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The reticulocyte binding-like proteins of *P. knowlesi* locate to the micronemes of merozoites and define two new members of this invasion ligand family

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Abstract

Members of the Reticulocyte Binding Protein-like (RBL) family are merozoite-expressed proteins hypothesized to be essential for effective invasion of host erythrocytes. Proteins of the RBL family were first defined as merozoite invasion ligands in *Plasmodium vivax*, and subsequently in *P. falciparum* and other malaria parasite species. Comparative studies are providing insights regarding the complexity and evolution of this family and the existence of possible functionally alternative members. Here, we report the experimental and bioinformatic characterization of two new *rbl* genes in the simian malaria parasite species *P. knowlesi*. Experimental analyses confirm that a *P. knowlesi* gene fragment orthologous to *P. vivax reticulocyte binding protein-1 (pvrbp1)* represents a highly degenerated pseudogene in the H strain as well as two other *P. knowlesi* strains. Our data also confirms that a gene orthologous to *pvrbp2* is not present in the *P. knowlesi* genome. However, two very diverse but related functional *rbl* genes are present and are reported here as *P. knowlesi normocyte binding protein Xa* and *Xb* (*pknbpxa* and *pknbpxb*). Analysis of these two *rbl* genes in Southern hybridizations and BLAST searches established their relationship to newly identified members of the RBL family in *P. vivax* and other species of simian malaria. Rabbit antisera specific for recombinant PkNBPXa and PkNBPXb confirmed expression of the prospective high molecular

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Note: Nucleotide sequence data reported in this paper are available in the EMBL, GenBank™ and DDBJ databases under the accession numbers **AY151130**, **EU867791**, **EU867792**, **DQ973816**, and **DQ973815**.

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weight proteins and localized these proteins to the apical end of merozoites. Their precise location, as determined by immuno-electron microscopy (IEM), was found to be within the microneme organelles. Importantly, PkNBPXa and PkNBPXb are shown here to bind to host erythrocytes, and discussion is centered on the importance of these proteins in host cell invasion.

Keywords

Plasmodium knowlesi; malaria; merozoite invasion; reticulocyte binding proteins; erythrocytes; apicomplexa

1. Introduction

Malarial merozoites gain entry into red blood cells through specific receptor-ligand interactions and a cascade of molecular interactions that are largely still undefined (reviewed in [1]). Moreover, merozoites can be characterized by their ability to invade erythrocytes of either all stages of maturation or by an evident restriction, only reticulocytes. The reticulocyte host cell specificity of human malaria *P. vivax* merozoites has been well known and attributed to the action of the Reticulocyte Binding Protein (PvRBP1 and PvRBP2) complex located at the invasive apical end of the merozoite [2–4]. Homologs of the *P. vivax* RBPs have since been characterized in the human malaria *P. falciparum* [5–9], non-human primate [10,11] and rodent malaria species [12]. This family of invasion ligand proteins is now known as the Reticulocyte Binding-Like (RBL) family. *Rbl* genes have a characteristic small exon encoding a signal peptide, a short intron, and a second exon encoding a large (230 – 350 kDa) predominantly hydrophilic protein with a transmembrane domain and short cytoplasmic tail (reviewed in [1]).

P. cynomolgi, which is a simian malaria parasite closely related to *P. vivax,* also invades predominantly reticulocytes and the orthologous genes for *pvrbp1* and *pvrbp2* have been reported [2,10]. In the rodent parasite *P. yoelii*, the Py235 gene family was shown to be most related to *pvrbp2* [4,13]. In *P. falciparum*, genomic and experimental studies revealed homolog genes that have been termed normocyte binding proteins (*nbp1, nbp2a, nbp2b*) [5,6], also referred to as reticulocyte-binding homologs 1, 2a and 2b (*rh1, rh2a* and *rh2b*) [5–7]. Other similar *P. falciparum* genes were subsequently characterized as *rh3/nbp3*, *rh4/nbp4*, and *rh5/nbp5*, with *rh3/nbp3* confirmed to be a pseudogene [8,9,14,15]. Interestingly, the chimpanzee parasite, *P. reichenowi*, has an identical composite of family members orthologous to the *P. falciparum* genes. However in *P. reichenowi*, *nbp1* is clearly a pseudogene without an open reading frame (ORF), while *nbp3* has an ORF and appears to be functional [16]. The RBL proteins known to date, though quite divergent in sequence, have shared structural and biological features that are presumed to be essential for the targeting of host cells and effective invasion. The initial identification and characterization of the *P. vivax* RBPs supported the hypothesis that merozoites require molecules to identify the appropriate target cells and then may signal the activation of subsequent receptor-ligand interactions involved in invasion [2–4]. It is now known that several other more distantly related *rbl* genes are present in *P. vivax* [17] and it remains to be determined whether the encoded proteins serve as alternative ligands, or if some are in fact pseudogenes, as suggested by the current gene sequences reported in the *P. vivax* genome database. The characterization of *P. falciparum* RBLs has provided additional information regarding the potential complexity of the RBL family and their role in merozoite invasion. *In vitro* experiments focused on generating *P. falciparum rbl* knock out parasites have begun to clarify the role of *rbls* in the cascading events of merozoite invasion. PfRh1/NBP1 is an important player in invasion through a sialic acid-dependent interaction [5,18]; however, this gene can be disrupted with continued survival of the parasites *in vitro* [18]. Disruption of *pfrh2a/pfnbp2a* and *pfrh2b/pfnbp2b* genes is also possible with the retrieval of

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live parasites, indicating that in some *P. falciparum* strains merozoite entry is mediated through a novel, alternative invasion pathway [19]. Recent studies have also reported regions of *P. falciparum* RBLs implicated in binding to erythrocytes [20–22].

Phylogenetic analysis based on the known *rbl* genes and encoded proteins show that they form subgroups that reflect specific relationships and a degree of similarity to the PvRBP1 or PvRBP2 prototype molecules ([10] and unpublished data). Though the overall identity between ortholog molecules in widely divergent species such as *P. vivax, P. falciparum* and *P. yoelii* is very low (23% – 30%), within a genetic clade such as between *P. vivax* and the Asian simian malarias or between *P. falciparum* and *P. reichenowi*, the degree of identity between orthologs within the same RBL subgroup increases dramatically to high levels of 75% or greater.

To better understand the functional role(s) and fine interactions of the RBL proteins in host cell selection and entry, we sought to identify and undertake a thorough investigation of the *rbl* genes and encoded proteins expressed in a simian malaria species, *P. knowlesi*, known to also naturally infect humans [23–27]. *P. knowlesi* is closely related to *P. vivax* and its sister parasite *P. cynomolgi* [28]. It is also very closely related to the simian malaria parasite *P. coatneyi*, which in many respects is phenotypically similar to *P. falciparum* [29]. As such, and given the stability of its merozoites [30], *P. knowlesi* has traditionally provided an exceptional instrument for the comparative study of the erythrocyte invasion mechanisms pertinent to both *P. vivax* and *P. falciparum*.

The *P. knowlesi* genome has recently been completed with 8-fold sequence coverage, yet the *P. knowlesi rbl* gene repertoire in this species remained in question, with a number of partial sequences annotated as fragments of putative *rbps* on chromosome 14, and no other genes recognized as members of this family [31]. Here, using a combination of experimental investigation and bioinformatics we definitively report the presence of three *rbl* genes in the *P. knowlesi* genome, and confirm that one is a pseudogene. The two expressed RBL members, PkNBPXa and PkNBPXb, have been investigated with regards to their structures and functions and localized by IEM to the microneme organelles of mature merozoites. The functional importance of these proteins in the context of the RBL superfamily, and the importance of *P. knowlesi* as a model for further investigations of the RBLs are discussed.

2. Materials and Methods

2.1. Parasite propagation and acquisition

P. knowlesi (H strain) infected erythrocytes were obtained fresh from *Macaca mulatta* monkeys or grown *in vitro* from cryopreserved samples and processed as described previously [32]. *P. vivax* Belem and Salvador I strain parasites were acquired from blood-stage infections in *Saimiri boliviensis* monkeys and processed to remove leukocytes and platelets prior to purification of infected erythrocytes. *P. coatneyi* originating from a 1961 isolation [33], *P. cynomolgi* (Berok strain) and *P. fragile* (Nilgiri strain), were expanded in *M. mulatta* monkeys for DNA isolation after removal of host cellular elements. All non-human primate experimental studies, infections and associated protocols were performed with the official approval of Emory University or CDC's Institutional Animal Care and Use Committee.

2.2. Nucleic acid isolation

Genomic DNA (gDNA) was prepared from blood-stage parasites using the QIAamp DNA Blood Extraction kit (Qiagen, Valencia CA), as described previously [2]. Total RNA was prepared from mature schizonts using TRIzol (Invitrogen, Carlsbad CA) following the manufacturer's protocol and treated with RNAase-free DNAase (Roche, Indianapolis IN).

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2.3. Polymerase Chain Reaction (PCR) amplification and cloning of *rbl* genes in *P. knowlesi*

PCR amplification using the Expand High Fidelity System (Roche, Indianapolis IN) was performed with gene-specific primers on gDNA from *P. knowlesi* following the manufacturer's instruction. Fragments were cleaned using the Qiaquick purification system (Qiagen, Valencia CA), cloned into the pCR2.1 vector (Invitrogen, Carlsbad CA) and sequenced using the ABI Prism BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City CA). DNA fragments for *pvrbp* and *pknbp* radiolabeled probes were generated by PCR amplification using primers based on the published *pvrbp1* (**M88097**) and *pvrbp2* (**AF184623**) gene sequences, data retrieved from database BLAST searches and new sequencing data. *Pknbp1* pseudogene (ψ*pknbp1)* amplicons were generated using three *P. knowlesi* strains as templates and primers were designed according to the sequence of a 1.5 kb DNA fragment identified from the H strain genome (F 5′ GGT CGA AAC ATA ATA CGG TG 3′ and R 5′ GGA ATT CGA TGG AGT TGA TT 3′). The complete *pknbpxa* and *pknbpxb* gene structures were constructed and finalized through a combination of genome database BLAST searches of *P. knowlesi* contigs and shotgun sequences and new sequence data generated from amplified DNA fragments corresponding to the *pknbp* genes.

