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Development of a multifunctional tool for drug screening against Plasmodial protein-protein interactions via Surface Plasmon Resonance

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Abstract

We have developed an expression system capable of producing large quantities of low cost, specific peptides that are either His₁₂-tagged, biotinylated or unlabeled. The flexibility of this peptide system is suitable for interaction studies via Surface Plasmon Resonance (SPR), co-crystallization and ELISA. Gene blocks containing peptide sequences of interest in addition to a 15 amino acid AviTag, were cloned into a vector expressing an N-terminal maltose binding protein (MBP). The constructs were expressed, purified, and the molecular weights of the recombinant proteins estimated by analytical size exclusion chromatography. Successful *in situ* biotinylation of the AviTag was confirmed by anti-biotin Western blot and used for coupling to the SPR chip. We were able to validate, as a proof of concept study, the specific protein-protein interaction of *Plasmodium falciparum* Aldolase (PfAldolase) with three different cytoplasmic adhesin tail peptides (TRAPs) and determine their affinities. This method of peptide production enables high yield production of peptides in a two-day, cost effective manner. This tool will allow us to screen for protein-protein interaction inhibitors directed towards the liver stage and blood stage complexes of the glideosome in *Plasmodium* species. Adaptation of this tool will allow researchers to pursue their own studies of protein-protein interactions.

Keywords

Surface Plasmon Resonance; AviTag; Protein-protein interaction; Crystallography; purification; Plasmodium; drug screening

Introduction

Malaria has been a human health concern for centuries, particularly in tropical and subtropical regions of the world. Nevertheless, our repertoire of measures to fight the disease has been very limited (Baird 2005), and emerging resistance of the malaria parasite *Plasmodium* has further restricted the use of current therapies. The most recent reports indicating artemisinin resistance in Cambodia (Jambou and others 2005; Phyo and others 2012; Saralamba and others 2010) are alarming, and underscore the critical importance of exploring novel pathways for interfering with the life cycle of the malaria parasite.

Plasmodium, and other protozoan parasites belonging to the phylum Apicomplexa, progress through their life cycle by invading new host cells. The underlying mechanism of invasion is

a complex process involving the glideosome, a multi-protein molecular machine that governs parasite-gliding motility through an Actin-Myosin motor complex (Baum and others 2006; Sibley 2010). Our structural and inhibitor design studies performed with recombinant *Plasmodium* proteins have visualized important protein interfaces within the glideosome (Bosch and others 2007a; Bosch and others 2012; Bosch and others 2006; Bosch and others 2007b).

The glideosome is highly conserved within apicomplexan species (Baum and others 2006; Sibley 2010) and essential at all stages of the parasite life cycle. A tetrameric complex of Aldolase connects the motor to the cytoplasmic portion of its plasma membrane anchor, a transmembrane adhesive protein of the TRAP family (thrombospondin-related anonymous proteins) (Bosch and others 2007a). In this study we mimic the cytoplasmic tails by binding recombinant peptides to a Surface Plasmon Resonance (SPR) chip surface and measure the binding properties of *Plasmodium falciparum* Aldolase (PfAldolase) to each peptide (Figure 1A). We investigated the binding of TRAPs involved in different stages of the parasite's life cycle and from multiple species. The TRAP tails studied included *P. falciparum* and *P. vivax* TRAP (PfTRAP/PvTRAP), utilized by sporozoites in liver stage invasion, and *P. falciparum* merozoite TRAP (PfMTRAP), utilized by merozoites in blood stage invasion.

