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Differences in biochemical properties of the Plasmodial falcipain-2 and berghepain-2 orthologues: Implications for in vivo screens of inhibitors

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Abstract

Falcipain-2A, the cysteine protease of *Plasmodium falciparum* has been proposed as a good drug target. This study evaluated the suitability of *Plasmodium berghei* as the animal model and reports the first functional expression and characterization of the falcipain-2A orthologue, berghepain-2. Comparative studies revealed that the orthologues exhibited different biochemical properties. Berghepain-2 demonstrated optimal activity at a narrower pH optima of 5.5–6 and a lack of preference for substrates with leucine at position 2. Mutagenesis studies revealed roles for residues Val63 and Arg230 of berghepain-2 in contributing to its distinctive biochemical properties. This warrants re-evaluation of employing *P. berghei* as the murine model for the in vivo screening of falcipain-2A inhibitors. More importantly, these findings stress the underlying importance of establishing the functionality of relevant genes of *P. falciparum* with concomitant relevance to its murine counterpart prior to its use as the animal model for the screening of potential antimalarials.

Keywords: Falcipain; Berghepain; Animal model

1. Introduction

Falcipain-2A (FP2A), the cysteine protease of the most virulent strain of the malaria-causing parasite *Plasmodium falciparum*, has been receiving growing support as a potential anti-malarial drug target [1]. This is of even greater significance due to increasing drug-resistance in malarial parasites [2,3]. Drug discovery and development have thus gained more attention and emphasis over the last few years. Safe and affordable anti-malarials are urgently needed and FP2A inhibitors offer potential chemotherapeutic treatment to the millions afflicted with the disease.

The well-characterized hemoglobin-degrading function of FP2A is essential to the survival of the parasite [4,5]. Moreover, the low sequence identity (37%) together with several key differences between FP2A and its human homologues allow for differential inhibition of the host and parasite targets, making it an attractive drug target [1]. In vitro studies of inhibitors against FP2A using cultured human erythrocytes have shown their ability to block hemoglobin processing and correlate with retarding parasite development [6–9]. Several in vivo studies of FP2A inhibitors have also shown potential in curing mice infected with the plasmodial parasite [9–11].

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A critical stage in drug development is the use of animal models for evaluating the in vivo efficacy, potency and possible toxicity of drug candidates. *Plasmodium vinckei*-infected murine models have been employed in the screening of FP2A inhibitors, but growing evidence suggests that it may be unsuitable as the animal model. First, the in vitro and in vivo screens of several inhibitors have shown discrepancies in data [9,10]. Moreover, recent studies have demonstrated the existence of key biochemical differences between the human and rodent orthologues [12]. In a study by Lee et al., synthetic peptidyl aldehyde and α-ketoamide cysteine protease inhibitors were active against recombinant FP2A at nanomolar concentrations in vitro, but were significantly less efficacious against recombinant vinckepain-2, the FP2A orthologue of *P. vinckei* [9]. Since many of these drugs are synthesized to target the substrate binding pocket of FP2A, the discrepancy in data obtained could be attributed to the differences in substrate specificity between FP2A and vinckepain-2. This underscores the importance of establishing an accurate animal model for the in vivo screens of FP2A inhibitors. Hence, a further analysis of the rodent counterparts is warranted to select a suitable rodent plasmodial parasite bearing an orthologue as similar to FP2A as possible to conduct screens with greater accuracy.

Since *Plasmodium berghei* is currently most commonly used for in vivo screens of anti-malarials [13–16], we sought to evaluate its suitability as the rodent model used for screening FP2A inhibitors. This is the first functional expression and characterization of the *P. berghei* FP2A orthologue, berghepain-2 (BP2). It was found to exhibit different substrate specificities and a narrower range of pH optima as compared to FP2A. Further mutagenesis studies revealed that amino acid substitution at positions 63 and 230 (BP2 numbering) contribute to their different biochemical properties. These dissimilarities might lead to different sensitivity to inhibitors and must be taken into consideration during the drug screening process.

