Exploiting the *Burkholderia pseudomallei* Acute Phase Antigen BPSL2765 for Structure-Based Epitope Discovery/Design in Structural Vaccinology

Louise J. Gourlay, 1, 8 Claudio Peri, 3, 8 Mario Ferrer-Navarro, 4 Oscar Conchillo-Solé, 4 Alessandro Gori, 3 Darawan Rinchai, 6 Rachael J. Thomas, 7 Olivia L. Champion, 7 Stephen L. Michell, 7 Chidchamai Kewcharoenwong, 6 Arnone Nithichanon, 6 Patricia Lassaux, 1 Lucia Perletti, 1 Renato Longhi, 3 Ganjana Lertmemongkolchai, 6 Richard W. Titball, 7 Xavier Daura, 4, 5 Giorgio Colombo, 3, * and Martino Bolognesi 1, 2, *

1 Department of Biosciences
2 Consiglio Nazionale delle Ricerche, Institute of Biophysics
University of Milan, 20133 Milan, Italy
3 Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, Via Mario Bianco 9, 20131 Milano, Italy
4 Institute of Biotechnology and Biomedicine (IBB), Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain
5 Catalan Institution for Research and Advanced Studies (ICREA), 08010 Barcelona, Spain
6 Center for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, 40002 Khon Kaen, Thailand
7 College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK
8 These authors contributed equally to this work
* Correspondence: g.colombo@icrm.cnr.it (G.C.), martino.bolognesi@unimi.it (M.B.)
http://dx.doi.org/10.1016/j.chembiol.2013.07.010

SUMMARY

We solved the crystal structure of *Burkholderia pseudomallei* acute phase antigen BPSL2765 in the context of a structural vaccinology study, in the area of melioidosis vaccine development. Based on the structure, we applied a recently developed method for epitope design that combines computational epitope predictions with in vitro mapping experiments and successfully identified a consensus sequence within the antigen that, when engineered as a synthetic peptide, was selectively immunorecognized to the same extent as the recombinant protein in sera from melioidosis-affected subjects. Antibodies raised against the consensus peptide were successfully tested in opsonization bacterial killing experiments and antibody-dependent agglutination tests of *B. pseudomallei*. Our strategy represents a step in the development of immunodiagnostics, in the production of specific antibodies and in the optimization of antigens for vaccine development, starting from structural and physicochemical principles.

INTRODUCTION

*Burkholderia pseudomallei* is a soil-borne Gram-negative bacterium that causes melioidosis, a debilitating and frequently fatal disease endemic in the subtropical and tropical regions of the world (Cheng and Currie, 2005). Melioidosis-affected individuals display a range of infections, including pneumonia, acute septicemia, localized abscesses, and chronic disease. Such diversity of symptoms, many of which are common to other pathologies, can make it difficult to diagnose the disease. The present treatment for melioidosis relies primarily on antibiotic administration, but *B. pseudomallei* exhibits resistance to the main antibiotic classes (Cheng and Currie, 2005). The combined problems of timely diagnosis and treatment of the disease contribute to a high fatality rate. A vaccine against melioidosis would be an effective option for preventing infection and in controlling relapse and reinfection, which often occur following antibiotic treatment (Peacock et al., 2012). However, several vaccination initiatives have been launched without success (Sarkar-Tyson and Titball, 2010).

Potential protein antigens are likely to be located or excreted at the cell surface, where they represent the first port of contact for the molecules that govern the host immune response. In this context, a recent study employed a protein microarray to display over a thousand *B. pseudomallei* proteins, identified via a reverse vaccinology approach, which was probed with sera from melioidosis patients to identify immunoreactive proteins and hence potential antigens (Felgner et al., 2009). As expected, half of the candidates were designated as outer-membrane or surface-located proteins and included putative outer-membrane protein A (OmpA) family members, which have known roles in bacterial virulence and immunity in other pathogens (Li et al., 2009; Weiser and Gotschlich, 1991). With regards to OmpAs from *B. pseudomallei*, 12 OmpA genes were identified for subsequent immunogenic characterization as potential vaccine candidates, two of which (*BPSL2522* and *BPSL2765*), produced as soluble recombinant proteins, were confirmed to be immunogenic in both mice and melioidosis patients (Hara et al., 2009). Antibodies against BPSL2765 were found at 10-fold-higher levels in patients who had only one episode of melioidosis, in comparison with patients who had suffered recurrent episodes. However, in mice challenged with ten times the lethal dose of *B. pseudomallei*, immunization with recombinant BPSL2765 offered only limited protection (50%), with disease progression...
B. pseudomallei agglutination. OPK results indicated that antibodies generated against the epitope were in fact bactericidal, functioning to enhance neutrophil function, eliminating the bacteria possibly via an oxidative burst pathway, whereas agglutination assays confirmed that the antibodies induced B. pseudomallei-specific agglutination. Notably, both the OPK and bacterial agglutination tests showed that antibodies raised against the synthetic epitope were significantly more active than those generated against the recombinant protein, adding a second validation stage to our structural vaccinology pipeline and underlying the potential use of this epitope in melioidosis vaccine development.