We developed this tool as a proof of concept for short peptide expression in *E. coli* that is a cost effective alternative to commercial peptide synthesis. Peptides are expressed at the C-terminus of the maltose binding protein (MBP) with a 15 amino acid AviTag, allowing for biotinylation *in situ* at a single lysine (Ashraf and others 2004; Beckett and others 1999). Our construct can be used with NTA-coated SPR chips, due to an additional His₁₂-tag insertion, for preliminary binding experiments and to establish mild regeneration protocols for the dissociation of the two interacting partners. Once suitable conditions are established, the His₁₂-tag and the MBP-tag can be removed using tobacco etch virus (TEV) protease, resulting in a shorter, biotinylated peptide. Using immobilized Neutravidin or Streptavidin on a CM5 chip (GE Healthcare), the biotinylated peptide can be captured for further interaction studies via SPR. If the desired peptide is to be utilized for crystallization purposes, removal of all N-terminal residues, except of a glycine and proline, can be achieved through use of Prescission protease (Figure 1A).

Materials & Methods

Development of a multifunctional tool for expression of short peptides in *E. coli*

A previously modified pRSF 1-b vector (Novagen), carrying the gene for MBP followed by a TEV recognition site and NcoI/BamHI restriction sites for cloning, was used as a vector to introduce short peptide sequences containing NcoI/BamHI restriction sites (see Supplemental Material). The resulting gene product, including His₁₂-tags, AviTag and Prescission and TEV sites, is described in Figure 1A. Three gene blocks, containing sequences for the cytoplasmic tails of *Plasmodium* parasite adhesins, were selected for this study and commercially synthesized (Figure 1B).

2 µg of pRSF-1b-MBP plasmid was double digested using NcoI/BamHI. Linearized plasmid was gel purified and quantified by absorption at 260 nm. The commercially obtained gene

blocks (IDT) were double digested with NcoI/BamHI and ligated into the linearized vector using an insert to plasmid molar ratio of 10. Clones carrying the desired insert were sequence verified prior to test expression.

50 ml of TB media was inoculated from a single colony of BL21(DE3) cells carrying one of the cytoplasmic tails fused to MBP. Cells were cultured in the presence of 1% glucose and 50 µg/ml kanamycin at 37°C and 250 rpm until the expression of the gene was induced at OD₆₀₀=3 with 0.5 mM IPTG. Culturing continued for a minimum of 10 hours at 20 °C and 250 rpm. Cells were lysed in PBS buffer (pH 7.4) at 15000 PSI using a cell disruptor (Avestin), and the cell debris was pelleted at 40000xg for 20 minutes at 4°C. The supernatant was allowed to incubate with 1 ml of PBS buffer equilibrated TALON resin (Clontech) in gravity columns for 40 minutes at 4°C. Bound protein was subsequently eluted with PBS buffer supplemented with 400 mM Imidazole. The purity of the proteins was verified using SDS-PAGE and biotinylation was confirmed through western blot using an anti-biotin antibody (Cell Signaling Technology). The fusion products were cleaved over night using TEV protease in a molar ratio of 1:100 (TEV:protein). Cleaved products were separated from MBP and uncleaved product using a 10 kDa MWCO concentrator (Amicon, Millipore) and collecting the flow through. The individual method of purification may vary depending on the size and hydrophobicity of the desired biotinylated peptide. Alternatively, peptides can be purified using reversed phase HPLC or size exclusion chromatography. Samples of each cleaved product were analyzed by a Superdex Peptide 10/300 column (GE Healthcare) to estimate their molecular weight using 20% Acetonitrile with 0.1% TFA (v/v) as running buffer.

Surface Plasmon Resonance (SPR) assay

Pilot studies of the interaction and follow-up regeneration protocols were established using the His₁₂-coupled peptides on an NTA-CM5 chip. If a regeneration protocol failed to result in at least 90% recovery of the initial response prior to injection of the analyte, the surface was stripped by chelating the Ni²⁺ from the NTA groups on the chip surface using a buffer containing 10 mM HEPES, pH 8, 100 mM EDTA, 1% SDS. The pre- scouting of regeneration conditions using an NTA-CM5 chip can save valuable time and resources prior to covalently linking the ligand to a chip.

