reactivity with antibodies (Fig. 1).

Within epitope VII, the critical residues involved in the recognition of the peptide from position 1287 to 1296 were T-1289, Q-1290, L-1292, and L-1293 (Fig. 2D). Replacement of S-1294, N-1295, and R-1296 had no significant effect on the binding activity, and replacement of K-1288 and D-1291 led to a slight decrease in binding activity. Replacement of Q-1287 yielded greater reactivity. Truncation experiments indicated that the presence of flanking residues on both sides of the pentapeptide from position 1289 to 1293 contributed significant binding strength (Table 2).

The entire set of peptides was tested for reactivity with serum from rabbit 64, which was immunized with the residual membrane-bound form of SPA. Only epitope VIII was recognized, and the absorbance signal was much weaker (<1 optical

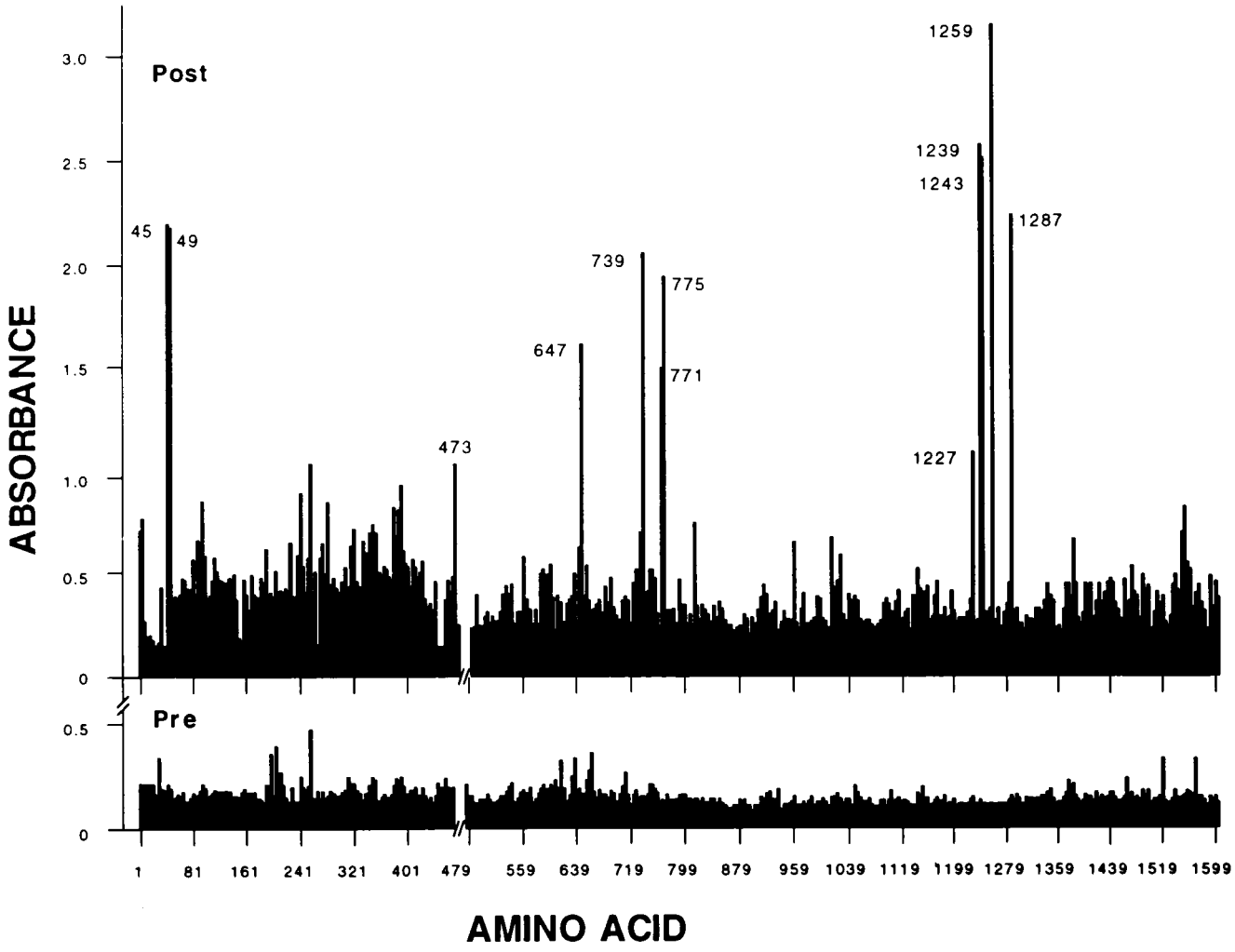

FIG. 1. ELISA profile of anti-SPA rabbit 51 pre- and postimmunization sera with overlapping synthetic decapeptides deduced from the sequence of the *spaP* open reading frame of *R. prowazekii* (excluding the signal peptide). The amino acid residue number indicated is the first amino acid of each decapeptide. Each successive decapeptide begins four residues after the preceding peptide. A total of 403 peptides were synthesized in two batches; the first batch covered amino acids 1 to 486, and the second batch covered amino acids 479 to 1612. The ELISA A_{405} values (against an air blank) for the reactions of each serum are shown.

density unit) than those of the dominant epitopes recognized by rabbit 51 (2 to 4 optical density units). Glycine substitution experiments indicated that N-1295 and L-1297 were the most important residues for recognition (Fig. 2E). Both glycine substitution and truncation experiments suggested that L-1300 might not be involved in antibody binding. The identities of L-1292, L-1293, and T-1299 were not critical, but the presence of amino acid residues at positions 1292 and 1299 was required for maximum binding. Elimination of either one reduced the binding activity to about half of that of the parent peptide (Table 2).

Chirality of epitopes. The chirality of the antibody-peptide binding was tested with peptides containing all D-amino acids or with peptides synthesized in the reverse direction (Table 2). In all cases, the binding activities for the modified peptides were almost completely lost, indicating the requirement of correct topological orientation in the antibody-epitope recognition.