2. Materials and methods

2.1. Cloning of berghepain-2 gene (bp2)

A search of the National Center for Biotechnology Information (NCBI) *Plasmodium* database identified an ORF coding for BP2 (Accession No. AY063763). The gene encoding mature BP2 was PCR-amplified from *P. berghei* genomic DNA using specific primers (OL772 5′-GGATCCAAACTAAAAGAAAACCAGAGC-3′ containing a *Bam*HI site and OL774 5′-CTGCAGTTATTCAATTATAGGAGCATA-3′ containing a *Pst*I site). The amplified fragment was recovered using MinElute™ Gel Extraction Kit (Qiagen) and ligated into the pCR®-Blunt II-TOPO vector using the Zero Blunt™ TOPO™PCR cloning kit (Invitrogen). Multiple positive clones were purified using Wizard®Plus SV Minipreps (Promega) and subsequent dideoxysequencing in both directions confirmed the recombinant plasmids. Sequences were analyzed with BLAST at NCBI and ClustalX method.

2.2. Expression and purification of recombinant BP2

The positive recombinant plasmids were double digested with *Bam*HI and *Pst*I, gel-purified and subcloned into pMAL expression vector predigested using the same enzymes [17]. The construct was transformed into *Escherichia coli* BL21 (DE3) and DNA was isolated and sequenced from multiple positive clones for verification. A positive clone was selected, grown at 37 °C in 50 ml LB medium with ampicillin until the $O.D._{600}$ reached about 0.8. Cells treated with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce protein expression were grown overnight at room temperature. The culture was harvested by centrifugation, washed and resuspended in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). Cells were disrupted via sonication at an intensity of 12 μm for seven cycles of 7 s pulses with a 20 s rest period between each burst and subsequent centrifugation at 12,000*g* for 15 min yielded the cell-free extract. The recombinant protein was purified by amylose affinity chromatography according to the manufacturer's instructions (New England Biolabs). To obtain the cleaved BP2 fusion protein, autocleavage at 4 °C was employed as Factor Xa interfered with the *Z*-Val-Val-Arg-AMC substrate used in subsequent fluorogenic assays. Protein concentrations were determined by the Bradford assay (Bio-Rad) with bovine serum albumin (Sigma) as a standard and adjusted to a concentration of 0.1 mg/ml. Cell-free extracts and purified protein fractions were separated and analysed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prestained protein standard (Bio-Rad) was used as a marker.

2.3. Construction of BP2 mutants

Two mutant BP2 constructs V63S and R230N were generated using site-directed mutagenesis by a PCR mutagenesis strategy using QuikChange site-directed mutagenesis kit (Strategene) according to the supplier's instructions [18]. Primers used were (OL808 5′-GCTACCGCTGGTagTGTCGCAGCCCAATATG-3′ and OL809 5′-TGGGCTGCGACActACCAGCGGTAGCAAATG- 3′ for V63S; OL810 5′-CGTGGTTACATAaaTCTTAAGACTAACG- 3′ and OL811 5′-GTTAGTCTTAAGAttTATGTAACCACGTTC-3′ for R230N). The fragments were PCR-amplified from

the pMAL recombinant plasmids, gel-purified and treated with restriction enzyme *Dpn*I (New England Biolabs). The mutant constructs were transformed into *E. coli* and DNA was isolated and sequenced from multiple positive clones to confirm the introduced mutation. Expression, purification and analyses of the recombinant mutant proteins were performed as previously described.

2.4. Assays of BP2 activity

To assay for BP2 activity, gelatin-substrate SDS-PAGE assay was used. Protein samples were mixed with non-reducing SDS-PAGE sample buffer and resolved in polyacrylamide gel co-polymerized with 0.1% gelatin at 4 °C. The gel was washed twice with 2.5% Triton X-100 for 30 min at room temperature and incubated overnight at 37 °C in 100 mM NaOAc, 10 mM DTT, pH 5.5 before staining with Coomassie Blue. For specific substrate analyses, autocleaved BP2 was used and its specific activity was evaluated against peptidyl amidomethylcoumarins (AMC). Rates of hydrolysis of two fluorogenic peptide substrates carbobenzyl-L-leucine–arginine-7-amino-methylcoumarin (*Z*-Leu-Arg-AMC) and carbobenzyl-L-valine–valine–arginine-7-amino-methylcoumarin (*Z*-Val-Val-Arg-AMC) were measured with constant enzyme concentrations to determine enzyme activity and substrate specificity. Fluorometric assays were performed using 5 μl of enzyme solution (0.1 mg/ml) and 55 μl of assay buffer (100 mM NaOAc, 10 mM DTT), incubated for 15 min at room temperature. Forty μl of substrate was added and the release of AMC (excitation 360 nm, emission 465 nm) was assessed for 30 min at room temperature using a Tecan spectrofluorometer. Varying dilutions of substrate were used to determine the enzyme kinetics. Assay buffer of varying pH was used to determine the pH optima. Enzyme activities were measured as fluorescence over time (RFU/min) and the assays were repeated at least twice to ensure reproducibility.