Although we report the specific case of BPSL2765 from B. pseudomallei, our structural vaccinology strategy is general and could be applied to produce peptides for the development of diagnostic tools, for the production of specific antibodies, and in the general context of antigen optimization for vaccine development.

RESULTS

Primary Structure Analysis
Based on a protein BLAST search, BPSL2765 exhibits high similarity to members of the peptidoglycan-associated lipoprotein (Pal) family, involved in maintaining outer membrane integrity and the import of select organic nutrients. At a primary structure level, Pals typically contain a hydrophobic N-terminal lipoprotein signal peptide synonymous with bacterial lipoproteins, followed by a cleavage/modification site and a highly conserved C-terminal Ompa-like domain. Accordingly, a 19-residue signal peptide containing the LAAC motif, typically found in protein precursors destined for proteolytic processing by signal peptidase II during translocation across the cytoplasmic membrane, was predicted using the signal peptide prediction program SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Furthermore, in agreement with other Pals, BPSL2765 (hereafter referred to as PalBp) possesses a highly conserved C-terminal Ompa-like domain (residues 65–168) that typically houses a peptidoglycan binding site. Multiple sequence alignment of the genomes of 28 diverse B. pseudomallei strains indicated that BPSL2765 is 100% conserved (data not shown).

PalBp 3D Structure
The crystal structure of PalBp was solved and refined at 2.3 Å resolution (Table S1 available online). There are three identical PalBp chains (A, B, and C; root-mean-square deviation [rmsd] values 0.30–0.36 Å) present in the crystal asymmetric unit (Figure 1). This trimer is a likely artifact of crystal packing given the absence of significant trimer interface interactions and the fact that Pal homologs, including E. coli Pal (Protein Data Bank [PDB] entry 1OAP) and Haemophilus influenzae Pal (PDB entry 2AIZ), with whom PalBp shares 52% sequence identity, are monomeric (Parsons et al., 2006). All structural comparisons were made using PalBp Chain A. As judged by superimposition of PalBp with E. coli Pal (rmsd of 0.53 Å), using the C-alpha match pairwise structural alignment program (http://bioinfo3d.cs.tau.ac.il/c_alpha_match/), the overall structure conforms to the canonical α-β sandwich Pal fold, organized in a helix-strand-helix topology (Figure 1).

Figure 1. Crystal Structure of PalBp
Ribbon representation of PalBp, illustrating three chains (labeled) present in the asymmetric unit and three acetate ions (spheres), bound at equivalent sites in each chain. Where visible, the N and C termini of the chains are labeled. This figure was produced using MacPymol (http://www.pymol.org).

Continuing from the acute to chronic stages of infection (Hara et al., 2009; Suwannasaen et al., 2011). Thus, to be effective, a potential vaccine may need to offer protection toward both phases of infection.

Structure-based antigen engineering could be used to specifically modify protein antigens of a pathogen to enhance their immunogenic properties, with the aim of improving their protective efficacy. Moreover, structural information could be used to design immunodiagnostics probes specific for melioidosis, overcoming current limitations in diagnosis. Such approaches may entail engineering only the portions of the protein that house the epitopes or simply the epitope sequences themselves in the form of synthetic peptides.

Focusing on the acute phase antigen BPSL2765 as a potential structural vaccinology candidate, we present a coordinated study that aims to connect the understanding of structural properties at the atomic level to the reactivity properties of the protein (or specific epitopes) in an immunological context.

Specifically, we first solved the X-ray crystal structure of the protein and used it as the basis for the application of a recently developed method for epitope design, which combines the results of computational epitope prediction with those of in vitro mapping experiments (Lassaux et al., 2013; Peri et al., 2013). On these bases, a consensus epitope was identified and a related synthetic peptide was synthesized. When the peptide was tested for immunoreactivity against sera from recovered melioidosis patients, it was recognized at levels comparable with those of the recombinant protein, thus validating our approach. The immunological properties of the epitope were further studied in opsonization killing (OPK) tests using human neutrophils and in assays of antibody-mediated agglutination.

Chemistry & Biology

PalBp Antigen as a Structural Vaccinology Target
There are three acetate ions bound at identical positions in each chain, coordinated by identical residues in a small cavity (vol 108 Å³) containing main- and side-chain atoms from residues F70, D71, T104, D105, R107, Y112, N113, L116, R120, and R162 (Figure 1). All three acetate ions show two alternate binding modes. The presence of bound acetate and sulfate ions was also observed in the crystal structures of E. coli Pal in complex with TolB (PDB entry 2HQ5) and alone, respectively.