Topological analysis of linear epitopes. To determine if these peptide epitopes are located on the surface of naturally folded soluble SPA and on the rickettsial cell surface, competition experiments with purified soluble native typhus SPAs, whole cells, and disrupted cell extracts were conducted (Table 1). The interaction of rabbit 64 antibody with peptides in epitope VIII could be completely inhibited by including 2 μg of *R. prowazekii* SPA in the incubation mixture of antibody with the peptides on the pins. *R. typhi* SPA was only slightly less efficient despite two changes in the amino acid sequence. This was expected, since glycine substitution for serine or threonine (Fig. 2E) and truncation of the peptide at threonine (Table 2) reduced antibody binding only 30 to 50%. Epitopes V and VI also were strongly inhibited by native *R. prowazekii* SPA, as demonstrated by a decrease in the absorbance signal of more than 70%. Since *R. typhi* SPA did not compete for binding to epitope V (Table 1) and the serine and arginine residues were not essential for antibody binding (Table 2), the replacement of asparigine by aspartic acid in *R. typhi* SPA appears to be responsible for the loss of antibody binding. Glycine substitution experiments also supported the crucial importance of this residue in epitope V (Fig. 2B). For epitope VI, glycine substitution suggested that proline was not essential but that the penultimate valine was important in antibody binding (Fig.

TABLE 1. *R. prowazekii* SPA Peptides strongly recognized by rabbit hyperimmune sera and competition of their binding with native antigens

Epitope[a]		Inhibition[b] by competing antigen from:					
		R. prowazekii			*R. typhi*		
No.	Peptide sequence (positions)	SPA[c]	WC[d]	FPC[e]	SPA	WC	FPC
I	NPITFNTPNGHLNS (45–58) *A********N***	—[f]	—	—	—	—	—
II	VAADPLNTNT (647–656) *****I**D*	—	ND	ND	ND	ND	ND
III	NGTTVKFLGD (739–748) **********	1+	ND	ND	ND	ND	ND
IV	TDHVESADNTGTLE (771–784) ***I**********	1+[f]	ND	ND	ND	ND	ND
V	ISRCLESTNTAAYN (1239–1252) *AI*****D*****	3+[f]	—	2+	—	—	—
VI	DPSDVATFVG (1259–1268) *S******I*	4+	—	3+	2+	—	1+
VII	QKTQDLLSNR (1287–1296) *******G**	2+	—	1+	1+	—	1+
VIII	DLLSNRLGTL (1291–1300) ***G****A*	4+	—	4+	3+	—	2+

[a] Two overlapping immunoreactive decapeptides were combined as one epitope for peptides I, IV, and V. In the lower sequences, asterisks indicate homologous residues in *R. typhi* SPA (30) that are identical to those in the *R. prowazekii* SPA peptide in the corresponding upper sequences.

