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Pseudomonas aeruginosa outer membrane protein OprF as an expression vector for foreign epitopes: the effects of positioning and length on the antigenicity of the epitope

(Recombinant DNA; genetic fusion; hybrid protein; surface exposure; peptide presentation)

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SUMMARY

OprF, the major outer membrane (OM) protein of *Pseudomonas aeruginosa*, has been proposed to be comprised of a series of β-strands separated by periplasmic or surface-exposed loop regions. In this study, a simple malarial epitope was used to demonstrate that OprF can be used as an expression vector to present foreign peptide sequences, namely, the 4-amino-acid (aa) repeating epitope (Asn-Ala-Asn-Pro = NANP) of the circumsporozoite protein of the human malarial parasite *Plasmodium falciparum*. Eight permissive sites, that allowed the expression and surface exposure of the malarial epitope, were identified throughout OprF. Using a monoclonal antibody (mAb) specific for the malarial epitope, we investigated the effects of positioning and length of the epitope on its antigenicity in the OprF expression vector system. It was demonstrated that the malarial epitope inserted at aa^{26} was significantly more reactive with the epitope-specific mAb (i.e., more antigenic) when assayed in the context of whole cells whereas those at aa^{213} and aa^{290} were more antigenic when assayed in the OM. The malarial epitope inserted at aa^{188} and aa^{196} was moderately antigenic, while this epitope inserted at aa^{215} and aa^{310} showed low antigenicity with the same mAb in both whole cell and OM assays. For two insertion sites, aa^{26} and aa^{213}, we demonstrated that the insertion of multiple copies of the epitope enhanced reactivity with the malarial epitope-specific mAb. These data are discussed with respect to the local OprF sequences into which the epitope was inserted.

INTRODUCTION

Previous studies have reported the expression of foreign epitopes in the context of specific *Escherichia coli* OM proteins (for review, see Hofnung, 1991). These studies have helped to define permissive sites in the target proteins, which are sites that can accommodate extra aa residues without causing extensive disruption to the biogenesis, final localization or folding of the protein (Charbit et al., 1991). These OM protein expression vector systems have important applications in the development of vaccines and diagnostic tests. Limited studies

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); Ap, ampicillin; bp, base pair(s); BSA, bovine serum albumin; *E.*, *Escherichia*; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IPTG, isopropyl thio-β-D-galactopyranoside; kb, kilobase(s) or 1000 bp; LPS, lipopolysaccharide(s); L-broth, Luria broth; mAb, monoclonal Ab; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; OM, outer membrane; OprF, outer membrane protein F; *oprF*, gene encoding OprF; *P.*, *Pseudomonas*; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (0.14 M NaCl/2.7 mM KCl/1.47 mM KH_2PO_4/20 mM Na_2HPO_4 pH 7.4); SDS, sodium dodecyl sulfate; wt, wild type.

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of two of these expression vector systems have shown that the position and the length of the inserted epitopes have effects on the antigenicity (i.e., the ability to interact with Ab) and the immunogenicity (i.e., the ability to stimulate an immune response) of the epitopes (Agterberg et al., 1990; Van der Werf et al., 1990). These findings suggested that investigations of the position and length effects of the epitope insertion in vector proteins will help us to exploit the effectiveness of such expression systems.

OprF, the major OM protein of *P. aeruginosa*, is a porin and also has a structural role in maintaining the integrity of the OM (Nikaido et al., 1991; Bellido et al., 1992). In a previous study, we performed linker-insertion mutagenesis on the *oprF* gene and created a series of *oprF* alleles with 12-bp insertions carrying a unique *Pst*I site at various positions within the gene (Wong et al., 1993). Ten permissive sites were identified in the OprF protein that allow the insertion of the 4-aa linkers.

In this study, a malarial epitope (Dame et al., 1984; Ballou et al., 1985) was employed as a model epitope to demonstrate that OprF could be used as an expression vector for foreign peptide sequences.