3. Results

3.1. Cloning, expression and purification of BP2

The mature *bp2* gene was amplified from *P. berghei* genomic DNA and cloned into a pMAL expression vector. Soluble expression of the MBP-BP2 fusion protein was achieved and purification was performed using amylose affinity chromatography (Fig. 1). The gelatin substrate SDS-PAGE assay revealed that the fusion protein was not functionally active (Fig. 2). The recombinant cleaved BP2 protein obtained by autocleavage was functionally active as determined by the gelatin substrate SDS-PAGE assay and was employed for subsequent kinetics and specific substrate analyses (Figs. 2 and 3).

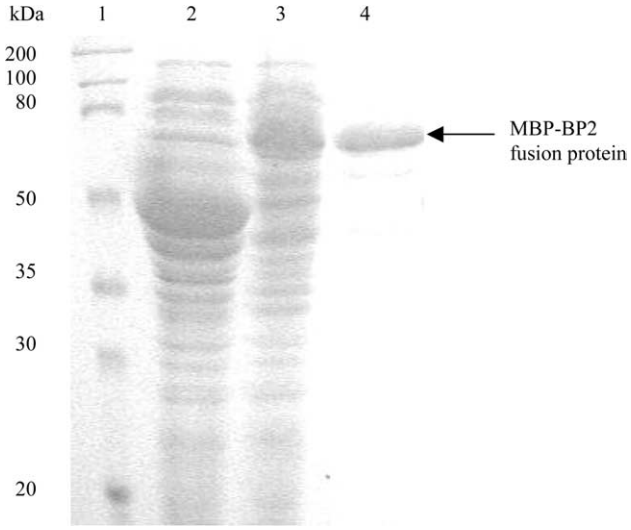

Fig. 1. SDS-PAGE analysis of expressed and purified fractions of berghepain-2 obtained from *E. coli* after expression at room temperature, overnight. Lane 1, molecular mass markers; lane 2, soluble cell-free extract of non-recombinant pMAL-c2x; lane 3, soluble cell-free extract of recombinant BP2; lane 4, purified soluble fraction of recombinant BP2 (70 kDa).

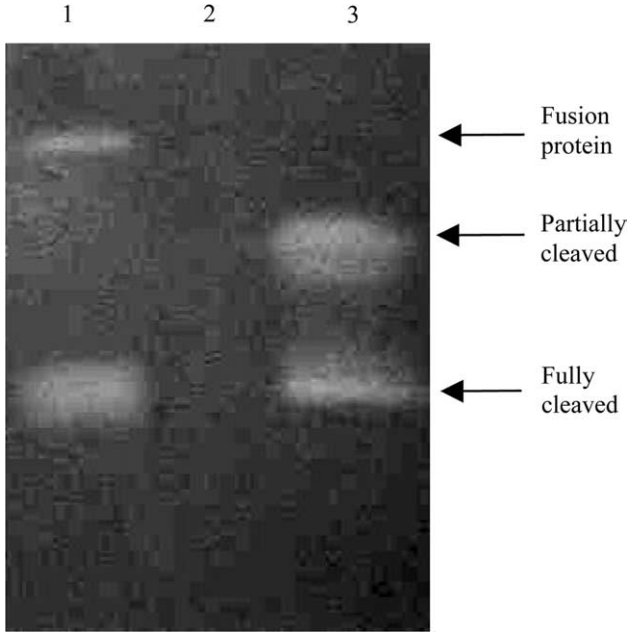

Fig. 2. Gelatin substrate SDS-PAGE assay of wild type berghepain-2 activity. Lane 1, FP2A; lane 2, wild type BP2 (inactive fusion protein); lane 3, wild type BP2 after storage at 4 °C for 4 days (autocleavage to active protein).