[b] 4+, more than 90% inhibition of the interaction of peptides and rabbit serum; 3+, 70 to 90% inhibition; 2+, 40 to 70% inhibition; 1+, 10 to 40% inhibition; —, no inhibition; ND, not done.

[c] Two micrograms of native SPA was included in the incubation mixture of peptides with the rabbit serum.

[d] Thirty micrograms of *R. prowazekii* or 50 μg of *R. typhi* purified intact cells (WC) was included in the incubation mixture.

[e] Thirty micrograms of *R. prowazekii* or 50 μg of *R. typhi* French pressure cell-disrupted cell extracts (FPC) was included in the incubation mixture.

[f] Similar inhibition results were obtained with either one of the two adjacent peptides.

2C). Consequently, the isoleucine substitution rather than that of serine was probably responsible for the lowered *R. typhi* SPA binding of antibody. Antibody binding was decreased 40 to 70% for epitope VII and only 10 to 40% for epitopes III and IV in the presence of *R. prowazekii* SPA. Surprisingly, *R. typhi* SPA was less efficient in competition with antibody binding to epitope VII even though glycine substitution (Fig. 2D) suggested that this change would not affect the epitope. However, truncation experiments had suggested that this residue contributed significantly to antibody binding (Table 2). The interaction with epitopes I and II was not inhibited at all by the native protein under these conditions. Similar inhibition results were obtained with heat-denatured *R. prowazekii* SPA, indicating that these epitopes are not affected by the folding of the protein (data not shown). Consequently, epitope I and the cluster of epitopes II, III, and IV appear not to be present on the SPA surface or accessible to antibody, while epitope cluster V, VI, VII, and VIII is present on the surfaces of both *R. prowazekii* and *R. typhi* SPAs.

Since the monomeric proteins constituting most S-layers interact with each other to form the morphological unit evident in the two-dimensional lattice, we also investigated the surface exposure of these epitopes in the SPA crystalline layer. The interaction of rabbit 51 antibody with epitopes V, VI, and VII and of rabbit 64 antibody with epitope VIII can be competitively inhibited by *R. prowazekii* rickettsial cell extracts but not by intact cells (Table 1). The extracts contain soluble SPA as well as all of the other antigens present in purified rickettsiae, while the SPA layer is intact in the whole-cell preparations. The presence of formaldehyde in the extracts and whole cells was apparently unimportant, as the extracts acted like purified SPA which had not been exposed to formaldehyde. Further, as would be expected, competition with *R. typhi* cell extracts was comparable to that obtained with purified SPA, while *R. typhi* whole cells were again noncompetitive (Table 1). Neither extracts nor whole cells of either species competed with antibody binding to epitope I. As a specificity control, *R. canada* cell extracts exhibited no competition for binding to epitope I, V, VI, VII, or VIII (not shown).

DISCUSSION

One of the limitations in the early application of the PEPSCAN pin technology was the nonuniformly grafted level of derivatized acrylic acid used as the linker for peptide synthesis. Results from this approach thus were usually considered to be qualitative rather than quantitative (6). However, with the improved grafting technique, at least semiquantitative or even quantitative data from the same lot were possible. We synthesized different sets of peptides from different lots of derivatized pins, and similar patterns of results were observed. It is very unlikely that the changes in signal in our epitope characterization experiments were due to different levels of derivatization which led to different amounts of peptide being synthesized. The consistent results obtained from glycine substitution experiments, truncation experiments, and determinations of the reactivities of adjacent overlapping peptides further indicate that the experimental data presented here were meaningful. The independent results obtained from antigen competition experiments using a homologous protein with known amino acid substitutions supported the conclusions obtained in the peptide synthesis experiments.