**Computational Epitope Predictions**

In order to predict the location of B cell epitopes from the experimental three-dimensional (3D) structure of PalBp, two different computational methods, matrix of local coupling energies (MLCE) and electrostatic desolvation profiles (EDP), were combined and applied to representative structures obtained from molecular dynamics (MD) simulations performed on chain B of PalBp (Fiorucci and Zacharias, 2010; Scarabelli et al., 2010). The MLCE approach was developed to identify antigenic epitopes, whereas EDP is programmed to identify, in addition to antigen-antibody interaction interfaces, more general protein–protein interaction interfaces (see Experimental Procedures and Supplemental Experimental Procedures). Starting from the idea that structure, dynamics and stability of a protein-antigen play a key role in the interaction with antibodies, MLCE integrates the analysis of the dynamical and energetic properties of proteins to identify nonoptimized, low-intensity energetic interaction-networks on the surface of the isolated antigens, which correspond to substructures that can aptly be recognized by a binding partner (the antibody). EDP calculates the free energy penalty for desolvation by placing a neutral probe at various protein surface locations. Surface regions with a small free-energy penalty for water removal may correspond to preferred interaction sites. Evidence suggests that it is easier for a partner to bind to an epitope when properties required for efficient binding, such as low desolvation penalty, are met.

The results individually produced by MLCE and EDP are reported in Table S2. The sequences of EDP predicted epitope regions broadly overlap with one another, and all MLCE-predicted residues were also found by EDP. Therefore, MLCE/EDP consensus regions are those composed of amino acids identified by both methods; moreover, as MLCE identified fewer residues than EDP, the resulting consensus regions simply correspond to the two MLCE-predicted patches (Table S2; Figure 3).

**Experimental Epitope Mapping**

In order to complement the computational predictions, epitope mapping experiments were carried out using recombinant PalBp and rabbit polyclonal antibodies (see Supplemental Experimental Procedures). To this aim, we adopted and extended an immunocapturing approach that is successfully used with monoclonal antibodies (Koehler et al., 2011; Soriani et al., 2010). The approach involves proteolytic digestion (using different proteases) of the target antigen prior to immunocapturing and subsequent analysis of antibody-bound peptides (containing the epitope) by mass spectrometry. Using this approach, one expects polyclonal antibodies to recognize the same epitope regions, regardless of the protease used to produce the peptide mixture.

Partial trypsin digestion of PalBp gave rise to two mass spectrometry peaks with m/z values of 3,284.673 Da (peak 1) and 3,440.791 Da (peak 2). To unequivocally assign the sequence of these peptides, tandem mass spectrometry (MS/MS) spectra were recorded. Both peaks corresponded to the same antigen region: peak 1 corresponds to residues 65 to 91, and Peak 2 corresponds to the same sequence but with an additional N-terminus residue (Table S2). For the LysC-obtained peptide mixture (Peak 3), a single peptide (m/z 3,440.818 Da) covering the sequence from R64 to K91 was immunocaptured and confirmed by MS/MS (Table S2). GluC digestion in ammonium bicarbonate shows a preference for cleavage at Glu residues; thus, the peptides immunocaptured from a partial GluC digestion in ammonium bicarbonate buffer should contain the same sequence of those captured from the trypsin and LysC digestions, but they should have a Glu residue at the C-terminal end. In fact, for GluC two different peptides were immunocaptured with m/z values of 4,137.148 (peak 4) and 4,667.003 (peak 5) Da, respectively. These peptides comprise residues 72 to 106 and 72 to 111 for peaks 4 and 5, respectively (Table S2). The sequence of these peptides could not be confirmed by MS/MS, so they were confirmed by peptide mass fingerprinting. Taken together, the experimental epitope mapping data show that all the immunocaptured peptides share a common sequence containing epitope residues that are key for the antibody-antigen interaction; such sequence covers PalBp residues 72 to 91 that, from a structural point of view, pertain to a loop region followed by α helix 3 (Figure 2A).

**Epitope Design**

The results of computational predictions and immunocapturing experiments were compared to search for common determinants for antibody recognition. Despite the very different conditions and approaches, the protein substructures individually identified by either MLCE or EDP overlap significantly with those derived by single protease degradation; for example, MLCE 2 overlaps with the GluC-derived peptides (peaks 4 and 5), while EDP predictions cover all protease-generated peptides (peaks 1–5; see Table S2).

Overall, the consensus of computational predictions, represented by MLCE 2, is also present in the experimental consensus epitope region. Based on this observation and on previous experience gained with the OppA antigen (Lassaux et al., 2013), we propose that the consensus region hosts key residues for antibody recognition and binding. It must be underlined, at this point, that the proposed overall consensus between the computationally predicted and the immunocaptured sequences is limited to residues 89–91 (YLK). Three residues are unlikely to elicit antibodies if used as an immunogenic peptide; however, the consensus sequence should be considered as starting information for the design/selection of longer synthetic peptide immunogens.

