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ATP/ADP-binding to a novel nucleotide binding domain of the reticulocyte binding protein Py235 of *Plasmodium yoelii*

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Running Title: Nucleotide-binding traits of the NBD 94 domain

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The mechanism by which a malaria merozoite recognizes a suitable host cell is mediated by a cascade of receptor ligand interactions. In addition to the availability of the appropriate receptors, intracellular ATP plays an important role in determining if erythrocytes are suitable for merozoite invasion. Recent work has shown that ATP secreted from erythrocytes signals a number of cellular processes. To determine whether ATP-signaling might be involved in merozoite invasion, we investigated whether known plasmodium invasion proteins contain nucleotide binding motifs. Domain mapping identified a putative nucleotide binding region within all members of the reticulocyte binding protein homologue (RBL) family analyzed. A representative domain termed here Nucleotide Binding Domain 94 (NBD94), has been expressed and demonstrated to specifically bind to ATP. Nucleotide affinities of NBD94 were determined by fluorescence correlation spectroscopy (FCS), showing increased binding of ATP- compared to ADP-analogues. ATP-binding was reduced by the known F1F0 ATP synthase inhibitor 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Fluorescence quenching and circular dichroism spectroscopy (CD) of NBD94 after binding of different nucleotides provide evidence for structural changes in this protein. Our data suggests that different structural changes induced by ATP/ADP binding to RBL could play an important role during the invasion process.

Malaria is caused by unicellular protozoan parasites and is considered one of the most important infectious diseases still affecting mankind today. The complex life cycle of the parasite is characterized by three invasive forms: the sporozoite and merozoite that invade hepatocytes and erythrocytes in the vertebrate host, respectively, and the ookinete inside the insect vector that penetrates the mosquito midgut epithelium (1-5). In the case of merozoites, specific organelles (rhoptries, micronemes, and dense granules) at the apical end of the parasite contain proteins that play an important role in the recognition and invasion of the host cell (6). Recognition of specific erythrocyte receptors by the merozoite is mediated by at least two gene families, the reticulocyte binding protein homologues (RBL) and the erythrocyte binding ligands (EBL) (7,8). Like the EBL, the RBLs are also found in varying numbers in all plasmodium species with each member believed to play a role in recognizing a different receptor (7-16). In general, the members of RBL are large transmembrane proteins with molecular masses above 200 kDa that get proteolytically cleaved off during the invasion process (14,17). In Plasmodium yoelii, members of the RBL termed Py235 (P. yoelii 235 kDa rhoptry protein) have been shown to be potential virulence factors that enable the parasite to invade a wider range of host erythrocytes (18-20). In addition, Py235 is also involved in clonal phenotypic variation of merozoites (21) enabling the parasite to evade immune responses and adapt to changes in the host environment during the invasion step (22). Recently, it has been demonstrated that variation in the amount of Py235 expressed in merozoites defines the host cell repertoire that a parasite can invade (20). Studies carried out on the RBLs of P. falciparum (PfRH1, 2a, 2b, 3 and 4) (10,14-16,23,24) have provided additional evidence that different RBL members interact with different receptors on the erythrocyte and that these interactions are crucial for invasion. Previously, work in P. vivax has indicated that RBL may have an initial sensing role preceding and possibly enabling the subsequent interaction of the EBL member with its corresponding receptor (25). In a more recent study the erythrocyte binding region of PfRH4 has been identified (26), showing that only a relatively small region of these proteins are actually involved in receptor binding. Recognition of the appropriate receptor on the erythrocyte by RBL is expected to lead to downstream effects that ultimately enable the parasite to continue the invasion process. Although all the studies done on RBL in the different plasmodium species have highlighted the importance of this protein family, they have given us little insight in terms of the underlying biochemistry that allows RBL to mediate its function. This problem is partly due to the fact that the overall sequence conservation between the different members of RBL is very low and that no conserved domains have been identified so far, that would indicate important functional regions within this class of proteins. While the availability of erythrocyte surface proteins is a key step in the recognition and
subsequent invasion by the merozoite it is not clear what and if other factors are important for the invasion process. ATP released by erythrocytes under normal physiological conditions has recently been shown to serve as a signalling molecule regulating vascular tone (27-29) and ATP receptors have now been implicated in a number of signalling pathways (30,31). Mechanical deformation of erythrocytes as encountered in capillaries forming the microvasculature lead to increased ATP release (29). Importantly, intracellular ATP is a requirement for merozoite invasion (32-35) with erythrocytes that have been depleted of ATP being refractory to invasion. These findings imply that merozoites can sense the intracellular ATP level of the erythrocyte. To explore this further, we investigated merozoite proteins that are thought to play a role in host cell recognition for the presence of putative nucleotide binding domains.