Genes encoding proteins with molecular masses of approximately 120 kDa from both *R. typhi* and *R. prowazekii* have been cloned and sequenced (5, 30). Homologous SPA proteins have also been identified in *Rickettsia rickettsii*, other spotted fever group rickettsiae, and *R. canada* (10, 20, 27, 28). In this study we used synthetic overlapping peptides to define some linear B-cell epitopes of SPA recognized by rabbit antibodies. SPA has been shown to be modified by multiple methylation at half of its lysine residues (13). Methylation of asparagine was also speculated to occur (8). Pin mapping would not detect the

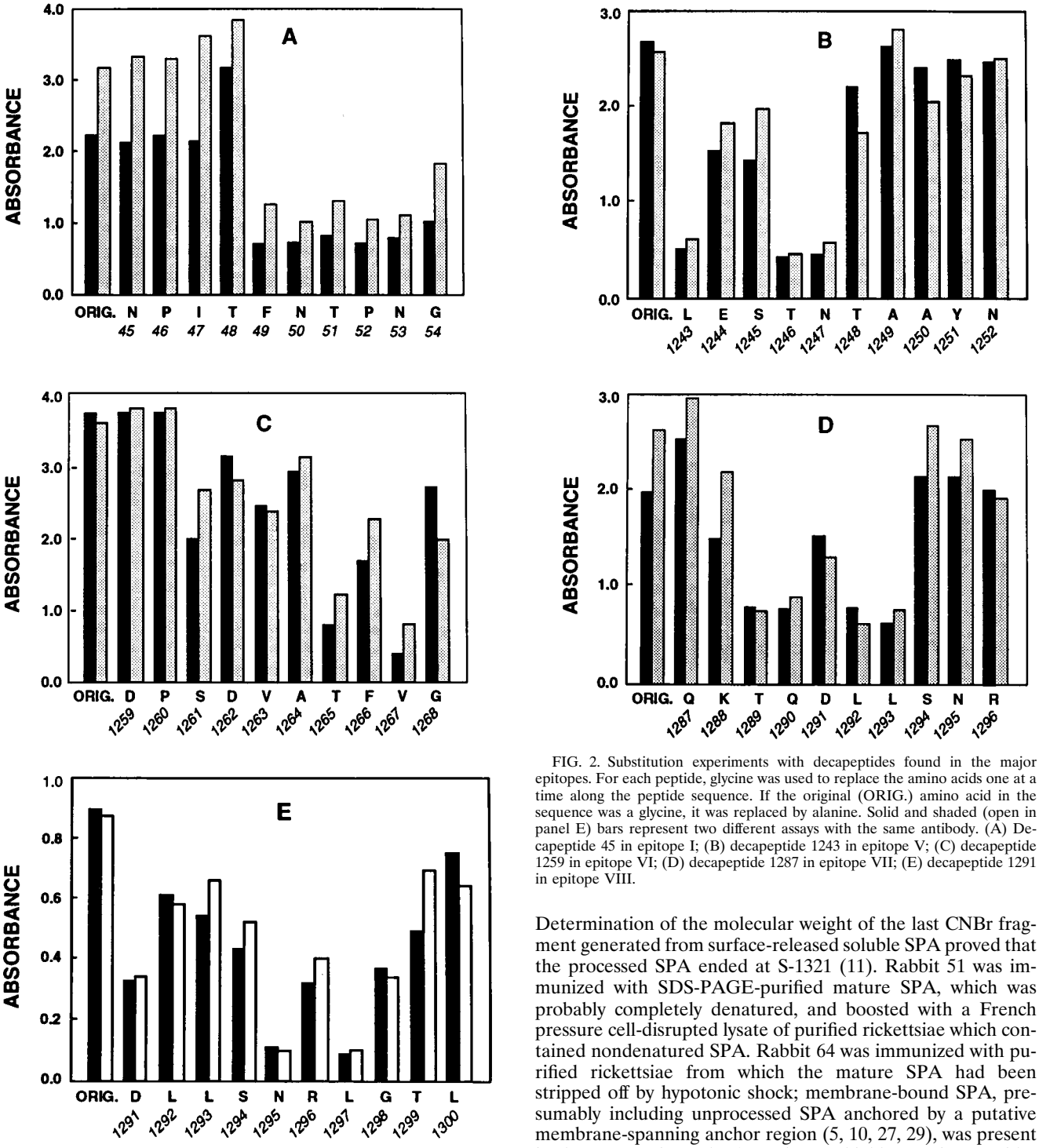

FIG. 2. Substitution experiments with decapeptides found in the major epitopes. For each peptide, glycine was used to replace the amino acids one at a time along the peptide sequence. If the original (ORIG.) amino acid in the sequence was a glycine, it was replaced by alanine. Solid and shaded (open in panel E) bars represent two different assays with the same antibody. (A) Decapeptide 45 in epitope I; (B) decapeptide 1243 in epitope V; (C) decapeptide 1259 in epitope VI; (D) decapeptide 1287 in epitope VII; (E) decapeptide 1291 in epitope VIII.

rabbit humoral response to epitopes which contain these modified residues if they are essential for binding. Consequently, the epitopes identified here may represent only those antigenic regions which lack essential posttranslational modification. However, lysine and asparagine were present in two and seven, respectively, of the eight epitopes we detected (Table 1).