EXPERIMENTAL AND DISCUSSION

(a) Construction of OprF::malarial epitope hybrid proteins

The hybrid proteins were constructed by inserting synthetic oligos encoding the malarial epitope sequence into the *Pst*I sites of the *oprF* alleles generated from a previous linker mutagenesis study (Wong et al., 1993). Fig. 1 shows the positions of epitope insertions in a topology model of the protein that was constructed, in part, from the results of this study. Three sets of oligos were required to accommodate the three possible reading frames at the *Pst*I sites (Fig. 2). Table I summarizes the series of plasmids expressing the hybrid proteins that carried the malarial epitope at different positions of OprF and the identities of the aa residues inserted.

(b) Expression and surface exposure of the inserted epitope

The hybrid plasmids bearing the epitope-encoding oligos at eight different sites expressed proteins that were reactive with both OprF-specific and malarial epitope-

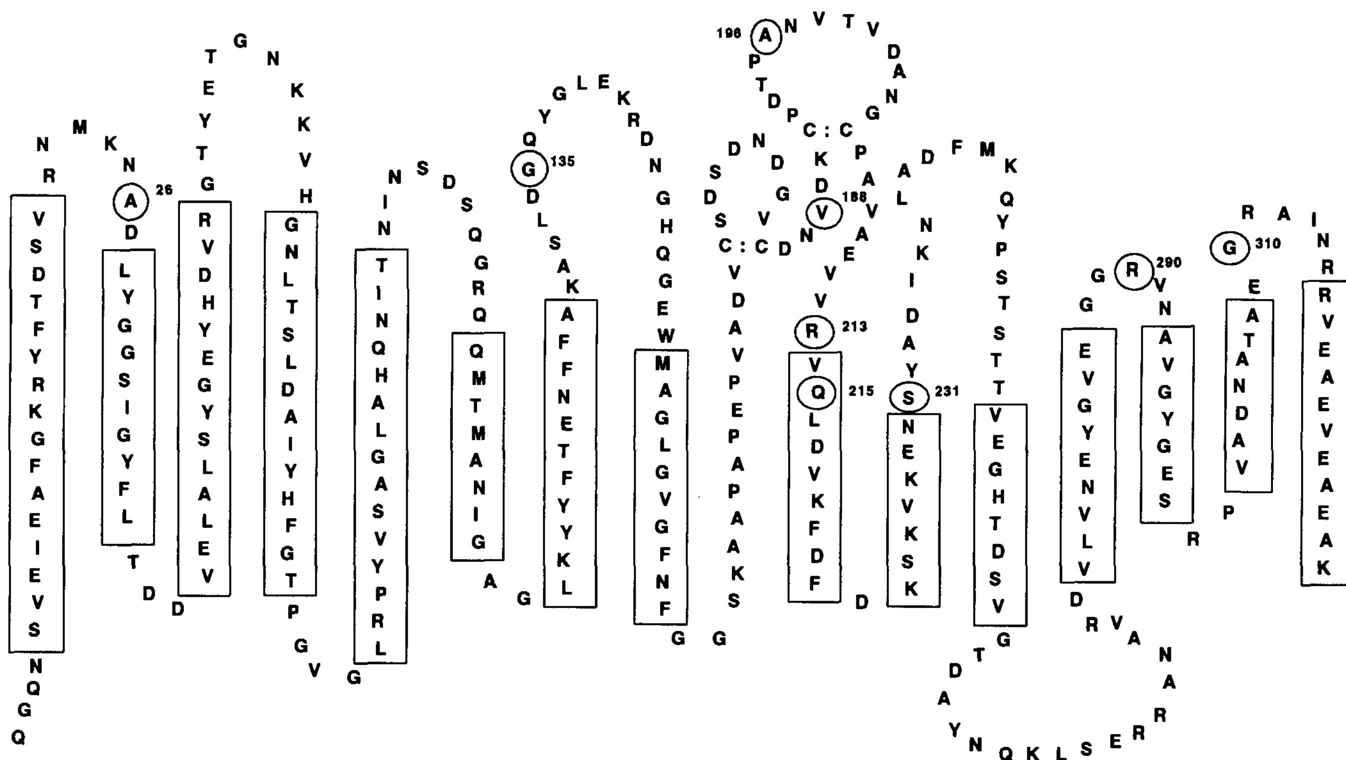

Fig. 1. A membrane topology model of OprF. The sites of malarial epitope insertion are marked by open circles and the transmembrane β-strands are indicated by rectangular boxes. The top of this model is proposed to face the exterior of the cell. This revised model is published in Rawling et al. (1995) and was drawn in part by utilizing data from this and previous studies (Wong et al., 1993). The placement of the aa sequences within 6 of the 8 surface exposed loops was suggested and confirmed by the insertion of the 4-aa linkers and the cell surface exposure of the inserted malarial epitope. The site at aa^{135} was found to be permissive for the expression of two copies of the malarial epitope insert, but we were unable to isolate a hybrid that carried a single copy of the epitope and therefore, did not include this site in the present study.

TABLE I

Summary of malarial epitope insertion mutants and antigenicity of the inserted malarial epitope when measured in OM and whole cell environments