Here a nucleotide binding domain that is highly conserved amongst the RBLs is described. We report for the first time the property of ATP/ADP-binding of this expressed domain, termed NBD94 (Nucleotide Binding Domain 94), independently observed by photoaffinity labeling and fluorescence correlation spectroscopy. Decreased tyrosine fluorescence and changes of circular dichroism spectra in the presence and absence of nucleotides are discussed in the light of structural alterations in NBD94 and its possible role in serving as an ATP/ADP sensor during the invasion process.

**Bioinformatics**

The nucleotide sequence data for the various Py235 members were obtained from PlasmoDB, Plasmodium genome resource, Version 4.4. ([http://www.plasmodb.org/](http://www.plasmodb.org/)). This site was also used to obtain nucleotide sequences of other Plasmodium erythrocyte and reticulocyte binding proteins. The protein sequences were then predicted based on the gene sequences obtained using PlasmoDB. Manipulation of nucleotide and protein sequences was carried out using Vector NTI and The Sequence Manipulation Suite ([http://bioinformatics.org/sms/](http://bioinformatics.org/sms/)). Alignment of these sequences was done using ClustalW (EMBL-EBI site, [http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)). The protein sequences were further analyzed using Normal SMART (Simple Modular Architecture Research Tool) program (36,37) ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)) and PSI Blast (Position-Specific Iterated BLAST) ([http://www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)) to search for conserved domain/motif.

**Parasite strains and genomic DNA extraction**

Frozen stabilitates of *Plasmodium yoelii* YM were separately taken up in 0.9% saline with glucose and injected intraperitoneally into 4-6 weeks old Balb/c mice bred under specific pathogen free (SPF) conditions as previously described (38). Genomic DNA was extracted from infected blood using the Easy DNA kit (Invitrogen) according to manufacturer’s protocol. Isolated DNA was stored at -20 ºC.

**Cloning of the NBD94 domain of PY01365 from Plasmodium yoelii**

The predicted nucleotide binding domain of PY01365 (hereafter termed NBD94) was amplified using approximately 100 ng of genomic YM DNA as a template and oligonucleotide primers 5'- GTGAGTCCCATGTTATCTGACAAAAATGAAAGATG-3' (forward primer) and 5'-AATTACGAGCTCTTAATCTTTACCACATCTACAG-3' (reverse primer). These primers were designed specially to include NcoI and SacI restriction sites respectively. After digestion with NcoI and SacI, the PCR products were ligated into pET9d-His6 vector (39) and the recombinant was then transformed into *Escherichia coli* DH5α competent cells. Successful transformants were

**Experimental Procedures**

**Biochemicals**

*Plasmodium yoelii* YM frozen stabilitates in liquid nitrogen were obtained from the National Institute of Medical Research (NIMR). *Pfu* DNA Polymerase and restriction enzymes were purchased from NEB. Ni²⁺-Sepharose™ High Performance chromatography resin was obtained from GE Healthcare Bio-Sciences AB (Upsalla, Sweden). Chemicals for gel electrophoresis were received from Biorad (Hercules, USA). All other chemicals were at least of analytical grade and purchased from Sigma-Aldrich (Deisenhofen, Germany), Amersham Bioscience (Buckinghamshire, UK), BIOMOL (Hamburg, Germany) and Merck (Darmstadt, Germany).
screened and verified by DNA sequencing. The correctly sequenced recombinant was then transformed into Rosetta-gami™ (DE3) cells (Novagen) and grown on Luria-Bertoni (LB) agar-plates, containing 30 µg/ml kanamycin and 34 µg/ml chloramphenicol.