Inspection of the PalBp 3D structure shows that the consensus YLK sequence is located in α helix 3, which is built by several residues (residues 76–93) that were also identified by experimental mapping (Figure 2A; Table S2). From a dynamical point of view, α helix 3 shows low flexibility (in the intact protein antigen), as evidenced from analysis of residue-based root-mean-square fluctuations (rmsf), obtained after projecting the MD trajectory along the eigenvectors, indicating the principal (nonrandom)
displacements (Figure 2B). Interestingly, the low-flexibility region includes a loop region, comprising residues 72–76, that are also present in the experimentally mapped peptide. This helix-loop motif in the full-length protein resists cleavage by three different proteases, indicating that it could behave as a unit that is recognized by polyclonal antibodies through a conformational selection mechanism. In this context, the conformational behavior of the isolated peptide, corresponding to residues 72–91, was characterized by MD simulations and circular dichroism (CD) spectroscopy. Long-timescale simulations in explicit solvent show that the peptide can populate ordered secondary structures, transiently populating helical conformations (data not shown). CD analysis in the presence of the secondary structure stabilizing solvent trifluoroethanol (TFE) experimentally confirmed that the peptide can populate helical conformations (Figure S2; Supplementary Experimental Procedures). On this basis, it is possible to hypothesize that sequence 72–91 can transiently populate helical structures in isolation that may be conformationally selected and recognized by an antibody elicited against it. Based on these considerations, we decided to synthesize three different peptides: two peptide sequences corresponded to MLCE-EDP computational predictions only and were labeled epitope 1 and epitope 2, and the third sequence was selected to match the 72–91 experimental peptide (epitope 3).

**Epitope Synthesis**

The peptides were synthesized using polyethylene glycol (PEG) units as spacers and coupled to human serum albumin (HSA) as carrier protein (see Experimental Procedures). When necessary, to synthesize peptides of appropriate size, epitopes were extended by incorporating flanking residues predicted by EDP. The resulting peptides were designated as follows: epitope 1 (residues 144–169), a linear epitope derived from the consensus patch MLCE 1 and EDP patches 4 to 6; epitope 2 (residues 132–141 and residues 89–99), a conformational epitope that houses the consensus tripeptide (residues 89–91) derived from both in silico and experimental data, extended around residues from MLCE patch 2 and EDP patches 1 and 2; and epitope 3 (residues 72–91), containing the same consensus tripeptide (residues 89–91), extended to include additional residues from α helix 3, identified by experimental mapping. The sequences, and locations of the final peptides with regards to the global PalBp structure, are displayed in Table S2 and Figures 3A–3C, respectively.

**Peptide Immunoreactivity to Human Plasma Antibodies**

Epitope peptides were tested for their antigenic properties in antibody recognition experiments in plasma samples collected from 19 healthy donors and 20 recovered melioidosis cases (Khon Kaen University and Srinakarin Hospital, Thailand) by indirect ELISA. Healthy donors were divided into two groups, seronegative and seropositive subjects, based on indirect hemagglutinin assay (IHA) antibody titers (see Experimental Procedures). As previously reported, control experiments showed that plasma antibodies to PalBp from the recovered group were significantly higher than those from the seronegative and seropositive groups (Mann-Whitney test; p < 0.001 and p < 0.05, respectively) (Figure 4) (Suwannasaen et al., 2011).
Neither epitope 1 nor epitope 2 was recognized, confirming that they are not antigenic. Such finding was not unexpected, given that the former peptide did not contain any residue identified in the in vitro mapping, and the latter contained only three residues (residues 89–91) that overlapped with the experimental epitope. On the other hand, the epitope 3 consensus peptide was successfully recognized, to the same extent as the recombinant protein, confirming that it represents a highly immunoreactive region capable of maintaining its structure and antibody recognition properties even in the form of an isolated sequence fragment. As previously mentioned, CD experiments on the isolated peptide support this view; no features of random coil “structure” were detected (Figure S2). Rabbit polyclonal antibodies raised against epitope 3 antibodies were raised for further validation through OPK and bacterial agglutination experiments (see Supplemental Experimental Procedures).

Neutrophil Opsonization Killing Experiments

We first investigated the effect of various concentrations of anti-epitope 3 antibodies on phagocytosis and oxidative burst functions of purified human neutrophils, in response to live *B. pseudomallei*. As shown in Figure 5A, approximately 95% of the harvested neutrophils produced superoxide when induced by phorbol 12-myristate 13-acetate (PMA; positive control) for 15 min. In a parallel group, approximately 50% of purified neutrophils could phagocytose *B. pseudomallei* within 60 min. The phagocytosis and oxidative burst by neutrophils (n = 3) exposed to *B. pseudomallei* was significantly enhanced (p < 0.05) by the presence of anti-epitope 3 antibodies at 10 μg/ml (Figure 5B). Complement was not added in all of these experiments, indicating that antibodies to epitope 3 alone are sufficient for enhancing phagocytosis and oxidative burst functions in human neutrophils in vitro. Notably, OPK tests carried out with antibodies raised against full-length recombinant PalBp did not enhance bacterial killing (results not shown).