Plasmid[a]	Insertion site[b]	Amino acids inserted[c]	Surface exposure of the epitope[d]	Antigenicity index[e]	
				OM[f]	Whole cell[g]
pRW3	-	wt	−	0	0
pRW302.1M	Ala^{26}	PAP(*ME*)GHAGP	+	7.06 ± 0.58	16.49 ± 1.24
pRW302.2M	Ala^{26}	PA{P(*ME*)GHA}$_2$GP	+	15.58 ± 1.19	25.60 ± 3.05
pRW307.1M	Val^{188}	DLQ(*ME*)LDVQV	+	7.18 ± 1.03	10.28 ± 3.22
pRW308.1M	Ala^{188}	PAP(*ME*)GHAGP	+	10	10
pRW309.1M	Arg^{213}	TCNP(*ME*)CRS	+	16.14 ± 1.82	5.84 ± 0.32
pRW309.3M	Arg^{213}	TC{NP(*ME*)C}$_3$RS	+	20.23 ± 1.91	12.82 ± 2.44
pRW310.1M	Gln^{215}	DLQ(*ME*)LDVQV	+	3.52 ± 0.63	3.93 ± 0.14
pRW311.1M	Ser^{231}	TCNP(*ME*)CRS	+	7.91 ± 0.74	6.57 ± 0.51
pRW312.1M	Arg^{290}	TCNP(*ME*)CRS	+	10.01 ± 0.67	4.74 ± 0.21
pRW314.1M	Gly^{310}	TCNP(*ME*)CRS	+	4.25 ± 0.31	3.83 ± 1.42

[a]Synthetic oligos encoding the epitope were cleaned by passing through a Sep-Pak C_{18} cartridge (Waters, Division of Millipore) and eluting with 20% acetonitrile. The sense and antisense strands of each set were annealed by heating an equal amount of each strand (100 μM) in 2 mM $MgCl_2$/50 mM NaCl/20 mM Tris pH 7.5 to 90°C for 15 min, followed by gradual cooling to 23°C. The annealed oligos, which carried *Pst*I-compatible ends, were ligated into the *Pst*I sites of the various *oprF* linker mutant plasmids (Wong et al., 1993). After transformation, the recombinants were screened by colony immunoblots with MA7-1 (Rawling et al., 1995) and pf2A.10 (Wirtz et al., 1987) separately. Plasmid DNA from transformants that demonstrated positive reactivities with both mAb were extracted and analyzed by restriction analysis and DNA sequencing.

[b]Position of the aa preceding the insertion. At insertion sites Ala^{196} and Ser^{231}, the preceding aa were replaced by a glycine and an arginine respectively.

[c]*ME* = NANPNANPNA, the outer two aa on both sides of the flanking sequences (i.e., PA_GP, DL_QV or TC_RS) are the results of the previous linker mutagenesis procedures.

[d] Determined by immunofluorescence labelling of whole cells with pf2A.10. − indicates negative reactivity, + indicates positive reactivity.