Expression and Purification of NBD94

To express His<sub>6</sub>-NBD94, liquid cultures were incubated with agitation in LB medium containing 30 µg/ml kanamycin- and 34 µg/ml chloramphenicol at 37 °C for about 3 h, 200 rpm, until an OD<sub>600</sub> of 0.6-0.8 was reached. In order to induce the production of His<sub>6</sub>-NBD94, the cultures were supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside) to final concentration of 1 mM and further incubated for another 3 h at 37 °C. The cells were then harvested at 8,000 × g for 12 min at 4 °C. These cells were then subsequently lysed on ice by sonication (Sonopuls HD2200, Bandelin, KE76, 20%) for 3 × 1 min in buffer A (50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 4 mM Pefabloc<sup>SC</sup> (BIOMOL) and 0.7 mM DTT). The lysate was cleared by centrifugation at 10,000 × g for 35 min, 6 °C and the supernatant was filtered through a 0.45 µm pore-size filter and added to the Ni Sepharose™ resin. Binding of the his-tagged proteins to the matrix was carried out for 90 min at 4 °C and eluted with an imidazole gradient (0 – 250 mM) in buffer A. Fractions containing His<sub>6</sub>-NBD94 were identified by SDS-PAGE (polyacrylamide gel electrophoresis) (40), pooled and applied on an ion-exchanger (DEAE column HiPrep<sup>™</sup> 16/10, GE Healthcare), which was equilibrated in 50 mM Tris/HCl, pH 7.5, 0.7 mM DTT, 1 mM PMSF. The protein was eluted using a linear gradient with Buffer B (50 mM Tris/HCl, pH 7.5, 1 M NaCl, 0.7 mM DTT, 1 mM PMSF) at 3 ml/min. Following elution, concentration was carried out using Centriprep YM-50 (50 kDa cut off) spin concentrators (Millipore) and the purity of the eluted protein was assessed by SDS-PAGE. The SDS-gels were stained with Coomassie Brilliant Blue G250. Western blot analyses using mouse anti-penta His (Qiagen) were performed as described (41). ATPase activity was measured according to Heinonen and Lahti (42) and Chang et al. (43), in which the spectrophotometric quantification of the phosphomolybdate-malachite green complex is used.

Cloning, production and purification of NBD94<sub>1-550</sub>

A truncated version of the full length NBD of PY01365 (hereafter termed NBD94<sub>1-550</sub>) was cloned, expressed and purified in the similar manner as above with the following changes. The reverse primer used for amplification is 5'-AATTACGAGCTCTTAATCTGTTACATCTTC TAA-3'. The recombinant plasmid was eventually transformed in BL21(DE3) cells (Novagen) and grown on LB agar-plates, containing 30 µg/ml kanamycin. NBD94<sub>1-550</sub> was purified using Ni<sup>2+</sup>-Sepharose followed by ion-exchanger as described for full length NBD94 (s. above). It was further purified via size-exclusion chromatography using Superdex<sup>™</sup> 75 10/300 GL (Amersham Biosciences) in 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM PMSF and 1 mM DTT.

Circular Dichroism Spectroscopy

Measurements of steady state CD spectra were carried out in the far UV-light (185-260 nm) using a CHIRASCAN spectropolarimeter (Applied Photophysics, UK). The CD spectroscopy measurements of NBD94 and NBD94<sub>1-550</sub> in Tris/HCl, pH 7.5, 160 mM NaCl, and 0.7 mM DTT and Tris/HCl, pH 7.5, 200 mM NaCl, and 1 mM DTT respectively were done using a 60 µl quartz cell (Hellma) at 18 °C with 1 nm step points. Independent of the presence or absence of nucleotides all samples were incubated 15 min rocking at 4 °C before the start of each measurement. The spectrum for the buffer was subtracted from the spectrum of the protein. The ellipticity values were calculated by the average of triple determinations for each sample with a bandwidth of 1 nm from 185 to 260 nm (1 nm step points). The CD data were converted to mean residue ellipticity (Θ) in units of deg x dmol<sup>-1</sup> x aa<sup>-1</sup> using the software Chirascan version 1.2.1 (Applied Photophysics). Baseline corrected spectra were used as input for computer methods to obtain predictions of secondary structure. In order to analyze the CD spectrum the following algorithms were used: Varselec (44), Selcons (45), Contin (46), K2D (47); all methods as incorporated into the program Dicroprot(48) and NeuralNet (49).
**Photoaffinity labeling**

Photoaffinity labeling was done as described by Schäfer et al. (50). Protein was incubated with $5 \times 10^{-3} \text{M}$ 8-N$_3$-3'-biotinyl-ATP and $5 \times 10^{-4} \text{M}$ of MgCl$_2$ in 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, and 0.7 mM DTT for 5 min on ice in the dark, followed by irradiation with a ultraviolet lamp at 366 nm for 30 min on ice. The samples were applied on SDS-PAGE, following which a western immuno-blot was carried out to identify the labeled (biotinylated) protein using streptavidin/POD conjugate. The competition assay was also done concurrently with nucleotide analogues (ATP, ADP and AMP-PNP).