Next, we investigated the effect of different antibody concentrations used to opsonize the bacteria on the ability of human neutrophils to kill *B. pseudomallei*. An increase of bacterial killing activity of purified neutrophils was observed in *B. pseudomallei* cells treated with high concentrations of anti-epitope 3 antibodies, compared to those in the absence of opsonin, confirming an enhancement of antibody-dependent bactericidal activity in the assay (Figure 5C).

Detection and Agglutination of *B. pseudomallei* by Antibodies Generated toward PalBp and Epitope 3

Agglutination by antibody is an additional important defense mechanism that aids clearance of pathogens (Bull, 1915; Pa’tsyn et al., 1999). We determined antibody-mediated agglutination of *B. pseudomallei* using anti-PalBp or anti-epitope 3-antibodies and found that both were able to agglutinate *B. pseudomallei*, but not *E. coli*, indicating that the antibody-mediated agglutination is bacteria specific (Figure 6). Interestingly, anti-epitope 3 antibodies agglutinated *B. pseudomallei* more efficiently than antibodies toward the whole protein, with the majority of the bacteria having formed aggregates after 30 min (Figure 6). Agglutination did not occur when *B. pseudomallei* was exposed to PBS, preimmune sera from the same rabbit (prebleed), or antibody toward the *B. pseudomallei* capsule (4V1H12).

DISCUSSION

In the vaccine design/development field, structural vaccinology has emerged as an innovative tool to help tailor protein antigens...
to match the properties required for the formulation of safe, effective, and, when necessary, cross-protective vaccines (Dormitzer et al., 2008). Antigens may be engineered to be biochemically more stable by isolating and producing only the minimal antigenic portions of the protein, which in some cases may be represented by domains or peptides. Furthermore, structure-based antigen engineering may be adopted to devise cross-protective vaccines that address pathogen antigenic variation or to develop multistage vaccines that take into account diverse pathogenic phases of a disease that may display different antigens (Aagaard et al., 2011; Nuccitelli et al., 2011).

Previous work has shown that antibodies against B. pseudomallei can play a key role in protection against melioidosis. In humans, the antibody titer to bacteria is broadly correlated with disease severity (Wiessinga et al., 2006). In small-animal models of infection (diabetic rats or mice), the passive transfer of antibodies against surface polysaccharides provides protection against a subsequent challenge; these antibodies appear to be opsonizing (Nelson et al., 2004; Zhang et al., 2011). Conversely, the role of antibodies to proteins in protective immunity is generally less clear. Antibody to flagellin has been shown to inhibit the motility of B. pseudomallei, and antibodies against PalBp are reported to promote complement-mediated killing by neutrophils (Brett and Woods, 1996; Su et al., 2010).

Our work presents and validates a 3D structure-based approach, combined with in vitro and immunological tests, to determine the location of B cell epitopes within PalBp, an acute antigen and peptidoglycan-associated lipoprotein from B. pseudomallei. Based on our results, three epitope peptides were identified, synthesized, and tested for their antigenicity against immune sera from melioidosis patients. The PalBp 2.3 Å resolution crystal structure, which displayed the canonical α/β sandwich fold of the Pal family, was used as a template for the application of MLCE and EDP epitope prediction methods. Cross-validating the computational predictions with proteolysis-based epitope mapping experiments, combined to the analysis of the protein's conformational dynamics, led us to identify one sequence stretch (epitope 3) that hosts key residues necessary for antibody recognition. In ELISA tests, the synthetic peptide corresponding to epitope 3 allowed to distinguish between healthy and melioidosis-affected individuals, displaying a reactivity profile comparable to that of the recombinant protein control.

In order to explore the potential of our integrated approach and the immunological properties of epitope 3 in more detail, we tested the ability of the peptide to raise opsonizing antibodies that induce bacteria killing. Since our method has recently proven that it is possible to use synthetic peptides to raise antibodies that retain full specificity for the epitope region and fully recognize the full-length protein (Peri et al., 2013), we expected specific anti-epitope 3 antibodies to enhance phagocytosis and oxidative burst. No complement components were present in the conditions and the anti-epitope 3 antibodies alone were seen to enhance bacterial activity of human neutrophils through increase phagocytosis and oxidative burst. It has been shown that the capsular polysaccharide of B. pseudomallei contributes to its resistance to in vitro phagocytosis by reducing C3b deposition on the bacterial surface (Reckseidler-Zenteno et al., 2005), which is necessary for killing the bacterium, via NADPH-oxidase induction by human neutrophils (Woodman et al., 2012). However, our studies show that antibodies can overcome bacterial resistance to neutrophil-mediated phagocytosis and killing, suggesting that treatment strategies to improve antibody-mediated opsonization, such as passive or active immunization, might be beneficial in preventing or altering the course of infections due to B. pseudomallei.