3.2. Biochemical properties of BP2

Consistent with FP2A and other members of the papain family, recombinant BP2 required reducing conditions for optimal activity [19]. However, fluorogenic assays performed revealed differing substrate specificities between the orthologues. Consistent with

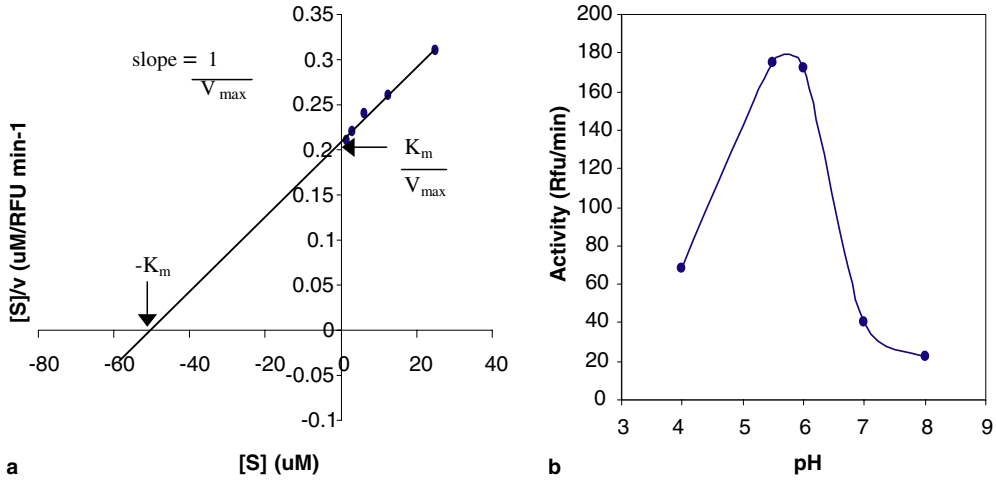

Fig. 3. (a) Substrate kinetics (Hanes–Wolff plot) of wild type BP2, K_m = 50 M, V_{max} = 240 RFU/min; (b) pH profile of wild type BP2, pH optima 5.5–6 as compared to FP2A, pH optima 5–7 [20].

previous reports, FP2A exhibited a preference for leucine at the P2 position [5], readily cleaving the peptide substrate *Z*-Leu-Arg-AMC. Activity against *Z*-Val-Val-Arg-AMC was distinctly lower. In contrast, BP2 readily hydrolyzed the tripeptide *Z*-Val-Val-Arg-AMC (160 RFU/min) but only little hydrolysis of *Z*-Leu-Arg-AMC was detected (30 RFU/min). Kinetic analyses of BP2 using its preferred substrate demonstrated K_m and V_{max} values of 50 M and 240 RFU/min, respectively (Fig. 3(a)). Moreover, in contrast to FP2A which has been reported to function optimally at both acidic and neutral pH [5,20], BP2 exhibited a narrower range of pH optima of 5.5–6 (Fig. 3(b)), with its activity decreasing sharply at pH 7 and above.

3.3. Substitutions of amino acid residues 63 and 230 contribute to the biochemical properties of BP2

Two BP2 mutants (V63S and R230N) were chosen after multiple alignment of BP2 with FP2A, vivapain-2 (VX2) and vinckepain-2 (VP2) using the ClustalX method (Fig. 4). As cysteine proteases VX2, VP2 and BP2 share a similar preference for the peptidyl substrate *Z*-Val-Val-Arg-AMC over *Z*-Leu-Arg-AMC, amino acids which were conserved among these orthologues but not in FP2A were identified as possible residues involved in preferential binding of the tripeptide. Alignment and analyses of the predicted secondary structures of FP2A and BP2 ensured that mutating the residues identified

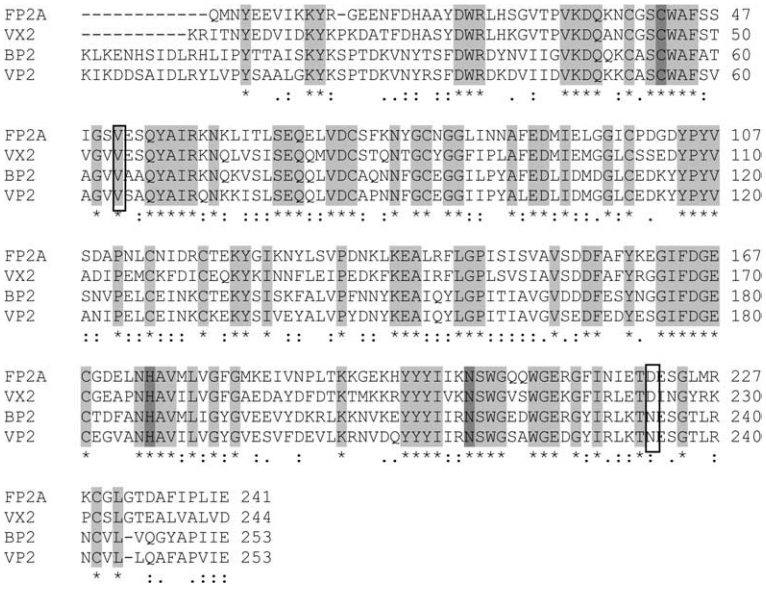

Fig. 4. Multiple alignment of the mature domains of berghepain-2 (BP2) with its orthologues falcipain-2A (FP2A), vivapain-2 (VX2), vinckepain-2 (VP2) using ClustalX. Conserved residues are shaded light grey. Conserved catalytic residues are shaded dark grey. Residues V63 and R230 of BP2 identified for mutagenesis studies are boxed.

would not disrupt the integrity of the protease (data not shown).