**In-gel tryptic protein digestion and mass spectrometric analysis**

The 8-N$_3$-3'-biotinyl-ATP labeled and unlabeled bands were cut out from the SDS-PAGE gel and destained with a solution of 25 mM ammonium bicarbonate and 50% acetonitrile for 12 h. The gel band was cut into small pieces of 1 mm$^3$, washed three times with acetonitrile, dried for 30 min in a speed-vacuum concentrator, and digested with trypsin overnight (51,52). For matrix assisted laser desorption-ionisation/time-of-flight (MALDI-TOF) spectrometry, aliquots of digested samples were applied to a target disk and allowed to air-dry. Subsequently, matrix solution (1% w/v $\alpha$-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Peptide mass mapping was performed using ABI 4800 MALDI TOF/TOF mass spectrometer. For interpretation of the protein fragments, the PEPTIDEMASS (53) program available at Expasy web site (www.expasy.ch/tools/peptide-mass.html) was used.

**Fluorescence correlation spectroscopy (FCS)**

Fluorescence correlation spectroscopy (FCS) was performed on a LSM-FCS system at room temperature (ConfoCor 3, Zeiss, Jena/Germany) using the ATP analogue EDA-ATP ATTO-647N (ATTO-TEC, Siegen/Germany). The FCS experiments were performed in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5 with 1 mM DTT. The 655 nm laser line of an 5 mW HeNe633 laser and focused into the aqueous solution by a water immersion objective (40 x / 1.2 W Korr UL-VIS-IR, Zeiss). FCS was measured in 15 µl or 50 µl droplets, which were placed on gelatin treated Nunc 8 well chambered cover glass according to Hunke et al. (54) Following filter sets were used: MBS (main beam splitter): HFT (Hauptfarbeiteiler, main color splitter) 488/543/633, EF1 (emission filter): LP 655, EF: None, DBS (dichroic beam splitter): None, 633 nm: 3% transmission. Out-of-focus fluorescence was rejected by a 90 µm pinhole in the detection pathway, resulting in a confocal detection volume approximately 0.25 fl. Fluorescence autocorrelation functions were measured for 30 sec each with 10 repetitions. Cy5 solutions in buffer were used as references for the calibration of the ConfoCor 3 system. In order to study the effect of NBD-Cl, protein solution, magnesium chloride and NBD-Cl were pre-incubated for 30 min at 4 °C. After addition of EDA-ATP ATTO-647N the solution was incubated for further 10 min. The following filter sets were used: MBS: HFT 514/633; EF1: BP 655-710R; EF: none; DBS: none; DBS1: plate; DBS3: mirror, 633 nm: 3% transmission. To analyze the autocorrelation functions, a standard model was used for fitting (FCS-LSM software, ConfoCor 3, Zeiss). The calculations were done by the ConfoCor 3-software 4.2, Microsoft Excel 2003, and Origin 7.5.

**Tyrosine fluorescence spectroscopy**

A Varian Cary Eclipse spectrofluorimeter (Australia) was used, and all experiments were carried out at 20 °C. The samples were excited at 277 nm, and the emission was recorded from 295 to 350 nm with excitation and emission bandpasses set to 5 nm.

**Preparation of P.yoelii YM culture supernatant**

YM parasites were harvested from BALB/c mice and schizonts were purified by centrifugation on a 50-80% step gradient of Nycodenz (Sigma). Purified schizonts were placed back into culture containing incomplete RPMI 1640 with 25% FBS (invitrogen). Cells were harvested by centrifugation after 16 h. To remove residual nucleotides the supernatant was dialyzed against incomplete RPMI 1640 at 4 °C overnight and stored in aliquots at -80 °C for further erythrocyte binding assay.
Erythrocyte binding assay (EBAs) using untreated and nucleotide-treated dialyzed *P. yoelii* YM parasite culture supernatant

Erythrocyte binding assays were performed with minor modifications as previously described (13,55). Briefly, 30ul of dialyzed supernatant were incubated with a final concentration of 3 mM Mg\(^{2+}\)ATP (ratio of 1:1), 3 mM Mg\(^{2+}\)ADP (ratio of 1:1) or no nucleotide addition, in incomplete RPMI 1640 at 4 °C for 15 min respectively, followed by the addition of 100ul packed BALB/c mice erythrocytes. The bound protein was eluted and separated by SDS-PAGE on a 6% polyacrylamide gel and detected by western blotting using mouse monoclonal antibody (MAb) 25.77 (18,19).