Bacterial agglutination is a defense mechanism whereby antibodies recognize and bind to surface antigens on multiple
highly immunogenic portion of PalBp is an important result and, abscess formation. Therefore, identification of epitope 3 as a bacterial clearance was demonstrated via splenomegaly and of mice prolongs median survival (Hara et al., 2009), incomplete epitope 3 represents a highly immunogenic region of Pal Bp microbes (Pal’tsyn et al., 1999). In support of our findings that also renders the bacteria, residing in the middle of the cluster, cytometry (B) and total killing of (B and C) Overall percentages of phagocytosis and oxidative burst by flow upon incubation with PMA. Phagocytosis is analyzed measuring the fluorescence intensity (FI) of SYTO 9 in human neutrophils after incubation with labeled bacteria. The positive control for oxidative burst is evaluated measuring the FI of hydroethidine (HE) in human neutrophils after incubation with labeled bacteria. The positive Phagocytosis is analyzed measuring the fluorescence intensity (FI) of SYTO 9 and (C) from three healthy subjects, assayed in the absence or presence of anti-epitope 3 are shown. Data represent means ± SE; *p < 0.05.

bacteria, which is observed microscopically as bacterial aggregates. Agglutination of bacteria, in this way, enables more efficient clearance of the bacteria by phagocytes, as well as hindering the bacteria from translocation through tissue barriers where they could cause more serious infection (Bull, 1915). It also renders the bacteria, residing in the middle of the cluster, devoid of oxygen and nutrients, thus facilitating death of the microbes (Pal’tsyn et al., 1999). In support of our findings that epitope 3 represents a highly immunogenic region of PalBp (being recognized to the same extent as the recombinant protein in sera from recovering human melioidosis patients and eliciting bactericidal antibodies), we also demonstrated that anti-epitope 3 antibodies specifically agglutinate B. pseudomallei. Interestingly, in both agglutination and OPK tests, antibodies raised against epitope 3 were active to a greater extent in comparison with antibodies generated against the recombinant protein. This could be due to the smaller recognition sequence, or this epitope could be more efficiently exposed to the host immune system. Although PalBp strongly reacts toward anti-sera from B. pseudomallei-infected humans and animals and immunization of mice prolongs median survival (Hara et al., 2009), incomplete bacterial clearance was demonstrated via splenomegaly and abscess formation. Therefore, identification of epitope 3 as a highly immunogenic portion of PalBp is an important result and, in contrast to the full-length protein, it may offer enhanced protection in mice, and ultimately humans, against B. pseudomallei.

Figure 5. Anti-EPITOPE 3 Increased Phagocytosis, Oxidative Burst, and B. pseudomallei Killing by Human Neutrophils

Since PalBp has been shown to offer only partial protection in mice immunization experiments, it is unlikely to represent a vaccine candidate alone; it is nevertheless an acute phase protective antigen and, together with other antigens, including chronic stage ones, its effects could be potentiated in a multi-stage vaccine, as exemplified by the recent example of the M. tuberculosis vaccine (Aagaard et al., 2011; Hara et al., 2009). Furthermore, our findings stress the concept that it is possible to (1) employ single immunogenic peptides as vaccine components, (2) use synthetic peptides to produce specific antibodies to be employed as adjuvant therapy in acute cases, and (3) exploit peptide-antibody recognition for diagnostic purposes when strong specificity and sensitivity is established. Thus, epitope 3 could be used as an alternative to full-length PalBp, considering that antigenic peptides may be easily conjugated to other antigens, bypassing more complex design schemes, such as the production of chimeric subunit vaccines based on whole proteins. Moreover, epitope peptides, coupled to gold nanoparticles that act as antigen-presenting platforms, provide an innovative area that is under consideration for vaccine development; such an approach may foster the design and application of whole libraries of antigenic peptides, pertaining to diverse antigens, for the production of multivalent vaccines (Fujita and Taguchi, 2011). For melioidosis, generating a library composed of antigenic peptides from acute and chronic phase antigens may prove the most fruitful approach.

As a final consideration, our structure-based in silico epitope prediction pipeline, coupled to validation experiments, could be applied to screening several antigen structures, determined directly or via homology modeling, in order to generate large databases of potential epitopes for subsequent synthesis as peptides and for immunological testing. Such an approach, which presently requires further validation on a larger set of antigens, would represent a significantly improved alternative to conventional means of epitope identification.

SIGNIFICANCE

Our key finding is the prediction, identification, synthesis, and immunological validation of a highly immunogenic epitope based on the combined experimental and computational analysis of the crystal structure of PalBp, an acute phase protein antigen from B. pseudomallei, the Gram-negative bacterium responsible for melioidosis. Using this approach, we identified a consensus epitope from the antigen structure that, when synthesized as a peptide, was selectively recognized in sera from melioidosis-affected subjects and elicited bactericidal antibodies with improved immunological activities relative to the native antigen. Such validation experiments on cell cultures show that our current knowledge on protein structure, dynamics, and physicochemical properties is mature enough to support productive experimentation in the structural vaccinology field. For PalBp, the next step will be to test the epitope in vivo immunization tests, either alone or conjugated to other acute and/or chronic stage antigens. Our structural vaccinology approach is of general validity and can immediately be extended to other known antigen structures to
create libraries of antigenic peptides not only for testing and validation in the discovery of vaccine candidates, but also for the development of immunodiagnostic tools.