[e]To allow comparisons between the two methods (OM and Whole cell), the antigenicity index of the epitope at the insertion site represented by pRW308.1M was used as an internal standard and arbitrarily set to 10 and the rest of the values were adjusted accordingly. Values were the means and standard errors from six independent experiments for OM ELISA and three independent experiments for dot blots. The indices that were discussed in the section c as being significantly different were confirmed by *F*-tests ($P < 0.05$).

[f]Antigenicity in OM was measured by ELISAs of OM samples. C158 strains containing the hybrid plasmids were grown as described in Wong et al. (1993) with supplements of 1.7% NaCl/0.1% glucose to suppress the production of OmpF and LamB respectively. OM samples were prepared as described by Hancock and Carey (1979). OM samples (0.5–20 μg/ml) in carbonate buffer (15 mM Na_2CO_3/35 mM $NaHCO_3$/3 mM NaN_3 pH 9.6) were used to coat the wells of microtiter plates and ELISA performed as described by Mutharia and Hancock (1983). To normalize the expression levels of the hybrid proteins, each index was the ratio of the $A_{450\ nm}$ readings when pf2A.10 was used as the primary Ab to the $A_{450\ nm}$ readings when the OprF-specific polyclonal Ab was used as the primary Ab.

[g]Antigenicity in whole cell was measured by dot blots. Mid-logarithmic phase cells of strain C158 (*ompA, ompC, phoE*) (CGSC 6047, Foulds and Chai, 1979) expressing hybrid plasmids were harvested, washed twice with PBS and diluted in PBS to 1×10^8, 2×10^7, 4×10^6 and 8×10^5 cells/μl. 1 μl of each cell resuspension was spotted onto nitrocellulose filters, and the procedures were performed as described in Mutharia and Hancock (1983). The intensities of the dots were quantitated by densitometry with the protein + dna Imageware (PDI, New York, NY, USA) systems using the Quantity One software. Each antigenicity index was the mean of the anti-malarial epitope/anti-OprF ratios obtained from the four sets of dots representing different numbers of cells (from 8×10^5 to 1×10^8 cells).

specific mAb on Western immunoblots (Fig. 3). The apparent molecular masses of these proteins were slightly higher than that of native OprF, consistent with the presence of additional malarial epitope sequences in the hybrid proteins. Plasmids pRW302.2M and pRW309.3M encoded proteins of higher apparent molecular masses than plasmids carrying a single copy of the insert at the same sites (Fig. 3, lanes 1 and 2; lanes 5 and 6). The lanes corresponding to plasmids pRW307.1M and pRW308.1M (lanes 3 and 4) showed a more prominent upper band which represented the heat-modified form of the protein. The increase in amount of the heat-modified form could conceivably be due to the presence of extra aa in the cysteine-containing region, affecting the formation of disulfide bonds (Hancock and Carey, 1979), and thus rendering the protein more susceptible to heat denaturation. Lanes 7 to 10 demonstrated an increase in abundance of the 28-kDa degradation product, which failed to react with the anti-malarial epitope Ab, suggesting that it represented the N-terminal part of these proteins lacking the malarial epitope sequences, a result consistent with previous findings that C-terminal pertubations rendered the proteins more susceptible to proteases (Finnen et al., 1992; Wong et al., 1993). However, Western immunoblots of OM samples containing the hybrid proteins demonstrated minor and comparable levels of protease degradation products in all of the samples, implying that the 28-kDa product was not associated with the OM fraction. Malarial epitope-encoding oligos were also inserted into sites corresponding to aa^2 and aa^{131} of