Extensive evidence that the encoded SPA protein is processed after translation has been obtained. An approximately 30-kDa peptide is cleaved from the C terminus (8, 10, 27, 29). Determination of the molecular weight of the last CNBr fragment generated from surface-released soluble SPA proved that the processed SPA ended at S-1321 (11). Rabbit 51 was immunized with SDS-PAGE-purified mature SPA, which was probably completely denatured, and boosted with a French pressure cell-disrupted lysate of purified rickettsiae which contained nondenatured SPA. Rabbit 64 was immunized with purified rickettsiae from which the mature SPA had been stripped off by hypotonic shock; membrane-bound SPA, presumably including unprocessed SPA anchored by a putative membrane-spanning anchor region (5, 10, 27, 29), was present in the preparation. The facts that no highly reactive peptides were found in the C terminus beyond residue 1300 (β-protein) for rabbit 51 antibody and only one epitope near residue 1300 was recognized by rabbit 64 antibody are consistent with either a lack of a significant amount of the β-protein region in the immunizing antigens or its low immunogenicity (Fig. 1). Partial digestion of soluble native SPAs with chymotrypsin or trypsin indicated that the region downstream from Y-1208 is highly susceptible to proteases (12). As soon as soluble SPA was exposed to these two proteases, the molecular weight of SPA decreased and disulfide-linked SPA polymers were lost. Se-

TABLE 2. Further characterization of epitopes by modified peptides

Epitope (positions) and sequence	% of A_{405} for parent peptide[a]
I (45–54)	
N P I T F N T P N G	100
T F N T P N G	84 ± 10
F N T P N G	102 ± 6
G N P T N F T[b]	30 ± 6
t f n t p n g[c]	24 ± 10
V (1243–1252)	
L E S T N T A A Y N	100
R C L E S T N	118 ± 9
C L E S T N T	104 ± 15
L E S T N T A	109 ± 18
R G L E S T N	113 ± 5
L E S T N	108 ± 6
A T N T S E L[b]	31 ± 7
l e s t n t a[c]	17 ± 2
VI (1259–1268)	
D P S D V A T F V G	100
S D V A T F V	100 ± 5
V A T F V	17 ± 8
V F T A V D S[b]	20 ± 11
s d v a t f v[c]	11 ± 4
VII (1287–1296)	
Q K T Q D L L S N R	100
K T Q D L L S	80 ± 2
T Q D L L	54 ± 8
S L L Q D T K[b]	17 ± 10
k t q d l l s[c]	17 ± 4
VIII (1291–1300)	
D L L S N R L G T L	100
D L L S N R L G T	118 ± 7
D L L S N R L G	51 ± 3
L L S N R L G T	83 ± 4
L S N R L G T	46 ± 24

[a] Percentages of absorbances detected with the parent peptides were calculated in order to normalize different assays obtained with the same antibody but performed on different days and on different sets of peptides. The A_{405}s for parent peptides of all epitopes except epitope VIII were above 2 optical density units (against an air blank). The signal of epitope VIII was less than 1.0 optical density unit (Fig. 2).

[b] Reversed sequence.

[c] D-Amino acids.