The mutant recombinants were obtained and assayed for activity (Figs. 5 and 6). Similar to wild type BP2, only the cleaved mutants were functionally active as determined by the gelatin substrate SDS-PAGE assay. Interestingly, R230N showed an absence of a zone of clearing at the position marked 'fully cleaved', indicating that the mutation introduced may have affected the protease's ability to fully autocleave its fusion partner. Fluorogenic assays performed revealed that both mutants exhibited low activity against *Z*-Leu-Arg-AMC, similar to that of wild type BP2. However, V63S exhibited a 70% decrease in activity against *Z*-Val-Val-Arg-AMC relative to wild type BP2 without a loss in general activity as demonstrated by the use of the gelatin assay. This indicates that the amino acid residue may be crucial for binding the tripeptide. The negligible activity of mutant R230N against *Z*-Val-Val-Arg-AMC can be attributed to the protease's inability to fully cleave off the fusion partner, where only the fully cleaved protein is active against the tripeptide. Together, these findings indicate critical roles for residues Val63 and Arg230 in substrate binding and the autocleavage of BP2, respectively.

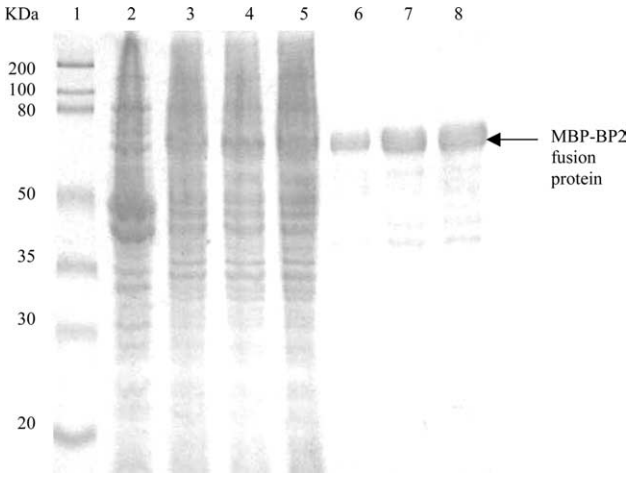

Fig. 5. SDS-PAGE analysis of expressed and purified wild type and mutant berghepain-2. Lane 1, molecular mass markers; lanes 2–5, soluble cell-free extracts of non-recombinant pMAL-c2x, wild type BP2, mutant V63S and mutant R230N, respectively; lanes 6–8: purified soluble fractions of wild type BP2, mutant V63S and mutant R230N, respectively.

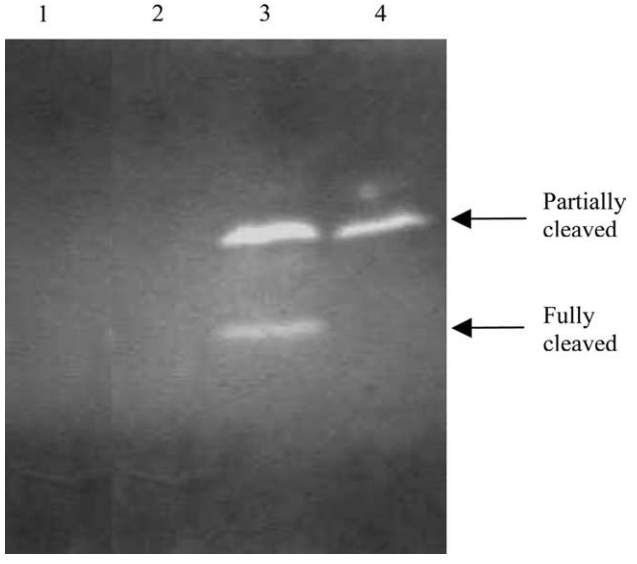

Fig. 6. Gelatin substrate SDS-PAGE assay of mutant berghepain-2 activity. Lane 1, BP2, lanes 2 and 3, V63S and R230N, respectively (before autocleavage); lanes 4 and 5, V63S and R230N after storage at 4 °C for 4 days (autocleaved protein).