Phase 1

P N A N P N A N P N A G H A 14 aa

CCGAACGCCAACCCGAACGCCAACCCGAACGCCGGGCATGCA 42 nt

ACGTGGCTTGCGGTTGGGCTTGCGGTTGGGCTTGCGGCCCGT

Phase 2

N P N A N P N A N P N A C 13 aa

ACCCGAACGCCAACCCGAACGCCAACCCGAACGCATGCA 39 nt

ACGTTGGGCTTGCGGTTGGGCTTGCGGTTGGGCTTGCGT

Phase 3

N A N P N A N P N A L D V Q 14 aa

GAACGCCAACCCAAACGCGAATCCGAATGCTCTAGACTTGCA 42 nt

ACGTCTTGCGGTTGGGTTTCCGCTTAGGCTTACGAGATCTGA

Fig. 2. Sequences of the three sets of oligos encoding the malarial epitope. All three sets of oligos carried *Pst*I compatible ends for the ligation into the *Pst*I sites generated by the linker-insertion mutagenesis procedures, the unique restriction enzyme sites engineered in the oligos are underlined (*Sph*I in frames 1 and 2, *Xba*I in frame 3). To avoid the problem of hairpin loop formation when more than one copy of the insert was ligated in opposite orientations, a different codon usage for the NANP repeats was chosen for the Phase-3 oligos. The aa sequences are indicated in one-letter code.

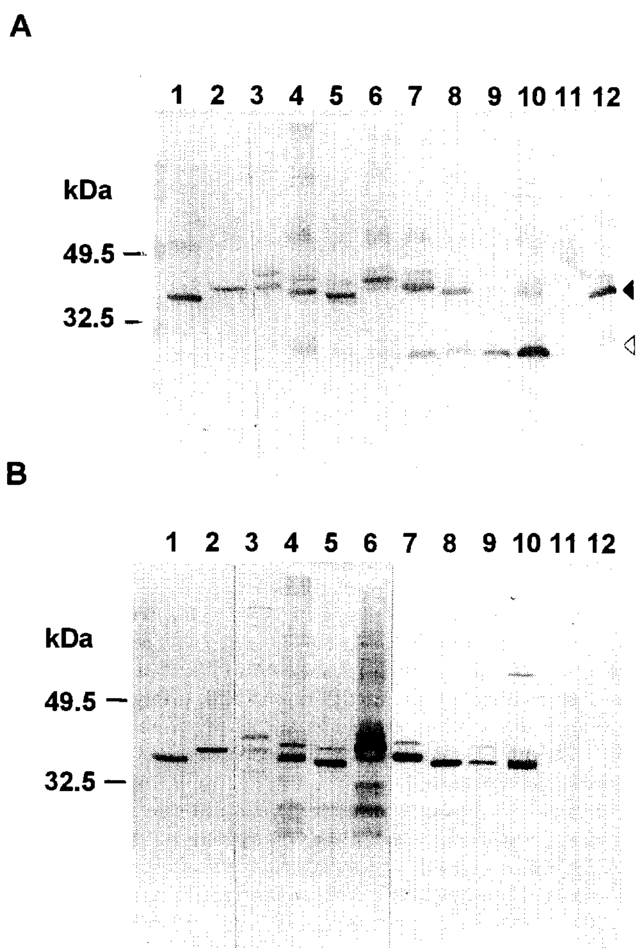

Fig. 3. Western immunoblots of whole cell lysates of *E. coli* DH5αF′ strains expressing various OprF::malarial epitope hybrid proteins after reaction with OprF specific mAb MA7–1 (**A**) or malarial epitope specific mAb pf2A.10 (**B**). Plasmids expressed in the bacteria samples from which these were generated were: Lanes: **1**, pRW302.1M; **2**, pRW302.2M; **3**, pRW307.1M; **4**, pRW308.1M; **5**, pRW309.1M; **6**, pRW309.3 M; **7**, pRW310.1M; **8**, pRW311.1M; **9**, pRW312.1M; **10**, pRW314.1M; **11**, pTZ19R; **12**, pRW3. The bands corresponding to wt OprF and the N-terminal degradation product are indicated by a solid and an open triangle, respectively. In some lanes, the bands corresponding to OprF oligomers are visible (Wong et al., 1993). The relevant molecular mass markers are indicated on the left-hand side of the blots. The procedures were as described in Wong et al. (1993).

OprF, but no hybrid proteins were detected on Western immunoblots. We speculate that these two sites were either non-permissive for the insertion of more than 4 aa (encoded by the linker insertion) or not compatible with the expression of the malarial epitope sequence.