2.4. Confirmation of *pknbpxa* and *pknbpxb* intron/exon junctions

The 5′ ends and the exon/intron junctions of the *pknbpxa* and *pknbpxb* genes were confirmed using a 5′RACE system and reverse transcriptase PCR, RT-PCR (Invitrogen, Carlsbad CA) following the manufacturer's recommendations. Two gene-specific primers were designed to produce cDNA, Nxa1R 5′ ATT CTG TCT ATC GTA GGA GC 3′ and Nxb2R 5′ TTG CTT CAC GGA TTT GCT 3′, followed by nested amplifications using the 5′ RACE Abridged Anchor Primer provided in the kit and gene-specific reverse primers, Nxa3R 5′ CCA ATA ATA ATT AAC AGA AG 3′ and Nxb4R 5′ TGG AGA TAG CCT CAA AT 3′.

2.5. Southern and northern blot analysis and library screening

1μg to 2μg aliquots of gDNA, digested overnight with designated restriction enzymes, were separated on 0.8% agarose gel by standard electrophoresis, essentially as described [2]. Fixation of DNA to the membranes was performed by either UV cross linking (Stratagene, La Jolla CA) or baking *in vacuo* at 80°C for 2 h. DNA fragments were labeled for hybridization reactions using the Prime-It II DNA labeling system (Stratagene, La Jolla CA) according to the manufacturer's instructions. Hybridization was performed as described previously [2]. Northern blots were prepared as described previously [34]. Blots were hybridized with radiolabeled probes in 7% SDS/0.5 M NaH_2PO_4, pH 7.2/2% dextran sulphate at 65°C overnight. The membrane was washed three times in 6XSSC/0.1% SDS, 2XSSC/0.1%SDS and 0.2XSSC/0.1%SDS for 15 min each time at 60°C. The signals were visualized by exposure to Kodak BioMax MS film (Kodak). *P. knowlesi Eco*RI-digested gDNA λ Zap II libraries were constructed and screened as described previously [2] and screened with a radiolabeled probe representing the 2.5 kb from the 5′ region of the *pvrbp*1 gene. Three positive clones containing a 1.5 kb *Eco*RI fragment were identified and sequenced, and subsequently compared and shown to be identical to sequence generated from the *P. knowlesi* genome sequencing project.

2.6. Pulse Field Gel Electrophoresis

P. knowlesi chromosomes were size fractionated by pulse field gel electrophoresis (PFGE) and sequential Southern blot analyses were performed using probes representing the central region of the *pknbpxa* and *pknbpxb* genes. Briefly, *P. knowlesi* chromosomes blocks were separated by PFGE in the CHEF-DR III system (BioRad, Hercules CA). Electrophoresis was performed in 0.8% chromosomal grade agarose using 1X TAE at 14°C. Chromosomes of *Hansenula wingei* were used as molecular mass standards. After electrophoresis the gel was denatured and transferred to a nylon support as described previously [2]. The *pknbpxa* and *pknbpxb* probes

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labeled by random priming were hybridized to the membranes following standard protocols and washed under high stringency conditions (2X SSC/0.1%SDS at 60°C).

2.7. Production of fusion proteins and rabbit antisera

Two recombinant His-tagged fusion proteins, rPkNBPXa and rPkNBPXb, were produced in the Gateway (Invitrogen, Carlsbad CA) and pET (Novagen, Madison WI) systems respectively. rPkNBPXa was produced as a 1.2 kb fragment by PCR amplification using primers rNBPXaF (5′ GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAC GTT GTT GAA AAC TGA A 3′) and rNBPXaR (5′ GGG GACCAC TTT GTA CAA GAA AGC TGG GTT GTT CAA TTT TCC TTG CAA ATC 3′). A positive recombinant clone was expressed by addition of 0.3 M NaCl for 3 h at 37°C. The inclusion bodies were purified as described previously [35], separated by SDS-PAGE and electro-eluted for 16 h. rPkNBPXb was produced as a 1.14 kb fragment by PCR amplification using primers rNBPXbF (5′ ctcgagAGCTTACGCAACATATTAAAC3′) and rNBPXbR (5′ggatccGTCATC ATCATCATTATCGTG3′). A plasmid with the expected sequence was expressed using 1mM IPTG in BL21-DE3 (Novagen, Madison WI) for 3 h at 37°C. The recombinant protein was bound to Ni-NTA agarose (Qiagen, Valencia CA) and purified under native conditions using 250 mM imidazol (Sigma, St. Louis MO). The purity of the eluted proteins was assessed by SDS-PAGE. The recombinant proteins were dialyzed against PBS before inoculation in New Zealand White Rabbits (Covance, Denver PA) for production of polyclonal antisera (Rab-anti-rPkNBPXa and Rab-anti-rPkNBPXb).

2.8. SDS-PAGE and western blotting

Infected erythrocytes containing mature, segmented schizonts where harvested and extracted with reducing sample buffer. The protein extracts were analyzed by SDS-PAGE on 5% polyacrylamide gels and transferred to protean nitrocellulose (Schleicher & Schuell, Keene NH) as described previously [2]. Membranes were probed with rabbit polyclonal antisera diluted 1:500, which had been depleted of antibodies against *E. coli* using standard adsorption procedures. Alkaline phosphatase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD) was used for detection (1:5000) and bands were visualized by adding NBT/BCIP substrate (Promega, Madison WI).

2.9. Immunofluorescence assays and immuno-electron microscopy

For indirect immunofluorescence assay (IFA), air-dried, thin blood smears prepared with mature schizont-stage parasites were incubated with rab-anti-rPkNBPXa, and rab-anti-rPkNBPXb polyclonal antisera diluted 1:500. Goat anti-rabbit IgG antibodies conjugated to FITC were added and the slides were visualized using a fluorescence equipped microscope. For immuno-electronmicroscopy, merozoites were added to rhesus RBC's, in the presence of 5 μg ml^{-1} cytochalasin B (Sigma, St. Louis MO), and were fixed in 0.1% (v/v) double-distilled glutaraldehyde and 2% (w/v) paraformaldehyde prepared in culture medium (RPMI, pH 7.2), for 20 min on ice, and then washed four times in ice-cold RPMI and dehydrated through a progressively low-temperature ethanol series before being infiltrated with LR White resin (EMSCOPE, London, United Kingdom). Resin polymerization was induced by ultraviolet light at room temperature for 48 h. Sections were immune-stained with the polyclonal sera anti-rPkNBPXa and rPkNBPXb, diluted 1:50 or 1:100 in 1% BSA/PBS, followed by Protein A conjugated to 10 nm gold particles, diluted 1:70 in 1% BSA/PBS (a kind gift by Dr Pauline Bennett, King's College London). Parallel samples were treated with pre-immunization serum antibodies for control purposes. Sections were stained for 4 min with 2% (w/v) aqueous uranyl acetate. Sections were viewed and digital images were taken using a Hitachi 7600 electron microscope.

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2.10. Erythrocyte Binding Assays

Erythrocytes infected with schizont stage parasites were purified by centrifugation on Percoll gradients, as previously described [36]. The purified schizont-infected erythrocytes were placed into culture *in-vitro* and allowed to grow overnight as previously described [1,5] until erythrocytes containing mature schizonts formed merozoites had completely ruptured. Cultures were centrifuged at 4000 rpm and the supernatants were removed and stored in liquid nitrogen. 500 mL of culture supernatants were rotated with 1×10^9 erythrocytes at room temperature for 4 h. The cells were washed twice by layering over and subsequent centrifugation through Dow Corning 550 silicone fluid. Bound proteins were eluted in 50 ul of 5× RPMI at room temperature and harvested by centrifugation. The resulting proteins were analyzed by SDS-PAGE on Pre-Cast 5% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules CA). Membranes were probed with rabbit polyclonal antisera diluted 1:500 for 2 h. Alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000 (Promega, Madison WI) was used for detection of bound antibodies. Bands were visualized by addition of NBT/BCIP substrate (Promega, Madison WI) to the nitrocellulose strips.

2.11. Bioinformatics and sequence analysis

TBlastN searches of preliminary and finalized *P. knowlesi* sequence database (8x coverage) at Sanger Center (http://www.sanger.ac.uk), the *P. vivax* sequencing project at TIGR Center (http://www.tigr.org) and PlasmoDB (www.PlasmoDB.org) database were performed by using translated sequence from *pvrbp1* and *pvrbp2* and the newly identified *pkrbl* genes. Signal peptide cleavage sites and transmembrane domains were predicted with SignalP V2.0 (www.cbs.dtu.dk/services/SignalPV2.1) and TMpred (www.ch.embnet.org/software/TMPRED_form.html) software, respectively. Multiple alignments of nucleic acid and protein sequences were generated using ClustalW 1.8.2 with all default parameters. Tree topology was generated using the Neighbor-Joining algorithm in the MEGA 2.2 software on distances calculated with a Poisson correction [37].

3. Results

3.1. The ortholog of *pvrbp1* is a highly degenerated pseudogene in *P. knowlesi,* but a functional full-length gene in the sister taxon *P. coatneyi*

To identify and characterize a *pvrbp1* ortholog in *P. knowlesi*, Southern hybridization assays were initially performed under high stringency conditions, and no hybridization signals were observed, suggesting that a highly related intact orthologous gene was not present in this species [2]. However, when a 2.5 kb *pvrbp1* probe representing the 5′ end of the gene was subsequently evaluated using lowered stringency conditions, clear distinct bands were detected in each gDNA sample digested with restriction enzymes (Fig. 1A). In contrast, *pvrbp1* probes representing other downstream regions of the gene, also evaluated at lower stringencies, did not result in hybridization signals (data not shown).