All four flow cells of a CM5 chip (GE Healthcare) were activated using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 7 minutes. A solution of 40 µg/ml Neutravidin in 10 mM sodium acetate, pH 5.0, was passed over the four flow cells for 7 minutes using a flow rate of 30 µl/min. Activated free binding sites were blocked using 1 M ethanolamine for an additional 7 minutes. Each of the three TEV-cleaved, biotinylated peptides was passed over an individual flow cell for 2 minutes at a concentration of 0.5 µg/ml, leaving one flow cell unmodified as a reference cell, only coated with Neutravidin.

All SPR assays were carried out in 10 mM HEPES, 150 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.2% Tween 20, adjusted to pH 7.5. Prior to performing the assay, solutions were sterile filtered and degassed for 1 h. Measurements were carried out at 25°C in triplicate. Samples were injected at 40 µl/min with 2 minutes of contact time followed by a 6 minute

dissociation and a single 25 μ l regeneration injection. 10 mM HEPES, pH 8.2 with 1% SDS was used as a regeneration buffer after each injection. The SPR data was analyzed with Scrubber 2.0 (BioLogic Software) using double referencing.

Results

Size exclusion characterization of the cleaved peptides

The molecular weights for the biotinylated peptides of PfTRAP (4586 Da), PvTRAP (4516 Da) and PfMTRAP (4722 Da), were estimated from an analytical peptide size exclusion column and compared to the calculated molecular weights. The resolution of the column was not sufficient to discriminate between the individual cytoplasmic tails; however, the peptides migrated at an estimated mass larger than 4200 Da (Figure 2A). Additionally, the Western blot of the purified samples confirmed biotinylation of the ~4.2 kDa peptides (Figure 2B). The yield from a 50 ml TB culture of the MBP-fused product ranges between 9.5 – 18.8 mg with purity of ~90% when using a tandem affinity purification step (TALON followed by Amylose capture, data not shown). Reduction of an anti-biotin positive, larger molecular weight species, indicates a near-complete TEV cleavage reaction in the samples treated with TEV protease (Figure 2B). After TEV cleavage and concentrator purification, purity of the peptide is >90% (Figure 2C).

Functional protein-protein interaction studies with cytoplasmic tails derived from *Plasmodium*

To validate that our recombinant peptides were correctly expressed, we investigated the established protein-protein interaction of PfAldolase with TRAP (Bosch and others 2007a; Pal-Bhowmick and others 2012). PfAldolase was expressed and purified as previously described (Bosch and others 2007a). Eight, three-fold dilution series ranging from 300 nM to 45 pM of PfAldolase, were passed over all four flow cells of the CM5 chip conjugated with either PfTRAP, PfMTRAP, PvTRAP or no protein, respectively. Our observed K_D of 115 nM for recombinant PfTRAP closely matches the previously measured K_D of 106 nM by Pal-Bhowmick *et al.* (Pal-Bhowmick and others 2012), despite using a slightly different buffer system. In their study, a synthetic, biotinylated peptide was used to determine the K_D . Our observed dissociation constants for PfMTRAP and PvTRAP are 91 nM and 140 nM, respectively. These dissociation constants confirm a specific interaction for all three recombinant peptides with PfAldolase (Figure 3).

Discussion

With successful measurement of a specific PfAldolase/PfTRAP interaction, we infer that C-terminal peptide degradation did not occur during expression or purification, as the last three C-terminal residues, Asp604-Trp605-Asn606, of PfTRAP are essential for interaction with PfAldolase (Bosch and others 2007a). We hereby demonstrate the functional expression and *in situ* biotinylation of short peptides suitable for SPR interaction studies using a simple, one-step purification method. Using this method, larger quantities of the peptides can be produced in a more cost effective manner compared to *de novo* peptide synthesis (Supplemental Material Table 1). After initial startup work and cost involving the synthesis

of the gene block, cloning and transformation, additional peptide production and purification can be accomplished in just two days compared to two weeks for commercial synthesis of peptides. Mutations in the cognitive part of these peptides may be used to functionally discriminate between essential and non-essential residues for the interaction. Generation of variants of the initial construct can be easily made through standard site-directed mutagenesis protocols. Peptide yield in this expression system is essentially limited only by the size of the culture that can be produced.