RESULTS

Domain mapping of merozoite invasion proteins using bioinformatics tools

The phosphate-binding loop (P-loop) is one motif commonly found in adenine nucleotide-binding proteins. The consensus sequence for the P-loop motif is normally quoted in the literature as GXXXXGK(T)X (56,57). Some ATP-binding proteins share another less conserved site with unknown function, originally called motif B (57). Although there are proteins known to bind ATP that lack a P-loop motif, like glycolytic kinases, El/E2-type ATPases, actin, tubulin and aminoacyl-tRNA synthetases(56), we nevertheless searched for the P-loop patterns using the program SMART (Simple Modular Architecture Research Tool) in known merozoite invasion proteins. As shown in table 1, a nucleotide binding domain (NBD) with an e-value of 2 x 10\(^{-35}\) was identified in the case of the Py235 family member PY01365 from *P. yoelii*. Similar values could be determined for other Py235 members, such as PY01185 and PY00649. It also became evident that the NBD is predicted with a very high level of confidence (1 x 10\(^{-12}\) – 2 x 10\(^{-35}\)) in all Py235 members. Additional confirmation came from another motif prediction program, PSI BLAST, which identified this region as a potential nucleotide binding domain for the Py235 members (data not shown). The same analysis was carried out on the highly diverse homologues of the RBL family in other *plasmodium* species as well as a range of other unrelated malaria invasion proteins. An NBD was also predicted with a high degree of confidence level in all members of the PfRh (2 x 10\(^{-11}\) – 2 x 10\(^{-20}\)) and PvRBP (6 x 10\(^{-22}\) – 6 x 10\(^{-25}\)) family. The location of the predicted NBD is similar within members of the RBL of the same species (Table 1). Importantly, no NBD domain was predicted for other types of malaria invasion proteins, such as AMA, MSP1-MSP7, the EBL families, RAP1-RAP3, RhopH1 and RhopH3, RAMA, SURFIN and ASP (data not shown). Only in the case of RhopH2 was a putative NBD domain identified, though with significantly reduced confidence (1 x 10\(^{-6}\)). Walker A and Walker B motifs were identified in the primary amino acid sequence of the predicted NBD domain of PY01365 (Figs. 1). A glycine-rich region, GTPKGNT, is seen in this sequence while a possible Walker B motif is also found.

Production, purification and spectroscopic characterization of NBD94

To investigate whether the predicted NBD domain indeed exhibit ATP binding traits the corresponding region from PY01365, named here Nucleotide Binding Domain (NBD) 94, was cloned and expressed. The SDS-PAGE of the expressed recombinant NBD94 protein revealed a prominent band of 94 kDa which was found entirely within the soluble fraction. A Ni\(^{2+}\)-Sepharose resin column and an imidazole-gradient (0 – 250 mM) in buffer consisting of 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 4 mM Pefabloc\(^{SC}\) and 0.7 mM DTT was used to separate NBD94 from the main contaminating proteins. NBD94 eluting at 125-200 mM imidazole was collected and subsequently applied to a DEAE column. Analysis of the isolated protein by SDS-PAGE revealed the purity of the protein (Fig. 2 insert). Like the purity, the identity of the protein was confirmed by MALDI-TOF mass spectrometry. The monodispersity of NBD94 has been proven by fluorescence correlation spectroscopy (see below). The secondary structure of NBD94 was determined from circular dichroism spectra, measured between 185-260 nm (Fig. 2). The minima at 222 and 208 nm and the maximum at 192 nm indicate the presence of \(\alpha\)-helical structures in the protein. The secondary structure content was calculated to be 65% \(\alpha\)-helix and 24% random coiled. This result is consistent with secondary structure predictions based on
NBD94 amino acid sequence. The molar ellipticity value at 208 nm and at 222 nm are -32,135.7 deg x cm² x dmol⁻¹ and -32,224.8 deg x cm² x dmol⁻¹ respectively, in a ratio of 1.0. Since non-interacting helices typically give ratios of around 0.8, whereas interacting ones have ratios close to 1.0 (58), the CD spectrum presented indicates that many of the residues in NBD94 are in strong interaction. An ATPase activity of the NBD94 protein in the presence of MgCl₂ could not be detected, when compared to the standard hydrolytic activity of 11-13 µmol of ATP hydrolyzed/mg · ml of the Escherichia coli F₁ ATPase (59).