To evaluate this putative related but divergent ortholog further, the 1.5 kb *Eco*RI fragment detected by Southern hybridization was cloned and sequenced. Instead of an expected single ORF, multiple ORFs were detected. In addition, 1200 bases of sequence present in *pvrbp1* and the orthologous genes in *P. cynomolgi, P. coatneyi* and *P. fragile* [10], though expected, were not present in the degenerate *pknbp1* sequence. In conclusion, a total of 626 bp of the 1.5 kb cloned sequence showed identities of 80.2% and 82.3% when aligned with the *pvrbp1* sequence. The remaining 900 bp of downstream sequence showed no apparent relationship (<28% identity) to *pvrbp1* (Fig. 1B). DNA samples from the Hackeri and Philippine strains of *P. knowlesi* also hybridized with the 1.5 kb DNA probe (data not shown). Although some single nucleotide polymorphism was observed, as would be expected between strains of parasites,

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amplified gene fragments corresponding to the 1.5 kb *Eco*RI clones were essentially identical (99% identity) and also showed no continuous ORF.

Contig sequences recently made available in the *P. knowlesi* (H strain) genome database (http://www.sanger.ac.uk/Projects/P_knowlesi/) [31] are consistent with our experimental analyses, supporting the presence of a degenerated pseudogene. Our searches of the genome database identified an additional 200 bp upstream corresponding to *pvrbp1* sequence, but no related sequences were present downstream. Thus, we conclude that the ortholog of *pvrbp1* in *P. knowlesi* (H strain, Hackeri and Philippine strains) is a highly degenerate pseudogene, with only short segments of the 5′ region maintained in the genome for each strain tested.

P. coatneyi is a sister taxon of *P. knowlesi* [29], yet it displays unique biological and phenotypic characteristics distinct from *P. knowlesi* (reviewed in [38]). In contrast to *P. knowlesi* data, the Southern hybridizations suggested that a complete ortholog of *pvrbp*1 existed in the *P. coatneyi* genome (data not shown). To characterize the *P. coatneyi rbp1* (*pcrbp1)* gene, overlapping gene regions were amplified by PCR using primers designed from the *pvrbp1* sequence, cloned and sequenced (Genbank Accession number **DQ973816**). This sequence proved to be highly homologous to the *pvrbp1* gene, showing that *pcrbp1* is an intact, likely functionally expressed gene. The *pcrbp1* homolog exhibits a nucleotide identity of 84% with the *pvrbp1* gene, and the deduced protein sequence shows 75 % identity to PvRBP1. *P. fragile*, also a genetically close parasite in the simian malaria clade, has an intact and presumably functional nbp1 gene (Genbank Accession number **DQ973815**).

3.2. A *pvrbp2* ortholog is not present in the *P. knowlesi* or *P. coatneyi* genomes

To determine if a *rbp2* ortholog was in *P. knowlesi* (and *P. coatneyi*), enzyme restricted *P. knowlesi* H strain and *P. coatneyi* DNA were analyzed by Southern blotting hybridization using 5′ through 3′-end amplicons of the *pvrbp2* gene as probes. No distinct bands could be discerned using conditions of lowered stringency, which previously permitted easy detection of the ψ*pknbp1* gene or the *pvrbp1* and *pvrbp2* orthologs in *P. cynomolgi* [10] (Fig. 1B). These data indicate that no highly similar counterpart or ortholog genes for *pvrbp2* exist in *P. knowlesi* or *P. coatneyi*, and are at least for *P. knowlesi*, in complete agreement with BLAST searches of the *P. knowlesi* genome database [31].

3.3. Characterization of two novel *P. knowlesi rbl* genes, denoted *pknbpxa* and *pknbpxb*

To search for possible distantly related *rbl* genes in *P. knowlesi*, which may serve as alternatives to *pvrbp2*, we used a combination of methods including library screening based on *pvrbp2* probes, PCR amplification based on *pvrbp2* gene sequences and database mining. A probe of about 1.5 kb representing the 3′ end of the *pvrbp2* gene hybridized weakly to *P. knowlesi* gDNA in Southern blots using low stringency conditions (data not shown). This sequence was used to BLAST the *P. knowlesi* sequencing project database using both nucleotide and translated sequences. ORFs on two separate contigs showed significant, but not particularly strong scores by BLAST based searching. The sequences were used as templates for further probe design and PCR based amplification of gDNA. Screening *P. knowlesi Eco*RI digested-gDNA λ Zap II libraries identified two clones, *pknbpxa* and *pknbpxb*. The sequences of the two cloned DNA fragments were utilized to search the *P. knowlesi* sequence database to extend the primary sequences and fill in the gaps.

The *pknbpxa* clone contains an *Eco*RI-fragment of 6641 nt with an ORF of 5742 nt but missing the expected exon I, intron, and 5′ end of exon II. PCR from lambda phage and BLAST search of the *P. knowlesi* database provided enough further sequence to design gene-specific reverse primers for RT-PCR experiments and determination of the exon/intron junctions. The 9578 nt contig encodes exon I from nt 130 to nt 184, a short intron of 186 nt, and an exon II from nt

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371 to nt 8679. Thus, the total coding sequence of *pknpbxa* is 8364 nt. The predicted PkNBPXa protein is 2788 amino acids, the putative signal peptide cleavage site located between S27 and E28 and the transmembrane region predicted to be 21 amino acids long (amino acid 2724 to amino acid 2745) (Fig. 2A).

The *pknbpxb* clone contains 9479 nt with a single ORF of 8681 nt, but no clear typical small exon I or intron consensus splice site sequence. We therefore generated 5′ RACE clones to verify the 5′UTR sequence, signal peptide-encoding exon I and the exon/intron junctions. Thus, PkNBPXb is encoded by exon I (nt 49 to nt 103) and by exon II (nt 347 to nt 8826) with an intron of 243 nt, which has a 3′ AAG splice site instead of the usual TAG or CAG 3′ splice motifs. Thus, the total coding sequence of *pknpbxb* is 8535 nt. The PkNBPXb protein has 2845 amino acid residues with a predicted signal peptide cleavage site between C21 and K22 and a hydrophobic transmembrane domain between amino acid 2784 and amino acid 2801 (Fig. 2A).

Overall, the gross structural characteristics of PkNBPXa and PkNBPXb proteins are remarkably similar to those of PvRBP1 and PvRBP2 and the other members of the RBL family. Similarly, these proteins are likely membrane-anchored as determined by a hydrophobic region at the carboxy-terminal ends (Fig. 2A). Chromosomal mapping localized *pknbpxa* in chromosome 12 and *pknbpxb* in chromosomes 3–4 (Fig. 2B). Currently, the *pknbpxa* gene is annotated in the *P. knowlesi* database as two unlinked fragments (PKH146970 and PKH146980). A contig containing the *pknbpxb* gene assembly is not currently retrieved through BLAST searches, although it is noted that shotgun sequences for this gene are present at in the Sanger database and, surprisingly, cover the complete gene.

3.4. The *pknbpxa* and *pknbpxb* genes are transcribed and expressed in mature schizonts

Transcription of both *pknbpxa* and *pknbpxb* was verified by RT-PCR using schizont stage RNA (data not shown). Northern blot analyses were then performed and demonstrated the schizont-specific nature of the transcripts. Total RNA was extracted over the 24-hour life cycle period from synchronous parasites representing ring stages (R), late trophozoite and early schizont stages with two nuclei (T), and mature schizonts with eight or more nuclei (S). Large transcripts (> 9 kb) were detected for both genes, suggesting the presence of long untranslated regions, and they were present only in the mature schizont-stage RNA samples (Fig. 2C).

Native and reduced protein was detected using rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera on mature schizont extracts, and culture supernatants harvested 14 h after erythrocyte rupture by western immunoblotting. Both PkNBPXa and PkNBPXb are predicted to be high molecular weight proteins of 324 kDa and 334 kDa, respectively. The two polyclonal antisera recognized proteins of ~300 kDa as predicted by the software algorithms without noting any migration differences between the reduced and non-reduced samples. However, rabbit anti-rPkNBPXa also recognized an additional band of 220 kDa which could be a processed fragment of PkNBPXa or attributed to spurious reactivity with the erythrocyte protein, spectrin (although there is no evidence of this from immuno-fluorescence or immuno-electronmicroscopy, see below). Rabbit anti-rNBPXb recognizes an additional band of ~140 kDa in these immunoblots (Fig. 2D). Soluble PkNBPXa and PkNBPXb of the expected size (~300 kDa) were also detected in culture supernatants by western blot by using rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera (Fig. 2E).

3.5. PkNBPXa and PkNBPXb localize to the apical microneme organelles of merozoites

Rabbit antibodies specific for rPkNBPXa and rPkNBPXb located the native proteins at the apical end of merozoites in segmented schizont stage parasites by IFA (Fig. 2F). A single robust dot of fluorescent signal sometimes seemingly capping the merozoite, which is typical for the PvRBPs at the apical pole of *P. vivax* merozoites [2], was also observed in *P. knowlesi* mature

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segmented schizonts with these two antisera. No reactivity was detected with pre-immune rabbit sera and with the *P. knowlesi* RBL antisera on earlier stages of parasite development. Immuno-electron microscopy of *ex-vivo* parasite preparations showed that anti-PkNBPXb was predominantly located in the micronemes (Fig. 3A–D). Anti-rPkNBPXa antibodies also reacted with micronemes, but less strongly (data not shown). Significantly, in the invading merozoite (Fig. 3D), very little labeling remains within the merozoite, mostly restricted to micronemes that have apparently failed to locate apically. This suggests that PkNBPXb had already been secreted, as would be expected if the protein is important in early red cell adhesion.