Moreover, if either longer peptides or small protein domains are required, this approach would be even more cost effective due to the limitations of conventional peptide synthesis platforms requiring chemical ligation, an added cost, to produce peptides over 30 amino acids in length. The current size limitation for the resulting peptide when using a single gene block is 113 amino acids plus tags and cleavage sites, without modifications to our plasmid. Insertion of the His₁₂-tag, AviTag, Prescission and TEV sites upstream of the NcoI restriction site, would facilitate cloning of longer sequences. Additionally, the gene block could be reorganized to facilitate tagging of the peptide at the C-terminus, in the case that the N-terminus needs to be available for interaction studies, whereas synthesized peptides are typically available with only an N-terminal biotin tag.

Pre-screening of optimal running buffer conditions and a suitable regeneration protocol for protein-protein interaction studies or peptide-protein interaction studies can be carried out by taking advantage of the His₁₂-tag and a Ni-NTA-CM5 chip. By doing so, a failure to regenerate the surface does not destroy a particular flow cell, as it can be stripped and fresh ligand attached. This regeneration ability is in contrast to a covalently linked peptide or protein, which might be permanently denatured under a chosen regeneration protocol and cannot be removed. Once mild regeneration conditions and optimal running buffers have been established, the recombinant peptide may be treated with TEV protease to remove the His₁₂-tag and subsequently be captured via the biotin tag on a Neutravidin coated chip.

Another advantage of the system is provided by the single site, *in situ* biotinylation of the target peptide or protein under physiological conditions. Harsh treatment of a peptide or protein through chemical modification is not needed with our system. Avoiding this treatment will be of most importance for biotinylation of folded domains. The ability to biotinylate a single site is also important for ensuring uniformly oriented peptides on the SPR chip. Moreover, specific attachment to Neutravidin via biotin can be carried out under near-physiological conditions, without the need for pH scouting, limiting the chances of protein unfolding or inactivation. The constraints of this system to produce active, biotin conjugated proteins and peptides is only limited by the ability to recombinantly produce the protein in *E. coli*.

Conclusions

Low cost, high expression yields of single-site biotinylated recombinant peptides are possible with this system, thereby facilitating the use in protein-protein interaction studies such as SPR or ELISA assays. Recombinant TRAP peptides were successfully expressed, purified, and used in conjunction with SPR to determine the affinity of each peptide for

PfAldolase. The ability to measure dissociation constants matching those in the literature, indicate that this system can be used for further Pf(M)TRAP/PfAldolase interaction studies; furthermore, we will be able to study via SPR how small molecules from drug library screens can disrupt or promote protein-protein interactions with the aim to inhibit invasion by the parasite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

(A) Analytical size exclusion chromatography plot with commercial peptides of known molecular weight. The inset shows the volume at which the three peptides eluted from the Peptide SEC column and their approximate derived molecular mass. (B) SDS-PAGE and anti-biotin Western blot confirmation of the biotinylated peptides and successful TEV-protease cleavage. (C) SDS-PAGE confirmation of TRAP peptide purification via concentrator.

Figure 3.

PfAldolase SPR binding curves to three adhesins from the *Plasmodium* parasite. Each measurement was carried out in triplicate for eight concentrations of PfAldolase. SPR binding curves ranging from 45 pM to 300 nM of PfAldolase to a (A) PfTRAP, (B) PvTRAP and (C) PfMTRAP chip surface. The fitted curve is plotted as a thin line in each of the sensorgrams for each measured concentration. (D) Concentration-dependent binding of PfAldolase to each of the adhesins and their associated dissociation constants.