quence analysis of the partially digested SPA proved that the decrease in size was due to the degradation at the C terminus. The rapidity of the cleavage of SPAs at this region suggested that these areas are not tightly folded. The clustering of the linear epitopes that we identified near the C terminus and the similar extents of competition with native soluble *R. typhi* and *R. prowazekii* SPAs and denatured *R. prowazekii* SPA also suggest that this region was not buried inside the SPA but rather might be open or accessible protein domains. Whole cells of *R. prowazekii* and *R. typhi* could not compete with the rabbit antibody for binding to peptide epitope V, VI, VII, or VIII, while disrupted cell extracts and SPAs were competitive (except epitope V for *R. typhi* SPA). These results suggest that these epitopes are not accessible to antibody when SPA is present only in the regularly arranged S-layer on the cell surface. Two other observations are consistent with this conclusion. SPA is not digested by trypsin or chymotrypsin when present on intact rickettsiae. Cysteines responsible for linking disulfide-linked polymeric SPAs are also located in the carboxy region that is accessible to proteases (12). Apparently, SPA monomeric associations that result in the S-layer depend on domains in the carboxy-terminal region of the processed protein that are immunogenic and antigenic but not accessible to antibody in intact rickettsiae. The results also suggest that *R. typhi* and *R. prowazekii* SPAs associate similarly in forming their respective morphological units. If methylation is a host cell-dependent process, it is possible that it does not occur in this region.

In contrast to the cluster of epitopes V to VIII, epitope I and the cluster of epitopes II, III, and IV appear not to be present on the SPA surface or accessible to antibody even when the SPA is denatured.

The deduced amino acid sequence of *R. prowazekii* SPA has been subjected to a number of computer algorithms to predict secondary structure and antigenic determinants (5, 10). Among the eight dominant epitopes found by PEPSCAN, seven were predicted to have a high antigenic index. Only epitope III was predicted to have a very low probability of being antigenic. The hyperimmune rabbit serum was prepared by immunization of SDS-PAGE-purified SPA which was partially denatured. Epitope III may be buried inside the molecule and become exposed upon denaturation. Alternatively, initial binding of antibody to solvent-exposed amino acid residues may promote local side chain displacement and allow the interaction with additional residues which were previously buried (1, 34). The secondary structures of these dominant epitopes were predicted to be either β-sheets or turns, except for that of epitope VII, which was predicted to be an α-helix (located between two turns). This is not surprising, since the mature protein has more than 60% β-sheet structure as indicated by the circular dichroism spectrum (11).

In two cases when the original residue was replaced by the unnatural sequence residue glycine, the binding activity increased compared with that of the original sequence. In epitope I, when T-48 was replaced by glycine, the binding activity increased about 20 to 40%. Similarly, in epitope VII, upon replacement of Q-1287 by glycine, the reactivity increased about 25% on average. In both cases, T-48 and Q-1287 are located just before the minimum binding site in the decapeptide. It is known that substitution with glycine may induce β-sheet structure (16). The change in the secondary structure or the decrease in steric hindrance may account for the stronger binding in antibody recognition.

Evidence that the typhus and spotted fever group SPAs have potential use as diagnostic reagents or vaccines is now quite extensive. Human sera from patients with epidemic and endemic typhus have been shown to react with denatured SPAs of *R. typhi* and *R. prowazekii* on Western blots. However, our preliminary trials with a 1:500 dilution of one laboratory-infected patient serum and a pool of sera from naturally infected patients with these overlapping peptides indicated that human antibodies exhibited only weak reactivity with decapeptide 1227 and decapeptide 1243, where decapeptide 1227 was one of the weak reactive epitopes and decapeptide 1243 was among the dominant epitopes recognized by rabbit 51 antibody (data not shown). When the experiment was repeated for epitopes I, V, VI, VII, and VIII, all of them except epitope VI were definitely recognized by a mixture of five *R. prowazekii*-infected patient sera at a dilution of 1:40 (data not shown). The low titer may be due to immune regulation acting against antibodies reacting to epitopes which are not exposed on the surface of the S-layer as present on intact rickettsiae. Lysine residues and possibly asparagine residues are methylated in mature soluble SPAs (8, 13). In our experience both rabbit and human typhus sera react quite strongly with native folded but not denatured SPA and to a much lesser degree with SPA fragments derived by protease digestion as detected on Western blots or by ELISA (data not shown). However, these sera are very poorly

reactive with the synthetic SPA decapeptides employed here in PEPSCAN. The relative importance of protein methylation and protein folding might be addressed by studies of unmodified recombinant SPA expressed from the cloned gene, but it has not yet been possible to obtain undegraded and normally folded typhus SPA for these studies. Hypomethylated SPA from the *R. prowazekii* Madrid E strain does not react with some monoclonal antibodies which bind to normally methylated SPA from virulent *R. prowazekii* (7a), but similar experiments have not been conducted with polyclonal sera. In summary, the identification of nonrickettsial sources of antigens for use in typhus diagnosis remains an elusive goal.

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