3.6. PkNBPXa and PkNBPXb bind to host erythrocytes

The capacity of the two expressed RBL proteins to bind to rhesus macaque erythrocytes was determined by traditional erythrocyte binding assays. Intense bands were detected in immunoblots probed with rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera (Fig. 4A and 4B). No bands were detected with erythrocytes that were incubated without supernatant and subsequently washed and eluted under the same conditions as the samples incubated with culture supernatants. There were no differences detected in the migration of PkNBPXa and PkNBPXb proteins between reduced and non-reduced samples (data not shown). To ensure that the binding detected was not due to carryover contamination, immunoblots were also probed with antiserum against the 140kDa PkMSP3 [39]. PkMSP3 is known to be present in culture supernatants but does not bind to the surface of erythrocytes. As expected, antibody reactivity with PkMSP3 was detected when testing culture supernatants (~140kD) but not the erythrocyte binding assay eluates (Fig. 4C and unpublished data). This indicates the binding of expressed *P. knowlesi* RBLs to rhesus macaque erythrocytes is specific.

3.7. Identification of a *rbl* gene in *P. vivax* that is an ortholog to *pknbpxa*

P. vivax gDNA was examined by Southern hybridization with fragments of the 5′ and 3′ portions of the *P. knowlesi rbl* genes as probes. Weak bands of approximately 7.0 kb were noted on *P. vivax* digested gDNA when probed with a *pknbpxa* 5′ DNA fragment (Fig. 5A) or approximately 9.0 kb, when probed with a *pknbpxa* 3′ fragment (Fig. 5B). These data suggested the presence of at least one more *rbl* gene more closely related to *pknbpxa* in this species. No hybridization was observed when *pknbpxb* probes were used at low stringency on the *P. vivax* DNA. Database search of the *P. vivax* genome sequencing project (www.plasmodb.org/P.vivax) using *pknbpxa* sequences provided a contig of 11726 bp containing several large ORFs totaling about 8366 bp. Though the gene predicted for the protein has a potential consensus start methionine (TATAATG), the expected intron/exon 5′ and 3′ consensus splicing sequences were not readily observed, and two stop codons at positions 5503 and 7071 interrupt a potential 8366 bp ORF [17]. Although *pknbpxb* was not detected in *P. vivax*, homologous sequences to both *pknbpxa* and *pknbpxb* were detected by Southern hybridization in the simian malaria parasites, *P. cynomolgi* and *P. coatneyi* (Fig. 5A and 5B).

3.8. Phylogenetic relationship of *pknbpxa* and *pknbpxb* within the RBL family of proteins

To achieve a comprehensive updated phylogenetic analysis of known *rbl* genes, the *rbl* sequences from human, simian, chimpanzee, and rodent malaria parasites were aligned and analyzed. These alignments included orthologs of *pvrbp1* from *P. coatneyi* (GenBank accession number **DQ973816**) and *P. fragile* (GenBank accession number **DQ973815**). The analysis of the RBL proteins demonstrates that these proteins comprise a large family of two distinct groups, namely, PvRBP1-like and PvRBP2-like homologs. Functional *pvrbp1*-like genes have been identified in *P. falciparum, P. fragile, P. cynomolgi* and *P. coatneyi*, but are degenerated pseudogenes in *P. knowlesi* and *P. reichenowi*. In contrast, various paralogs and orthologs of *pvrbp2* have been identified in *P. vivax, P. falciparum, P. reichenowi, P. cynomolgi, P. knowlesi*, and *P. yoelii* (Fig. 6). Importantly, the genes designated here as

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pknbpxa and *pvrbpxb* share a paralogous relationship with *pvrbp2*, and *pknbpxa* may constitute a new *rbl2* subgroup sharing greater affinity with *pfnbp3/rh3* and *pfnbp2a-b/rh2a-b.* Though quite divergent, *pknbpxb* is clearly more related to *rbp2* of *P. vivax* and *P. cynomolgi* than is *pknbpxa*.

4. Discussion

Here we report the experimental characterization of the *rbl* family in *P. knowlesi* and show it is comprised of one pseudogene related to *pvrbp1* and two functional intact members, which we have called *pknbpxa* and *pknbpxb*. These two functional genes encode large RNA transcripts and high molecular weight proteins as predicted, and we have confirmed that they become expressed in the late schizont stage. Their products localize to the microneme organelles of mature merozoites and bind specifically to rhesus monkey erythrocytes.

Our initial identification of these genes was through hybridization of *pvrbp* gene fragments to restriction enzyme digests of *P. knowlesi* gDNA (and other species), followed by sequencing and RT-PCR to fully characterize the genes. These experimental findings have been important alongside the development of the *P. knowlesi* genome project [31], since this genome-wide sequencing and annotation effort alone failed to recognize the *ψpknbp1* pseudogene and the *pknbpxb* gene reported here, and annotate them accordingly. On the other hand, two fragments of the *pknbpxa* gene were annotated by the genome project, but not recognized as segments of one gene. Our experimental and bioinformatic work was required to identify unannotated members of the *rbl* family (ψ*pknbp1* and *pknbpxb*), correctly link annotated fragments of the *pknbpxa* gene, and confirm all the sequences, structures, and importantly, their expression and functional properties.

The *P. knowlesi* ortholog of *pvrbp1* is only a relic pseudogene (*ψpknbp1*) in the H, Hackeri, and Philippine strains of *P. knowlesi*. What is left of this pseudogene has a high level of identity with ~0.8 kb of the 5′ end of *pvrbp1* (a gene of ~8.6 kb) with no ORF. The *ψpknbp1* sequence downstream has been entirely lost in relationship with *rbp1* gene structure, presumably through an ancient recombination event, or extensive drift over time through mutations. Interestingly, this pseudogene is differentially transcribed as a small fragment that is consistent with the size of the gene remnant. This truncated transcript is primarily produced during ring-stage parasite growth, as opposed to schizont stage development for *bona fide* full-length *rbl* genes. The presence of *rbl* pseudogenes has been recognized before in *P. falciparum* and *P. reichenowi*, and different levels of degeneracy have been observed. For example, the *P. falciparum rbl3* (*pfnbp3*/*rh3*) gene transcript is present, yet not translated into protein in the 3D7 strain due to two frameshift mutations [8]. The *P. reichenowi rbl1* (*prnbp1/rh1)* gene, however, is much more degenerated containing numerous frameshifts leading to termination codons throughout the sequence [11].

Regardless of the mechanism by which *rbl* pseudogenes are generated and maintained, it has now become evident that *P. knowlesi* parasites can survive without an *rbl1* ortholog. Furthermore, this apparently extends also to a *pvrbp2* ortholog as our data shows that the *P. knowlesi* genome lacks completely a direct counterpart of *pvrbp2,* although an ortholog is in *P. cynomolgi*. Initial hybridization experiments were surprising, since the closely related simian malaria parasite *P. cynomolgi* [10], and the sister taxon *P. coatneyi* (unpublished), clearly had orthologs of *rbp1* and *rbp2* in their genomes. Thus, only two *rbl* genes function in *P. knowlesi*, *pknbpxa*, reported in the genome database as PKH_146970 and PKH_146980, and *pknbpxb* as reported here but remains unannotated. Clearly, the loss of the *rbp1* and *rbp2* orthologs in *P. knowlesi* is a recent evolutionary event occurring sometime after the divergence of *P. coatneyi* and *P. knowlesi*, perhaps two to three million years ago [40,41].

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Our data indicate *P. knowlesi* has evolved to maintain just two functioning members of the *rbl* gene family (*pknbpxa* and *pknbpxb*), while losing the *rbp1* and *rbp2* orthologs and perhaps others present in the other members of the vivax-simian malaria clade of *Plasmodium* species. In contrast, most other species of *Plasmodium* seem to have a larger repertoire of expressed *rbl* genes, including *P. vivax. P. yoelii* has at least a dozen *rbl* genes that may be functional; all falling within the *rbp2*-like grouping of *rbl* genes. *P. falciparum* has a group of six genes of which five are deemed functional and likely serve to interact with alternative receptors in whatever role they play in erythrocyte invasion. *P. vivax* also apparently has more than two functional *rbl* genes.

We have already noted here that the *P. vivax* (Sal I) genome has an *nbpxa* ortholog gene, although it seems to be a non-functional pseudogene. Hypothetical translation of the long ORF, across the stop codons and without frameshifts gives an amino acid identity of 63% between PkNBPXa and the *P. vivax* homologue. It remains to be demonstrated if the *pvrbpxa* gene is transcribed, as is the *nbp3/rh3* gene in *P. falciparum* [8]. The two frameshift mutations and lack of evident intron splice sites indicate this is likely a pseudogene and probably does not produce a functional protein. This gene has been designated as *pvrbp3* [16]. At least four other gene sequences in the *P. vivax* (Sal I) genome [17] are annotated as *rbp genes*, besides those discussed above (*pvrbp1, pvrbp2,* and *pvrbp3*), but none are orthologs of *pknbpxb*. Importantly, of these four additional genes designated as *pvrbp1b*, *pvrbp2a*, *pvrbp2b* and *pvrbp2d*, two, *pvrbp1b* and *pvrbp2d* are likely non-functional genes with mutations that disrupt the reading frame. Thus, *P. vivax* may have a larger set of functional *rbl* genes than previously realized, but less than recently reported [16], whereas *P. knowlesi* has reduced its *rbl* allotment to only two ligands. Nevertheless, *P. knowlesi* has compensated by retaining its invasion ligand diversity through three *dbl* genes, while *P. vivax* only has one.

The *pknbpxa* and *pknbpxb* genes have the signature structure of *rbl* genes but they do not exhibit a high degree of homology between them, as is also the case for the *rbp1* and *rbp2* genes in *P. vivax* or *P. cynomolgi* [2,4,10]. The absence of the *rbp1* and *rbp2* family members in *P. knowlesi* may simply be a reflection of the adaptation of *P. knowlesi* to invade all red blood cells and not be restricted to reticulocytes like *P. vivax* and *P. cynomolgi.* However, *P. coatneyi* does not preferentially invade reticulocytes and it has a complete *rbl1* gene structure that is functional from the perspective that there is a complete ORF. The presence of distant members in *P. knowlesi* may also simply be indicative of a dynamic evolutionary process and the parasite's strategies to maintain a minimum number of functional members of this gene family. Experiments aiming to disrupt *pknbpxa* and *pknbpxb* genes support the hypothesis that the encoded proteins are essential for *P. knowlesi* survival, as no parasites were retrieved after selection for disrupted parasite phenotypes in *in vivo* and *in vitro* experiments, although disruptions of other genes performed at the same time were successful in each case (unpublished data).