Interaction of the monofunctional label 8-N₃,3'-biotinyl-ATP with NBD94

There has been considerable interest recently in the use of photoaffinity analogues in order to characterize the structure-function relationship of ATPases and nucleotide-binding proteins from different sources (50,60,61). Here, 8-N₃,3'-biotinyl-ATP, which reacts predominantly with nucleophilic groups (50), has been supplemented with nucleotide depleted NBD94. After irradiation of NBD94 in the presence of Mg²⁺-8-N₃,3'-biotinyl-ATP, the bound photoaffinity label is detected by western blotting using streptavidin. The results are shown in lane 3 of Figure 3A. To test whether labeling could be protected by the natural substrates, Mg²⁺-ATP (lane 6) and Mg²⁺-ADP (lane 5) or by the non-hydrolysable analogue Mg²⁺-AMP-PNP (lane 4), these nucleotides were added prior to the labeling procedure. As can be seen, the presence of Mg²⁺-ATP, Mg²⁺-AMP-PNP as well as Mg²⁺-ADP prevent binding of Mg²⁺-8-N₃,3'-biotinyl-ATP to the protein. Also irradiation of NBD94 in the absence (lane 1) of the label or by incubation of the protein in the presence of 8-N₃,3'-biotinyl-ATP in the dark (lane 2) did not result in labelling of NBD94, indicating the specific covalent binding of 8-N₃,3'-biotinyl-ATP by the protein.

A more detailed location of the label in NBD94 was determined by MALDI-TOF mass spectrometry. For that the Mg²⁺-8-N₃,3'-biotinyl-ATP labeled protein band was subjected to in-gel tryptic digestion. Figure 3B shows a MALDI-TOF spectrometry map. The peptides were identified as bands deriving from NBD94. In addition an increase of 1079 Da for the fragment EKLKHYNFDDFVK₅₀₀ (numbering includes the His₆-tag; peak position 2761.85 m/z) was observed, suggesting that this peptide is labeled by 8-N₃,3'-biotinyl-ATP, bound as a tri-triethylamonium salt derivative.

Nucleotide-binding of NBD94 determined by fluorescence correlation spectroscopy

ATP- and ADP-binding of NBD94 and the strength of the nucleotide-binding were further examined by fluorescence correlation spectroscopy (FCS), which is a highly sensitive tool to determine binding/dissociation equilibria. The characteristic diffusion time, τ₀, for NBD94 was measured by binding of labeled nucleotides to the protein. The diffusion times of the NBD94 ATTO-647N complex were compared to the standard fluorophore Cyanine 5 (Cy5) and the ATTO-647N conjugated nucleotides without NBD94. The mean count rate per ATTO fluorophore bound to NBD94 was 56.4 kHz for ATP ATTO-647N (50 nM) and 31.2 kHz for ADP ATTO-647N (50 nM), respectively. Fitting the autocorrelation functions resulted in characteristic diffusion times of ATTO-647N and τ₀ = 62.4 µs for ATP ATTO-647N and τ₀ = 62.7 µs for ADP ATTO-647N.

Figure 4A shows the measured autocorrelation curves of the fluorescent ATP-analogue, ATP ATTO-647N, in the absence and presence of different concentrations of NBD94. The addition of protein resulted in a significant change in the mean diffusion time τ₀. The diffusion time of ATP ATTO-647N increased up to 14.2% with increasing concentrations of NBD94. This confirmed that nucleotides bound to NBD94 in the presence of Mg²⁺ (ratio of 1:1). A binding constant of 21 ± 3 µM bound ATP ATTO-647N to NBD94 was calculated (Fig. 4C). When the ADP analogue ADP ATTO-647N was tested with the full length protein NBD94 the mean diffusion time increased up to 14.9%. A binding constant of 26 ± 3 µM for bound ADP ATTO-647N to NBD94 was determined (Fig. 4D).

7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been found to inhibit the F₁F₀ ATP synthase by reacting with a Tyr residue in the central nucleotide-binding domain of the catalytic subunit β (62), resulting in the loss of the lowest affinity binding site for nucleotides (63). By comparing the autocorrelation curves of the NBD94-ATP-ATTO-647N bound form with
those, in which the protein has been preincubated with increasing amounts of NBD-CI, the diffusion time of ATP ATTO-647N was shown to decrease with increasing concentrations of NBD-CI, indicating that nucleotide binding to NBD94 became inhibited.