EXPERIMENTAL PROCEDURES

Chemicals
All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

PalBP Production, Crystallization, Data Collection, and Structure Determination
The BP3L2785 gene was amplified from B. pseudomallei strain K96423 genomic DNA, cloned into pET-14b (Novagen), and expressed as a His-tag fusion protein in BL21 Star (DE3) cells (Invitrogen) (Figure S1). The protein was purified by affinity chromatography on a 1 ml HisTrap FF column (GE Healthcare). Following thrombin cleavage of the His-tag, purified PalBP was crystallized using the sitting drop vapor diffusion method (see Supplemental Experimental Procedures for more details). Diffraction data were collected at a resolution of 2.3 Å at the European Synchrotron Radiation Facility (Grenoble, France) beamline ID14-1. The 3D structure of PalBP was solved by molecular replacement using the structure of the periplasmic domain of the Pal protein from E. coli (PDB entry 1OAP; unpublished data) as a search model (50% sequence identity), as described in Supplemental Experimental Procedures.

Molecular Dynamics Simulations and Analyses
The simulation and analysis of the PalBP crystal structure were performed using the GROMACS 4.51 software package (Hess et al., 2008), GROMOS96 force field (van Gunsteren et al., 2006), and the SPC water model (Berendsen et al., 1987). See Supplemental Experimental Procedures for details.

MLCE Epitope Predictions
Epitope predictions were carried out on the representative structure of the most populated structural cluster obtained using the method developed by Daura et al. (1999). The MLCE method (Scarabelli et al., 2010) is based on the eigenvalue decomposition of the matrix of residue-residue energy couplings, available as the free web tool BEPPE (http://bioinf.uab.es/BEPPE). For more detailed information, see Supplemental Experimental Procedures.

EDP Epitope Predictions
In order to increase the consistency of the epitope predictions, the MLCE output was intersected with the EDP output for consensus surfaces. The EDP method calculates the free energy penalty for desolvation placing a neutral probe at various protein surface positions. Surface regions with a small free energy penalty for water removal may correspond to preferred interaction sites. Evidence suggests that it is easier for an antibody to bind to an epitope when properties required for high-affinity binding like low desolvation penalty are met (Westhof et al., 1984).

Experimental Epitope Mapping with Polyclonal Antibodies
Peptide mixtures were obtained by digestion with trypsin and endoproteases, LysC and GluC, followed by immunocapture of the epitope-containing peptides on Dynabeads Pan Mouse IgG. Subsequent MALDI-MS analysis of captured fragments was carried out to identify their sequences. See Supplemental Experimental Procedures for a full description of the entire procedure.

Synthesis of Epitope Peptides
Epitope sequences for peptide synthesis were designed based on a comparison of the consensus results of the two computational predictions and experimental epitope mapping. All three peptides (epitopes 1–3) were produced via solid-phase synthesis.

In view of their use as immunogens to induce antibody production, the original peptides were elongated in two cases (epitopes 1 and 2) by including flanking residues at the N and C termini, based on the consideration that longer sequences induce better immunogenic responses (Purcell et al., 2007). A cysteine residue preceded the PEG group to facilitate conjugation to HSA, the carrier protein. All peptides were prepared in free- and HSA-conjugated forms. Epitope 2 is a discontinuous conformational epitope composed of two short peptides that are brought close together in the tertiary structure. Measurement of the average distance between the termini of patch 1 from MD simulations suggested that insertion of a PEG (molecular weight, 308.16) moiety would be sufficient to bridge the two short sequences.

Figure 6. Agglutination of B. pseudomallei after Exposure to Antibodies Raised against PalBP or Epitope 3
RFP-expressing B. pseudomallei or E. coli were incubated with 1 μg of antibodies for 30 min at 37°C. Bacterial agglutination was observed under epifluorescence microscopy, and the images are representative of three independent experiments. Red arrows indicate agglutination of B. pseudomallei after incubation with PalBP. Magnification = x 100. All controls were negative for agglutination with B. pseudomallei, and all samples incubated with E. coli showed the same amount of background agglutination, which can be attributed to natural agglutination.
Peptide Conjugation to Carrier Proteins
Epitope 3 peptides were specifically conjugated to freshly prepared maleimido-activated hemocyanin from Concholepas and rabbit serum albumin using sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) according to the procedure described by Liu et al. (1979).

Serum PalBP Antibody Concentration Determination
The concentration of antigen-specific antibodies in the terminal immune sera was determined by ELISA as described in the Supplemental Experimental Procedures.

Agglutination Assay
RFP-expressing K96243 (Wand et al., 2011) or E. coli 29522 (American Type Culture Collection), as negative control, were subcultured from overnight broths into LB and grown to log phase. Bacteria (1 × 10⁸) were incubated with either 1 µg PalBP anti-sera or 1 µg antibodies toward epitope 3 reconstituted in PBS. Controls were prebleed from the immunized rabbit, B. pseudomallei capsule antibodies 4V1H12 (Cuccui et al., 2012), and PBS alone. Incubation was performed static at 37°C for 30 min. A 30 µl aliquot of culture was placed onto a glass cover slip and allowed to air dry before fixing in 1% paraformaldehyde for 10 min. Coverslips were washed three times in PBS and mounted onto glass microscope slides using Vector shield hardset mounting medium with DAPI (Vector Laboratories). Epifluorescence microscopy was used to discern bacterial agglutination (Zeiss).