Phylogenetically, these two *rbl* genes have helped to define new ortholog genes in *P. vivax*, *P. cynomolgi*, *P. coatneyi* and *P. fragile*, which complement the *pvrbp2* subgroup of the RBL family (Fig. 6). The data presented here and contained in genome databases suggest that the *rbl* gene family in *Plasmodium* is dynamic, in that gene duplications, recombination events and mutations have been common occurrences; yet all species investigated to date seem to minimally maintain at least two *bona fide* family members.

A micronemal location is evident for PkNBPXb by IEM analysis, and a similar pattern is observed for PkNBPXa, though the antibody reactivity was most clear for PkNBPXb. The micronemal location of these proteins contrasts with the rhoptry localization reported for PfNBP2a/b [19] and Py235 [42]. IFA patterns in merozoites for RBP1 and 2, and for RH1 and RH4 that colocalize with micronemal invasion ligands such as DBP, EBA-175 and AMA1

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suggest a microneme location for these *rbl* gene products. Further studies are necessary to thoroughly evaluate the locations of different RBLs in and between different species of *Plasmodium* to reliably locate them by IEM. If RBLs are stored in different locations as seems to be the case (i.e. in the micronemes versus rhoptries), or potentially in different microneme organelles, this may reflect different functional roles in invasion attributable to specific family members. More so than rhoptries, the micronemes appear to store invasion ligands that interact with specific RBC receptors to initiate actions during the early stages of recognition and entry. Precise localization data could be helpful for formulating and testing hypotheses relating to the function of the RBLs in the cascade of events that dictate erythrocyte invasion.

RBL proteins have been hypothesized to be critical components in host cell identification and invasion, and accumulating evidence with counterpart molecules present in each malaria species examined supports this premise. As their names denote, reticulocyte or normocyte binding proteins are predicted to function by binding to (as yet undefined) receptors on erythrocytes. The specific adhesion of RBL proteins to host erythrocytes in *in vitro* erythrocyte binding assays has been demonstrated for *P. vivax* (with reticulocyte specificity) [2], *P. falciparum* [5,20–22], and *P. yoelii* [12], and here we demonstrate that the expressed RBLs of *P. knowlesi* bind to erythrocytes from the rhesus macaque model.

P. knowlesi is the traditional model for investigating *Plasmodium* merozoite invasion of erythrocytes [1,43,44]. *Ex-vivo P. knowlesi* merozoites have a biological half-life of about 20 minutes and remain viable in the extracellular milieu much longer than other species [43]. This trait allows for the unique experimental capture and analysis of merozoites during different stages of attachment and entry of erythrocyte host cells [1,43,44] (and see Fig. 3D). Of interest, our IEM data shown here, depicting an invading parasite suggests that the RBLs are released from microneme storage at a time before the merozoite begins to enter the host red blood cell (Fig. 3D). It is also worth noting that labeling was absent from the general surface of free merozoites, consistent with the notion that the RBLs are likely to function in apically-related events rather than initial red blood cell adhesion. Considering the likelihood that binding of the RBLs may be critical for invasion of erythrocytes, further investigation to define the binding properties and characteristics of these molecules is warranted.

With the goal of using the *P. knowlesi* model to improve our understanding of the invasion of red blood cells, we set out to define the *rbl* genes and proteins present in this species. Moreover, the importance of the RBL family as potential vaccine candidates is emphasized by the parasite's maintenance of functional members, ability to bind erythrocytes, proposed role in signaling release of other merozoite proteins early during invasion [1–4], and evidence suggesting their critical role in parasite survival. *P. knowlesi* has become a public health concern since a series of recent reports have confirmed the zoonotic transmission in human populations throughout South East Asia, and severe disease and four deaths have so far been attributed to these infections [25–27]. It is possible that expressed RBLs characterized in this report are the ligands used to gain entry into human erythrocytes, as well as the erythrocytes of simian hosts, be they the experimental rhesus monkey host (*Macaca mulatta*) or the natural host (*M. fascicularis* or *M. nemestrina*) in South East Asia. Further studies are in progress to define the host cell binding specificities of the *P. knowlesi* RBLs, identify the *P. knowlesi* RBL red blood cell binding domain(s), and maximize the use of this model parasite to pave the way for the possible inclusion of RBL molecules in future malaria vaccines.

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References

1. Galinski, MR.; Dluzewski, AR.; Barnwell, JW. A mechanistic approach to merozoite invasion of red blood cells: merozoite biogenesis, rupture, and invasion of erythrocytes. In: Sherman, IW., editor. Molecular approaches to malaria. ASM Press; 2005. p. 113-168.
2. Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of Plasmodium vivax merozoites. Cell 1992;69(7):1213–26. [PubMed: 1617731]
3. Galinski MR, Barnwell JW. Plasmodium vivax: Merozoites, invasion of reticulocytes and considerations for malaria vaccine development. Parasitol Today 1996;12(1):20–9. [PubMed: 15275304]
4. Galinski MR, Xu M, Barnwell JW. Plasmodium vivax reticulocyte binding protein-2 (PvRBP-2) shares structural features with PvRBP-1 and the Plasmodium yoelii 235 kDa rhoptry protein family. Mol Biochem Parasitol 2000;108(2):257–62. [PubMed: 10838229]
5. Rayner JC, Vargas-Serrato E, Huber CS, Galinski MR, Barnwell JW. A Plasmodium falciparum homologue of Plasmodium vivax reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. J Exp Med 2001;194(11):1571–81. [PubMed: 11733572]
6. Rayner JC, Galinski MR, Ingravallo P, Barnwell JW. Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion. Proc Natl Acad Sci U S A 2000;97(17):9648–53. [PubMed: 10920203]
7. Triglia T, Thompson J, Caruana SR, Delorenzi M, Speed T, Cowman AF. Identification of proteins from Plasmodium falciparum that are homologous to reticulocyte binding proteins in Plasmodium vivax. Infect Immun 2001;69(2):1084–92. [PubMed: 11160005]
8. Taylor HM, Triglia T, Thompson J, Sajid M, Fowler R, Wickham ME, Cowman AF, Holder AA. Plasmodium falciparum homologue of the genes for Plasmodium vivax and Plasmodium yoelii adhesive proteins, which is transcribed but not translated. Infect Immun 2001;69(6):3635–45. [PubMed: 11349024]
9. Kaneko O, Mu J, Tsuboi T, Su X, Torii M. Gene structure and expression of a Plasmodium falciparum 220-kDa protein homologous to the Plasmodium vivax reticulocyte binding proteins. Mol Biochem Parasitol 2002;121(2):275–8. [PubMed: 12034462]
10. Okenu DM, Meyer EV, Puckett TC, Rosas-Acosta G, Barnwell JW, Galinski MR. The reticulocyte binding proteins of Plasmodium cynomolgi: a model system for studies of P. vivax. Mol Biochem Parasitol 2005;143(1):116–20. [PubMed: 15990180]
11. Rayner JC, Huber CS, Galinski MR, Barnwell JW. Rapid evolution of an erythrocyte invasion gene family: the Plasmodium reichenowi Reticulocyte Binding Like (RBL) genes. Mol Biochem Parasitol 2004;133(2):287–96. [PubMed: 14698440]
12. Ogun SA, Holder AA. A high molecular mass Plasmodium yoelii rhoptry protein binds to erythrocytes. Mol Biochem Parasitol 1996;76(1–2):321–4. [PubMed: 8920020]
13. Keen JK, Sinha KA, Brown KN, Holder AA. A gene coding for a high-molecular mass rhoptry protein of Plasmodium yoelii. Mol Biochem Parasitol 1994;65(1):171–7. [PubMed: 7935623]
14. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006;124(4):755–66. [PubMed: 16497586]
15. Rodriguez M, Lustigman S, Montero E, Oksov Y, Lobo CA. PfRH5: a novel reticulocyte-binding family homolog of plasmodium falciparum that binds to the erythrocyte, and an investigation of its receptor. PLoS ONE 2008;3(10):e3300. [PubMed: 18827878]
16. Rayner JC, Huber CS, Barnwell JW. Conservation and divergence in erythrocyte invasion ligands: Plasmodium reichenowi EBL genes. Mol Biochem Parasitol 2004;138(2):243–7. [PubMed: 15555736]
17. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, Cheng Q, Coulson RM, Crabb BS, Del Portillo HA, Essien K, Feldblyum TV, Fernandez-Becerra C, Gilson PR, Gueye AH, Guo X, Kang'a S, Kooij TW, Korsinczky M, Meyer EV, Nene V, Paulsen I, White O, Ralph SA, Ren Q, Sargeant TJ, Salzberg SL, Stoeckert CJ, Sullivan

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SA, Yamamoto MM, Hoffman SL, Wortman JR, Gardner MJ, Galinski MR, Barnwell JW, Fraser-Liggett CM. Comparative genomics of the neglected human malaria parasite Plasmodium vivax. Nature 2008;455(7214):757–63. [PubMed: 18843361]