4. Discussion

FP2A inhibitors have been identified as potential chemotherapeutic agents in combating malaria and are currently being developed as drugs. A crucial stage in drug development is the in vivo screens commonly employing murine models to evaluate the efficacy, potency and possible toxicity of the drug candidate. However, biochemical similarities and differences between human and rodent orthologues must be taken into account when interpreting in vivo experiments. Therefore, we sought to characterize BP2, the FP2A orthologue in *P. berghei* to assess its suitability as a rodent model for the screening of FP2A inhibitors.

We have cloned and solubly expressed BP2 and performed comparative studies with its closest orthologue in *P. falciparum* FP2A. Both proteases require an acidic pH optimum and a preference for reducing conditions, consistent with their principle function in the acidic food vacuole of the parasite. However, the unique ability of FP2A to function at neutral pH was not observed in BP2. The pH profile of BP2 displayed a narrower optimum range of pH 5.5–6, decreasing sharply at pH 7 onwards. As FP2A has been proposed to play a role in erythrocytic rupture by cleaving cytoskeletal proteins such as ankyrin and protein 4.1 at neutral pH, these findings indicate that BP2 may not be involved in the cleavage of cytoskeletal proteins and thus, erythrocytic rupture. It appears that BP2 may not fulfil the battery of functions of its counterpart FP2A. Consistent with the study done by Humphreys et al. [15], it appears that *P. berghei* may not represent an ideal model system for the in vivo analysis of inhibitors targeted against *P. falciparum*.

Another key biochemical difference observed between the proteases is their unique substrate specificities. In congruence with several other members of the papain family, FP2A demonstrates an optimal hydrolysis of substrates with leucine at position 2. Contrastingly, BP2 exhibited a preference for the tripeptide

Z-Val-Val-Arg-AMC. This difference in substrate profiles between the orthologues would have profound implications on the in vitro and in vivo screens of FP2 inhibitors, many of which target the substrate-binding pocket of the protease. Consistent with *P. vinckei*-infected murine models, our studies indicate that differences in biochemical properties between FP2A and BP2 may pose problems for drug screens and complicate the evaluation of the in vitro and in vivo data obtained.

In the absence of a crystal structure for FP2A and BP2 together with the inability to model BP2 currently, a rational approach was employed to identify residues potentially involved in conferring substrate specificity to the proteases [21]. Mutant V63S showed little hydrolysis of *Z*-Leu-Arg-AMC, similar to wild type BP2. However, a 70% decrease in hydrolysis of *Z*-Val-Val-Arg-AMC was observed in V63S relative to the wild type BP2, indicating a critical role of V63 in binding the tripeptide substrate. Valine, which contains a hydrophobic side chain, may be critical for forming specific hydrophobic interactions with the valine residues of the tripeptide substrate. Intriguingly, R230N was shown to be involved in autocleavage. Substitution of the positively charged arginine residue with an uncharged asparagine abolished the autocleavage ability of BP2, indicating that the hydrophilic residue is important in the complete autocleavage of the fusion partner. R230 may also be crucial for cleaving the prodomain of BP2 as its ability to autocleave in vivo has been documented [22].

Collectively, the mutagenesis studies performed may account for the unique biochemical properties of BP2. We propose that converting the substrate specificity of BP2 to a FP2A-like profile may involve a concerted mutation of multiple residues. Nevertheless, our data has provided new insights into the functional and biochemical properties of BP2 and provides the groundwork for future studies.

The finding that BP2 possesses key biochemical properties distinct from its orthologue FP2A highlights the need to re-evaluate *P. berghei* as the animal model for screening FP2A inhibitors. The differences in substrate specificity between the two proteases may complicate the evaluation of the data obtained from in vitro and in vivo screens. This underscores the need to characterize the other rodent plasmodial orthologues and identify the key intrinsic differences between the rodent and human orthologues. Nevertheless, as current animal models available for in vivo screening of FP2A inhibitors are limited, *P. berghei* might still prove to be invaluable. Key differences between the human and rodent orthologues however, must be taken into account when interpreting the in vivo screens. Together, these data stress the value of evaluating the suitability of a *Plasmodium* species as a murine model prior to its use for screening FP2A inhibitors. This would then positively aid in the establishment of more accurate in vivo screens for drug development.

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