Since the presence of OmpA appeared to mask the binding of mAb to OprF in intact *E. coli* cells, the hybrid plasmids were expressed in an OmpA-deficient strain C386 (Wong et el, 1993) and examined for surface exposure by indirect immunofluorescence labelling (Fig. 4). The malarial epitope expressed at all eight permissive sites was detectable on the cell surface (Table I), which corroborated the placement of the insertion sites in the surface-exposed loop regions of OprF that are more likely to be flexible enough to accommodate foreign peptide sequences (Hofnung, 1991), as proposed in the latest topology model (Fig. 1). Although site aa^{215} was placed within the membrane, examination of the inserted sequence indicated the possibility that it maintained the amphipathic β-strand character, sufficiently to extend this strand and permit the malarial epitope to be exposed on the surface of cells.

(c) Position and length effects on antigenicity

The antigenicity of the malarial epitope expressed at different positions of the vector protein OprF was compared by whole cell dot blot analysis and OM ELISA (Table I). Both assays indicated that the epitope had different relative affinities for the malarial epitope-specific mAb when expressed at different positions. For example, malarial epitope inserted at aa^{215} (pRW310.1M) or aa^{310} (pRW314.1M) consistently demonstrated low antigenicity; whereas insertions at aa^{188}, or aa^{196} (represented by pRW307.1M and pRW308.1M, respectively) were significantly more antigenic in both assays. The epitope inserted at aa^{26} was more antigenic in whole cell dot blots; while the epitope inserted at aa^{213} and aa^{290} was significantly more antigenic when assessed by OM ELISA. Although we believe that the hybrid proteins were in their native configuration in the OM preparations, the isolation procedures utilized might have removed part of the surface moieties such as lipopolysaccharides (LPS). The presence of cell surface LPS could promote the presentation of the inserted epitope for Ab binding at aa^{26} while reducing the accessibility for Ab

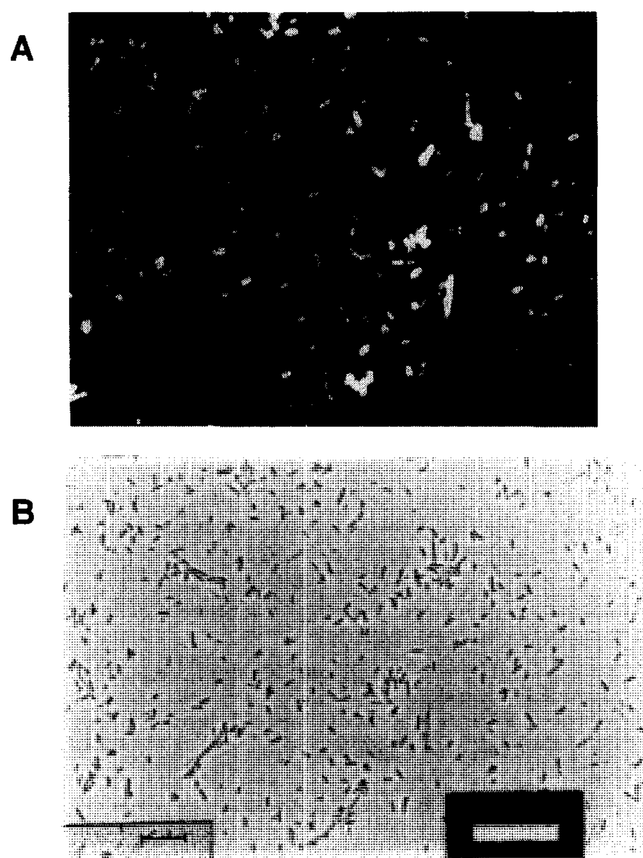

Fig. 4. Representative result of indirect immunofluorescence labelling of *E. coli* cells expressing an OprF::malarial epitope hybrid plasmid (pRW302.1M) with the malarial epitope specific mAb pf2A.10. (**A**) labelled cells observed under fluorescence microscopy; (**B**) the same field observed under phase contrast. The scale bar in the bottom left corner indicates 10 μm. Experimental procedures were as described in Wong et al. (1993).

binding at aa^{213} and aa^{290}, thus resulting in the significant dissimilarities in antigenicity indices when assayed in whole cell and OM environments.