18. Triglia T, Duraisingh MT, Good RT, Cowman AF. Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by Plasmodium falciparum. Mol Microbiol 2005;55(1):162–74. [PubMed: 15612925]
19. Duraisingh MT, Triglia T, Ralph SA, Rayner JC, Barnwell JW, McFadden GI, Cowman AF. Phenotypic variation of Plasmodium falciparum merozoite proteins directs receptor targeting for invasion of human erythrocytes. Embo J 2003;22(5):1047–57. [PubMed: 12606570]
20. Gao X, Yeo KP, Aw SS, Kuss C, Iyer JK, Genesan S, Rajamanonmani R, Lescar J, Bozdech Z, Preiser PR. Antibodies targeting the PfRH1 binding domain inhibit invasion of Plasmodium falciparum merozoites. PLoS Pathog 2008;4(7):e1000104. [PubMed: 18617995]
21. Gaur D, Singh S, Jiang L, Diouf A, Miller LH. Recombinant Plasmodium falciparum reticulocyte homology protein 4 binds to erythrocytes and blocks invasion. Proc Natl Acad Sci U S A 2007;104 (45):17789–94. [PubMed: 17971435]
22. Hayton K, Gaur D, Liu A, Takahashi J, Henschen B, Singh S, Lambert L, Furuya T, Bouttenot R, Doll M, Nawaz F, Mu J, Jiang L, Miller LH, Wellems TE. Erythrocyte binding protein PfRH5 polymorphisms determine species-specific pathways of Plasmodium falciparum invasion. Cell Host Microbe 2008;4(1):40–51. [PubMed: 18621009]
23. Chin W, Contacos PG, Coatney GR, Kimball HR. A Naturally Acquited Quotidian-Type Malaria in Man Transferable to Monkeys. Science 1965;149:865. [PubMed: 14332847]
24. Fong YL, Cadigan FC, Coatney GR. A presumptive case of naturally occurring Plasmodium knowlesi malaria in man in Malaysia. Trans R Soc Trop Med Hyg 1971;65(6):839–40. [PubMed: 5003320]
25. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet 2004;363(9414):1017–24. [PubMed: 15051281]
26. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis 2008;46(2):165–71. [PubMed: 18171245]
27. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? Trends Parasitol 2008;24(9):406–10. [PubMed: 18678527]
28. Waters AP, Higgins DG, McCutchan TF. Evolutionary relatedness of some primate models of Plasmodium. Mol Biol Evol 1993;10(4):914–23. [PubMed: 7689135]
29. Vargas-Serrato E, Corredor V, Galinski MR. Phylogenetic analysis of CSP and MSP-9 gene sequences demonstrates the close relationship of Plasmodium coatneyi to Plasmodium knowlesi. Infect Genet Evol 2003;3(1):67–73. [PubMed: 12797974]
30. Johnson JG, Epstein N, Shiroishi T, Miller LH. Factors affecting the ability of isolated Plasmodium knowlesi merozoites to attach to and invade erythrocytes. Parasitology 1980;80(3):539–50. [PubMed: 6771738]
31. Pain A, Bohme U, Berry AE, Mungall K, Finn RD, Jackson AP, Mourier T, Mistry J, Pasini EM, Aslett MA, Balasubrammaniam S, Borgwardt K, Brooks K, Carret C, Carver TJ, Cherevach I, Chillingworth T, Clark TG, Galinski MR, Hall N, Harper D, Harris D, Hauser H, Ivens A, Janssen CS, Keane T, Larke N, Lapp S, Marti M, Moule S, Meyer IM, Ormond D, Peters N, Sanders M, Sanders S, Sargeant TJ, Simmonds M, Smith F, Squares R, Thurston S, Tivey AR, Walker D, White B, Zuiderwijk E, Churcher C, Quail MA, Cowman AF, Turner CM, Rajandream MA, Kocken CH, Thomas AW, Newbold CI, Barrell BG, Berriman M. The genome of the simian and human malaria parasite Plasmodium knowlesi. Nature 2008;455(7214):799–803. [PubMed: 18843368]
32. Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P. Plasmodium vivax, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated with the surface of merozoites. Exp Parasitol 1999;91(3):238–49. [PubMed: 10072326]
33. Eyles DE, Dunn FL, Warren M, Guinn E. Plasmodium Coatneyi from the Philippines. J Parasitol 1963;49:1038. [PubMed: 14084189]

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34. Kyes S, Pinches R, Newbold C. A simple RNA analysis method shows var and rif multigene family expression patterns in Plasmodium falciparum. Mol Biochem Parasitol 2000;105(2):311–5. [PubMed: 10693754]

35. Altman JD, Henner D, Nilsson B, Anderson S, Kuntz ID. Intracellular expression of BPTI fusion proteins and single column cleavage/affinity purification by chymotrypsin. Protein Eng 1991;4(5): 593–600. [PubMed: 1716367]

36. Barnwell JW, Howard RJ, Miller LH. Altered expression of Plasmodium knowlesi variant antigen on the erythrocyte membrane in splenectomized rhesus monkeys. J Immunol 1982;128(1):224–6. [PubMed: 6172478]

37. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17(12):1244–5. [PubMed: 11751241]

38. Galinski MR, Corredor V. Variant antigen expression in malaria infections: posttranscriptional gene silencing, virulence and severe pathology. Mol Biochem Parasitol 2004;134(1):17–25. [PubMed: 14747139]

39. Johnson JG, Epstein N, Shiroishi T, Miller LH. Identification of surface proteins on viable Plasmodium knowlesi merozoites. J Protozool 1981;28(2):160–4. [PubMed: 7277254]

40. Escalante AA, Cornejo OE, Freeland DE, Poe AC, Durrego E, Collins WE, Lal AA. A monkey's tale: the origin of Plasmodium vivax as a human malaria parasite. Proc Natl Acad Sci U S A 2005;102 (6):1980–5. [PubMed: 15684081]

41. Mu J, Joy DA, Duan J, Huang Y, Carlton J, Walker J, Barnwell J, Beerli P, Charleston MA, Pybus OG, Su XZ. Host switch leads to emergence of Plasmodium vivax malaria in humans. Mol Biol Evol 2005;22(8):1686–93. [PubMed: 15858201]

42. Oka M, Aikawa M, Freeman RR, Holder AA, Fine E. Ultrastructural localization of protective antigens of Plasmodium yoelii merozoites by the use of monoclonal antibodies and ultrathin cryomicrotomy. Am J Trop Med Hyg 1984;33(3):342–6. [PubMed: 6731669]

43. Aikawa M, Miller LH, Johnson J, Rabbege J. Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J Cell Biol 1978;77(1):72–82. [PubMed: 96121]

44. Mitchell GH, Bannister LH. Malaria parasite invasion: interactions with the red cell membrane. Crit Rev Oncol Hematol 1988;8(4):225–310. [PubMed: 3064934]

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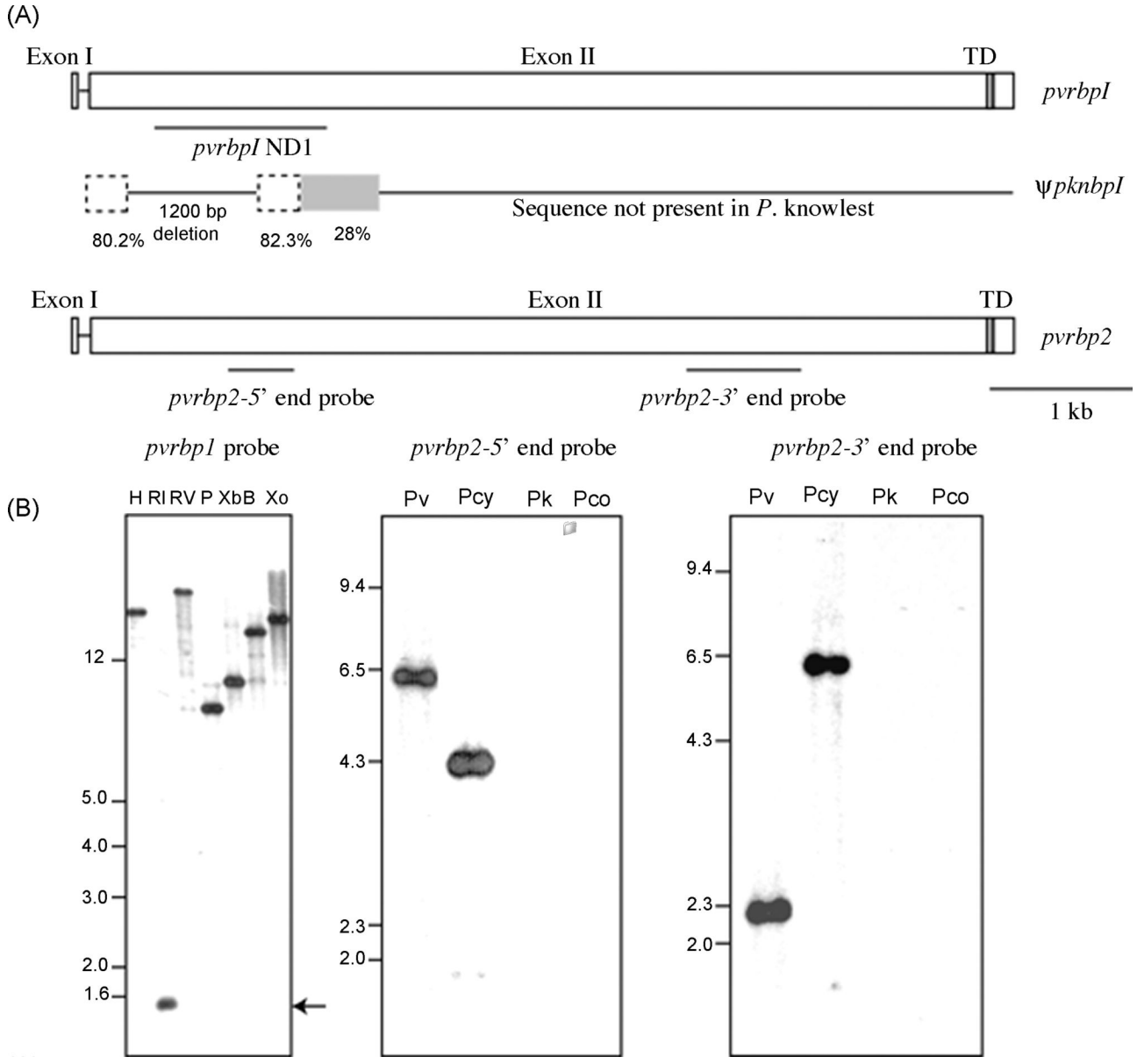

Figure 1.