Human Plasma Sample Preparation
Plasma samples were collected from healthy volunteers (n = 19) at the Blood Bank Center, Khon Kaen University, and from recovered melioidosis patients (n = 20) at Sirinakarin Hospital, Khon Kaen University and Ubon Ratchathani, Northeast Thailand, with informed consent. Ethical permission was obtained from Ethical KKU research no. HE470506. For details, see Supplemental Experimental Procedures.

Indirect ELISA to Detect Antibodies to B. pseudomallei
Antibody recognition of the three synthesized peptides (epitopes 1–3) was determined using indirect ELISA using 96-well microtiter plates (Nunc Maxisorp) with either 1 µg PalBP anti-sera or 1 µg antibodies toward epitope 3 reconstituted in PBS. Controls were prebleed from the immunized rabbit, B. pseudomallei capsule antibodies 4V1H12 (Cuccui et al., 2012), and PBS alone. Incubation was performed static at 37°C for 30 min. A 30 µl aliquot of culture was placed onto a glass cover slip and allowed to air dry before fixing in 1% paraformaldehyde for 10 min. Coverslips were washed three times in PBS and mounted onto glass microscope slides using Vector shield hardset mounting medium with DAPI (Vector Laboratories). Epifluorescence microscopy was used to discern bacterial agglutination (Zeiss).

Human Plasma Sample Preparation
Plasma samples were collected from healthy volunteers (n = 19) at the Blood Bank Center, Khon Kaen University, and from recovered melioidosis patients (n = 20) at Sirinakarin Hospital, Khon Kaen University and Ubon Ratchathani, Northeast Thailand, with informed consent. Ethical permission was obtained from Ethical KKU research no. HE470506. For details, see Supplemental Experimental Procedures.

Neutrophil Isolation
Human neutrophils were freshly isolated from heparinized venous blood by 3.0% dextran T-500 sedimentation and Ficoll-PaquePLUS centrifugation (Amersham Biosciences). In general, neutrophil purity was >95%, as determined by Giemsa staining and microscopy, while cell viability was >98%, as determined by trypan blue exclusion (Egan and Gordon, 1996).

Labeling of Bacteria with SYTO9
Live B. pseudomallei at a concentration of 1 × 10⁷ CFU/ml were incubated with 5 mM SYTO9 Green-Fluorescent Nucleic Acid Stains (Syto9) (Invitrogen) in the dark at room temperature for 30 min, and Syto9 labeled live B. pseudomallei intensity was analyzed by flow cytometry (FACSCalibur; BD Biosciences) prior to use. Syto9-labeled bacteria were used only once and discarded after use.

Phagocytosis and Oxidative Burst Assayed by Flow Cytometry
Phagocytosed neutrophils were treated with medium alone or anti-epitope 3 antibody at concentrations of 0.1, 1, and 10 µg/ml for 30 min, before incubation with Syto9-labeled live B. pseudomallei, at a multiplicity of infection (MOI) of 1:1 for 60 min or with 800 ng/ml PMA (Sigma) as a positive control for oxidative burst for 15 min at 37°C; then 25 µl of a 2,800 ng/ml hydroethidine (Sigma) solution was added and the preparation was incubated for 5 min at 37°C. Cells were washed twice and fixed with 10% paraformaldehyde for decontamination prior to analysis by flow cytometry (FACSCalibur; BD Biosciences) (Chanchamroen et al., 2009).

Total Bacterial Killing Assay
Purified neutrophils were treated with anti-epitope 3 antibodies as described above prior to infection with B. pseudomallei at a MOI of 1:1 and incubated at 37°C for 60 min. Then, infected cells were lysed by 1% Triton X-100 and total bacterial killing was determined by standard bacterial plating on Luria-Bertani agar plates and count the numbers of bacterial colony after 24 hr.

ACCESSION NUMBERS
The Protein Data Bank (http://www.pdbe.org) accession code for atomic coordinates and structure factors relative to the crystal structure of PalBP, reported in this paper, is 4B5C.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.07.010.

ACKNOWLEDGMENTS
This project was supported by Fondazione CARIPLO (Progetto Vaccini, contract number 2009-3577) and by MIUR PMN 2008 grant 2008K37RHP. L.J.G. is a recipient of Assegno di Ricerca (2012) from the University of Milan.

Received: April 15, 2013
Revised: June 14, 2013
Accepted: July 23, 2013
Published: August 29, 2013

REFERENCES

Chemistry & Biology 20, 1147–1156, September 19, 2013 ©2013 Elsevier Ltd All rights reserved

1155