Secondary structural alterations due to nucleotide-binding probed by spectroscopy

To obtain additional information on possible secondary structure alterations in NBD94 after nucleotide-binding the fluorescence emission of the protein with and without ligands was studied. Since NBD94 lacks any tryptophan residue the fluorescence spectrum was monitored using the intrinsic tyrosine fluorescence (Fig. 5, curve A). Addition of Mg\(^{2+}\)ADP (curve b) decreased the quantum yield (up to 18\%) with a concurrent shift of the maximum to a longer wavelength, which might be caused by the reorientation of a Tyr residue(s) inside the binding pocket to a more polar environment. The fluorescence intensity was further reduced in the presence of Mg\(^{2+}\)ATP (curve c). In contrast, NBD94 in the presence of Mg\(^{2+}\)GTP (curve x) displayed a spectrum similar to that obtained in the absence of nucleotides. The data show that the tyrosine fluorescence spectrum of NBD94 is sensitive to ATP and ADP binding and offered the possibility to investigate the binding behavior of the protein to MgATP and MgADP. The results of both titration experiments are shown in Figure 5B, revealing a hyperbolic shape of the binding curve. A binding constant of 25 ± 2 \(\mu\)M and 31 ± 2 \(\mu\)M could be determined for MgATP and MgADP, respectively.

The secondary structure of NBD94 in the presence of nucleotides was determined from circular dichroism spectra (Fig. 2). Figure 2 indicates that when Mg\(^{2+}\)ATP is bound to NBD94, the protein showed a slightly lower helical content (62\%) and a lower \(\theta_{222}/\theta_{208}\) ratio (0.98). By comparison, the \(\theta_{222}/\theta_{208}\) ratio of the Mg\(^{2+}\)ADP form of NBD94 is 0.9 and the \(\alpha\)-helical amount is 69\%. The identical values could be observed in repeated experiments. In summary the data imply significant secondary structural alterations due to nucleotide binding.

Purification and spectroscopic traits of the truncated 65 kDa form of NBD94

In order to determine what forms the minimal nucleotide-binding core of the NBD94 protein, the C-terminal truncated form of NBD94 (NBD94\(_{1-550}\)), including both the Walker A and B motif, was constructed. The expressed protein was purified by metal chelate affinity chromatography, followed by ion-exchange chromatography (DEAE; Fig. 6A). In the final step the protein was eluted from a size-exclusion column (Superdex\(^{\text{TM}}\) S-75 10/300 GL column) in a single peak. Analysis of the isolated protein by SDS-PAGE revealed the high purity of the 65 kDa protein NBD94\(_{1-550}\) (Fig. 6B). To explore the nucleotide-binding traits of NBD94\(_{1-550}\) FCS experiments were performed. Figure 4B illustrates the measured autocorrelation curves of Mg\(^{2+}\)ATP ATTO-647N in the absence and presence of the truncated form of NBD94\(_{1-550}\). The addition of the truncated form resulted in a significant change of the mean diffusion time \(\tau_D\), whereby the diffusion time of Mg\(^{2+}\)ATP ATTO-647N increased up to 31.9\%. A binding constant of 51 ± 3 \(\mu\)M for bound Mg\(^{2+}\)ATP ATTO-647N to this protein could be calculated (Fig. 4C). By comparison, the diffusion time of Mg\(^{2+}\)ADP ATTO-647N in the presence of the truncated protein yielded an increase of 31.6\%. A binding constant of 53 ± 3 \(\mu\)M of Mg\(^{2+}\)ADP ATTO-647 to NBD94\(_{1-550}\) was established (Fig. 4D). The data showed that NBD94\(_{1-550}\) is still able to bind nucleotides, albeit at a lower affinity compared to the entire NBD94 protein. In addition, when CD-spectroscopy was used to elucidate the effect of Mg\(^{2+}\)ADP- or Mg\(^{2+}\)ATP-binding on the secondary structure of NBD94\(_{1-550}\) the spectra were found to be similar to the one done in the absence of nucleotides (Fig. 7).