The *P. knowlesi* gene orthologous to *pvrbp1* in *P. vivax* is a pseudogene. (A) Schematic representation of *pvrbp1* and *pvrbp2* genes and the remnant *pknbp1* locus with locations of DNA probes used in Southern blot analysis. The comparison of the 1.5 kb fragment of the *nbp1* locus in *P. knowlesi* with *P. vivax rbp1* revealed two remnant regions (dashed lines) in the *P. knowlesi* gene *ψpknbp1* comprising part of the intron and 5′ end of coding region. (B) Southern blot analysis (bottom left) of *P. knowlesi* H strain gDNA digested with *Hind*III (H), *Eco*RI (RI), *Eco*RV (RV), *Pst*I (P), *Xba*I (Xb), *Bam*HI (B) and *Xho*I (Xo) hybridized with a 5′-end *pvrbp1* probe identifying a 1.5 kb fragment after *Eco*RI restriction analysis (arrow). *P. vivax (Pv), P. cynomolgi (Pcy), P. knowlesi (Pk),* and *P. coatneyi (Pc)* gDNA digested with *Hind*III restriction enzyme was analyzed by Southern blot analysis using a 5′-end (*pvrbp2*–5′ end) or a 3′-end (*pvrbp2*–3′end) probe from the *pvrbp2* gene, only *P. cynomolgi* showed cross-hybridization under moderately stringent conditions.

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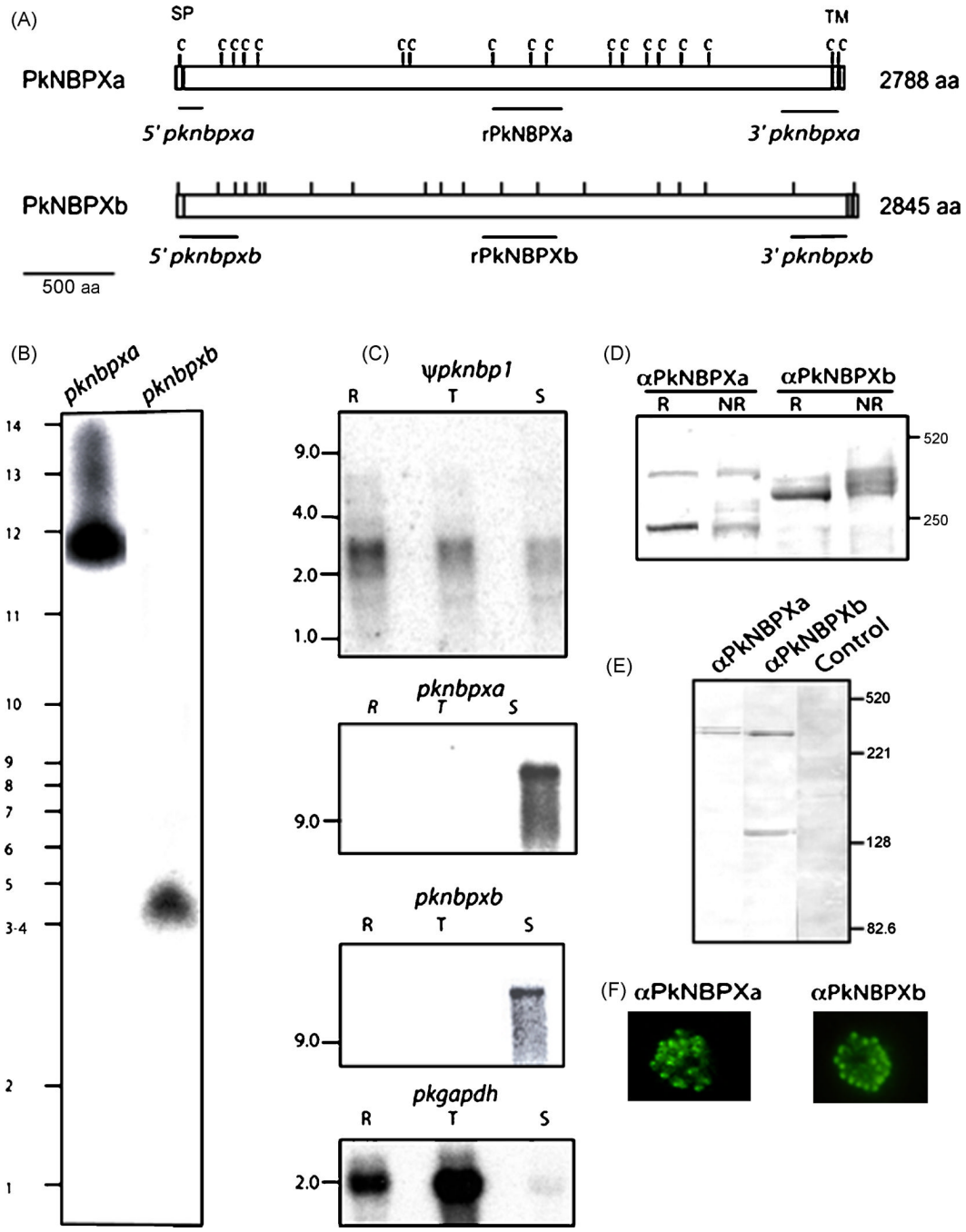

Figure 2.

(A) Schematic of the *P. knowlesi* PkNBPXa and PkNBPXb RBL proteins with cysteine residues (tick marks), positions of the signal peptides (SP), a large extracellular domain and a transmembrane domain (TM) shown. Probes used for chromosomal Southern and Northern blot hybridizations are indicated in italics and the fragments expressed as recombinant proteins are indicated in bold letters. (B) The *pknbpxa* and *pknbpxb* genes were localized in chromosomes 3–4 and 12, respectively, by PFGE using the *pknbpxa* and *pknbpxb* probes. (C) Total RNA from ring stages (R), late trophozoites and early schizonts (T) and late schizonts (S) was hybridized with radiolabeled probes representing the 5′ end of the *ψpknbp1* gene, and the central regions of *pknbpxa* and *pknbpxb* genes using high stringency conditions. Apparent

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ψpknbp1 partial transcripts (~3 kb) were detected in ring, trophozoite and schizont stages, compared to *pknbpxa* and *pknbpxb* transcripts of >9kb, which were detected only in the matured schizonts. The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as positive control (*pkgapdh*). PkNBPXa and PkNBPXb were detected by western blot in *P. knowlesi*-schizont-infected erythrocytes solubilized with SDS-PAGE sample buffer with (R, reduced) or without (NR, non-reduced) 2-mercaptoethanol (D), and in supernatant collected after schizont rupture (E). The NBPXa1 antisera recognized specific bands of ~300 kDa and 250 kDa, and the NBPXb1 antisera recognized bands of ~300k Da and 140 kDa (D and E). PkNBPXa and PkNBPXb native proteins were identified by IFA using rabbit antisera PkNBPXa1 or PkNBPXb1, respectively. The robust single dot pattern typical for the PvRBPs at the apical pole was observed in segmented schizont-stage parasites; pre-immune sera were used as negative control (F).

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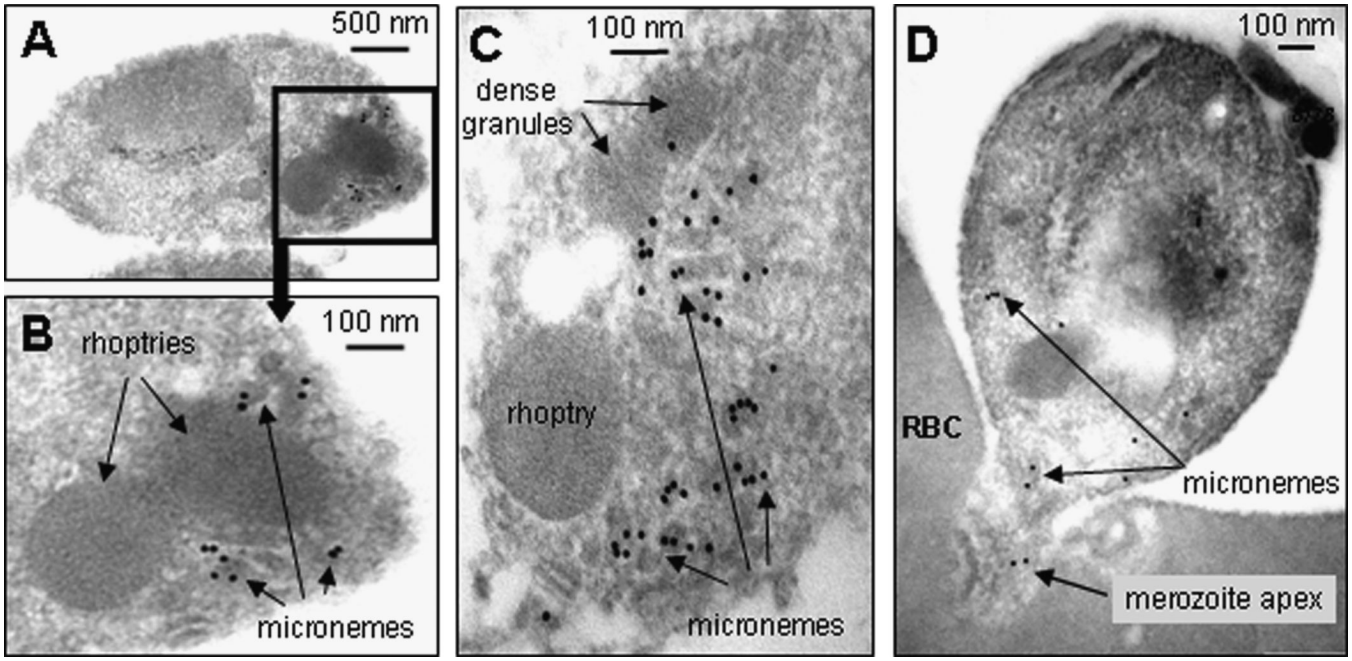

Figure 3.