Insertion of multiple copies of the epitope at aa^{26} and aa^{213} resulted in a significant increase in antigenicity (Table I, rows 2 and 3; 6 and 7). The presence of an additional copy of the epitope might improve the exposure of the epitope and thus its accessibility for Ab binding, or it might increase the valency of the Ab binding. The lesser influence of multiple insertions at aa^{213} on the antigenicity might imply that this site is already well-exposed in the OprF protein, or that the effects of multivalency in this case might have induced steric hindrance which limited the accessibility of the epitope to Ab.

(d) Antigenicity and flanking aa sequences

The larger number of sites examined in this study permitted us to attempt to correlate the measured antigenicity of the epitope at the various insertion sites with the primary and secondary structures at these sites. The possible structures of each insertion site were analyzed using various structure prediction methods. We analyzed the structures at each insertion site in the context of the entire protein, as well as in a segment of the sequence including the six OprF aa residues flanking either side of the insertion. When only the flanking residues were taken into consideration, the Gascuel and Golmard Basic Statistical Method analysis (Gascuel and Golmard, 1988) predicted that three or more aa were in extended conformation on at least one of the flanking sequences of insertion sites aa^{196}, aa^{290} and aa^{213}. The antigenic determinant program of Hopp and Woods (1981) predicted that these sites have low to medium hydrophilicity on both sides of the flanking regions. When analyzed in the context of the entire aa sequence, the insertion sites that exhibited high relative antigenicity were found in regions that were generally predicted to be more flexible in their local secondary structure and to have higher coil propensity (Karplus and Schulz, 1985). Although the correlations were not universal, the general predicted properties (e.g., extended conformation and high flexibility) of the local sequences suggest that these features might improve the accessibility of the epitope.

(e) Conclusions

(*1*) It was demonstrated that the major OM protein of *P. aeruginosa* can be used as an expression vector system to display foreign peptide sequences. Eight permissive sites were identified which could express a model malarial epitope sequence. Insertion of the epitope sequence in the disulfide-containing region of OprF increased the heat sensitivity of the hybrid proteins, while insertions in the C terminus of the protein rendered the hybrid proteins more susceptible to degradation by cellular proteases.

(*2*) The malarial epitope inserted at different positions of OprF displayed different binding affinities for an epitope-specific mAb. For example, the malarial epitope inserted at aa^{188} or aa^{196} was consistently more antigenic than that inserted at aa^{215} or aa^{310}; while insertions at aa^{26} or aa^{213} were significantly more antigenic in whole cell and OM environment, respectively. Insertion of multiple copies of the epitope at aa^{26} and aa^{213} resulted in higher levels of Ab binding; possibly due to the increase in valency and/or better presentation of the binding epitope.

(*3*) The OprF exposure vector system described here provides a number of insertion sites and therefore a variety of flanking aa sequences for the expression of foreign epitopes. Studies of other expression vector systems have provided preliminary data supporting the conclusion that flanking aa sequences also had an effect on the immunogenicity of the epitope (Agterberg et al., 1990; Van der Werf et al., 1990). In these prior studies, it was hypothesized that flanking regions provided protease cleavage

sites for antigen processing and/or affected the terminal charges of the peptide for presentation by MHC (major histocompatibility complex) molecules to T cells of the immune system (Van der Werf et al., 1990). On the other hand, in a study of a different expression vector system (Martineau et al., 1992), it was shown that the site of insertion had an effect on the antigenicity but not the immunogenicity of the epitope. The availability of a larger number of insertion sites in this system allowed us to establish relationships between the ability of the epitope to bind Ab and the nature of the flanking aa sequences. A further study of the immunogenicity of the malarial epitope in the context of different flanking sequences in OprF will improve our understanding of the relationship between flanking sequences and the immunogenicity of the epitope.

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