Immuno-electron microscopic localization of the PkRBL protein, PkNBPXb. In A – C, PkNBPXb is shown to be strongly localized to the apical micronemes of free *P. knowlesi* merozoites, using rabbit anti- NBPXb anti-serum (1:50) as primary antibody, and Protein A -10 nm gold for detection.. In an invading merozoite (D), very little labelling remains (rabbit anti-NBPXb anti-serum 1:100 dilution used), and is mostly detected on micronemes that have apparently failed to locate apically, suggesting that the RBL had already been mostly secreted and lost, as expected if the protein is important in red cell adhesion. Note the absence of general labelling around the merozoite perimeters in all examples. RBC – red blood cell.

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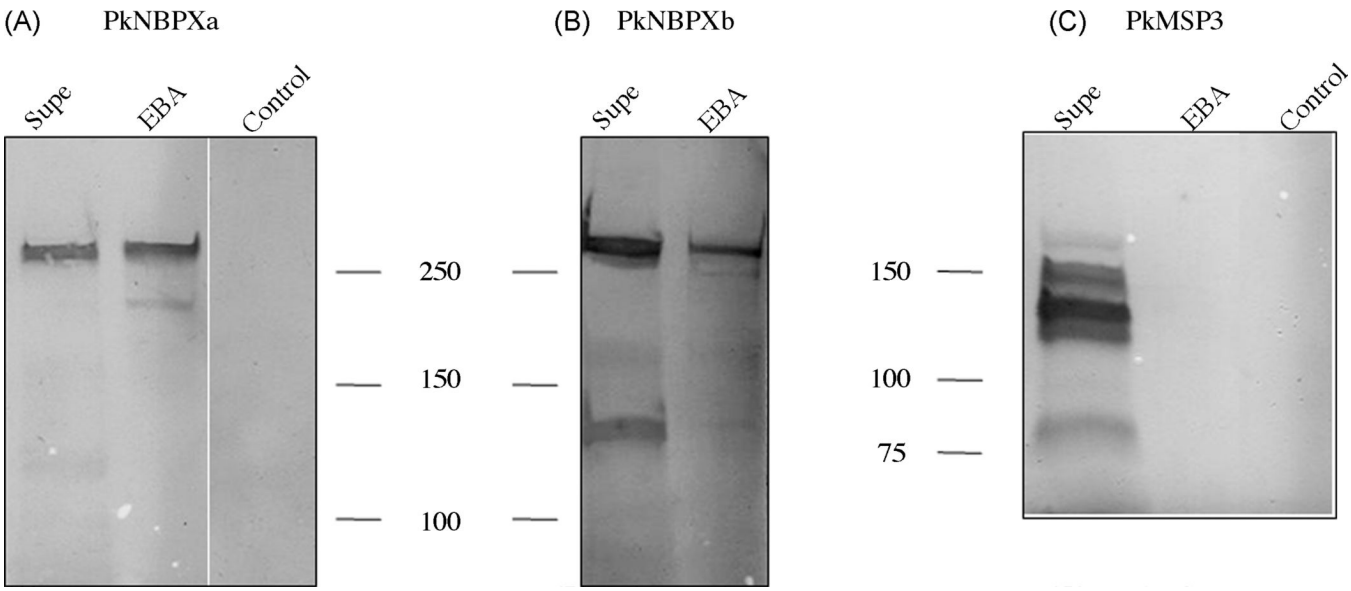

Figure 4.

PkNBPXa and PkNBPXb bind to host erythrocytes. Culture supernatants (Supe) containing soluble proteins released from free merozoites were electrophoresed by SDS-PAGE and transferred to nitrocellulose membranes as were protein samples eluted from rhesus macaque erythrocytes after incubation with supernatants in binding assays (EBA). Western blots using NBPXa1 or NBPXb1 antisera demonstrate that PkNBPXa and PkNBPXb bind to rhesus erythrocytes. PkMSP3 140 was abundantly detected in the culture supernatants by PkMSP3 140 rabbit antisera but was not detected in the eluted protein samples. Rhesus monkey erythrocytes were incubated within culture medium (Control) and eluted under the same conditions as erythrocytes incubated with culture supernatants. No protein bands were detected by any of the antisera (data not shown for PkNBPXb).

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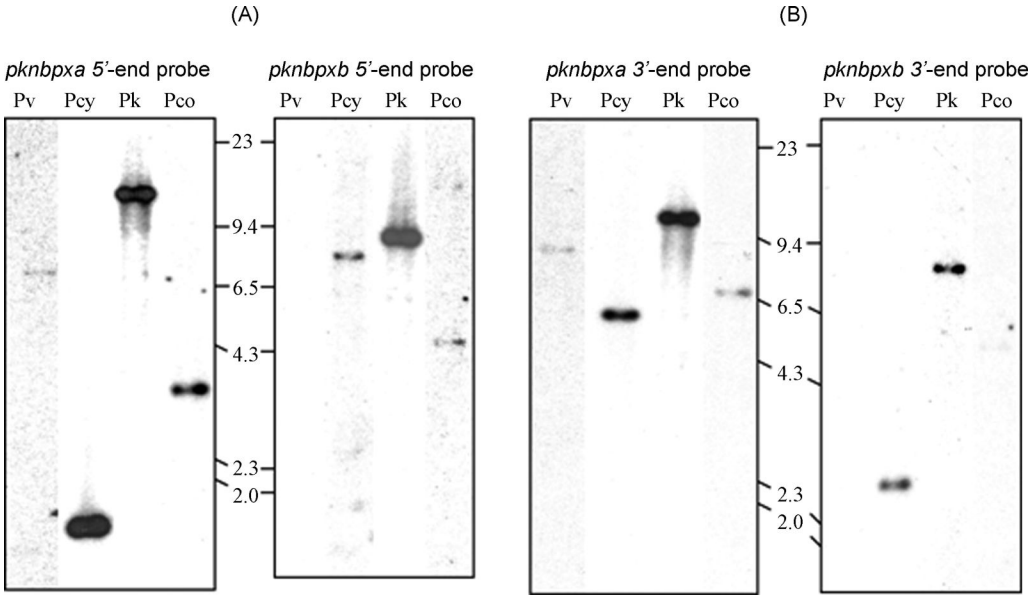

Figure 5.

Genes homologous to *pknbpxa* and *pknbpxb* are in *P. vivax* and other related simian malaria species. *P. vivax* (Pv), *P. cynomolgi* (Pcy), *P. knowlesi* (Pk), and *P. coatneyi* (Pco) gDNAs were digested with the restriction enzyme *Hind*III, blotted and probed with the 5′ (Panel A) and 3′ (Panel B) probes from *pknbpxa* and *pknbpxb* genes as indicated in Fig. 2. Sizes are in kilobases. The hybridization temperature was 60°C followed by low stringency washes as described in the materials and methods. The 5′ and 3′ probes from *pknbpxa* hybridized strongly with *P. vivax* and *P. cynomolgi*, and with less intensity to *P. coatneyi* gDNA. The 5′ and 3′ probes from *pknbpxb* hybridized with *P. cynomolgi* and *P. coatneyi* gDNA but not with *P. vivax* gDNA.

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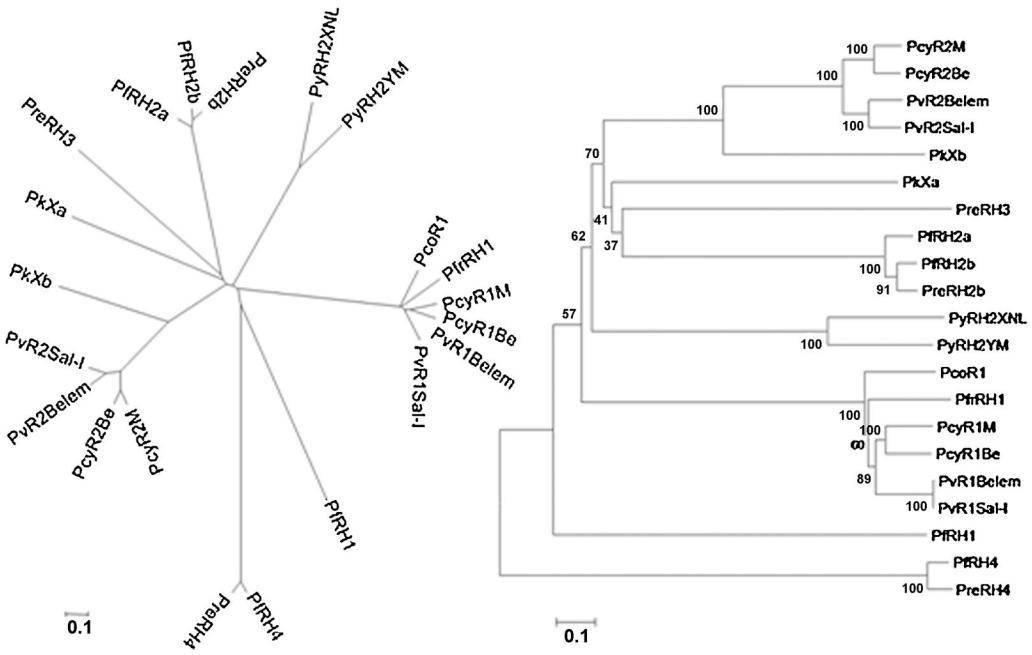

Figure 6.

Phylogenetic relationships among members of the RBL invasion ligand family. Unrooted neighbor-joining tree obtained from the alignment of RBL protein sequences from *P. vivax*, *P. cynomolgi*, *P. coatneyi*, *P. knowlesi*, *P. fragile*, *P. yoelii*, *P. falciparum*, and *P. reichenowi*. The cladogram (right) was generated using the Neighbor-Joining method following a Poisson model with pairwise deletion from MEGA 4 software. Bootstrap values for 1000 replicas are indicated in the tree. Distinct groups can definitely be identified for RBL1, RBL2, RBL3 and RBL4.