Impact of ATP/ADP on erythrocyte binding of full length Py235 protein presence

To investigate whether ATP or ADP had a direct impact on the ability of Py235 to bind to erythrocytes, erythrocyte binding assays (EBA) in the presence of either no nucleotide, ATP or ADP were carried out (Fig. 8). Bound Py235 was detected using the well characterized monoclonal antibody MAb 25.77 (18) which recognizes the full length in the parasite supernatant (Fig. 8, lane 4). Binding of Py235 to erythrocytes could be inhibited by treatment of the erythrocytes with neuraminidase, while treatment with trypsin or chymotrypsin had no effect (data not shown), confirming the previously described binding properties of Py235 (13). EBA shows some
binding of Py235 to erythrocytes in the absence of any additional nucleotide (Fig 8A, lane 3). Binding of Py235 increases significantly in the presence of additional MgATP (Fig. 8A, lane 2) while there is a noticeable reduction of binding in the presence of additional MgADP, as compared to no nucleotide added (Fig. 8A, lane 1). This is confirmed by quantitation of the scanned bands using the program AIDA 2.40 (advanced image analyzer, (Fig. 8B).

DISCUSSION
The initial interactions between the merozoite and the erythrocyte that lead to junction formation and invasion involve a cascade of receptor-ligand interactions. In *P. yoelii*, the Py235 rhoptry protein multigene family has been implicated in host cell selection and is strongly linked to parasite virulence. Clonal phenotypic variation of *py235* in *P. yoelii*, where each merozoite originating from a single schizont expresses a distinct *py235* member, has also implicated Py235 in playing a role in host cell adaptation and immune evasion (21,22). Currently, a single member of Py235 has been shown to directly bind to erythrocytes and while at this stage it is believed that the other Py235 members have also red cell binding activity this still awaits confirmation (13,64). The interaction of Py235 with the erythrocyte is mediated by a specific trypsin and chymotrypsin sensitive erythrocyte receptor (13), which is in line with the PfRH1 protein of *P. falciparum*, shown to bind to a specific erythrocyte receptor (14,16). These findings indicate that part of the protein sequence of Py235 would encompass a receptor binding domain that mediates the specific interactions needed for erythrocyte recognition. A gap in our understanding is the molecular events that lead up to the binding of Py235 to its receptor and the subsequent events that lead to the downstream signaling that enables the merozoite to continue the invasion process. Considering the large size of Py235, it made it likely that other functional domains besides a receptor binding region could be found in the full length protein.

Here analysis of the known Py235 protein sequences by bioinformatics approaches predicted the presence of a nucleotide binding domain with relatively high confidence. Importantly, this domain was only predicted in all Py235 members and in other members of the RBL family as well. In addition, the NBD was not predicted for any other key malaria invasion protein we analyzed, indicating an important role of nucleotide binding in the function of RBL. Based on the initial prediction, we have successfully expressed a 94 kDa domain of Py235 and shown that it indeed binds the nucleotides ATP and ADP with high specificity. Based on FCS and tyrosine fluorescence analysis, binding to ATP is stronger than that observed for ADP. Interestingly, ATP-binding is inhibited in the presence of NBD-Cl, a potent inhibitor of F-ATP synthases (65). In this case the inhibitor binds to a Tyr residue of one catalytic β subunit of F1F0 ATP synthases and prevents this so-called empty β subunit from being changed into a nucleotide-binding state (62). Because of the protein traits described above, we have termed our recombinant protein the nucleotide binding domain 94 (NBD94). There were significant changes observed in the intrinsic tyrosine fluorescence spectra upon binding of NBD94 to either ATP or ADP respectively, indicating that these two nucleotides induce slightly different structural changes in the adenine-binding site of the binding pocket upon nucleotide binding. Additional evidence for the changes in secondary structure comes from the CD analysis of NBD94 in the presence of either ATP or ADP. The changes in the spectra are consistent with a significant change in the helical contents of the secondary structure in response to binding either ATP or ADP. Using the truncated version of NBD94 (1-550) that still retains the ATP/ADP binding activity though with slightly reduced affinity clearly shows that the observed structural changes due to nucleotide binding can be contributed by the C-terminal part of NBD94. Our findings are consistent with this C-terminal region acting like a hinge that changes conformation depending on whether ATP or ADP is bound. Such hinge structures have previously been described for the nucleotide-binding subunits β (66) and B (67) of the F- and A-ATP synthases, respectively, where the C-terminal domain of the nucleotide-binding subunit undergoes a relative rotation, effecting the coupling with the neighboring subunits γ and ε, and F, respectively, thereby resulting in rotational movements in the entire enzyme complex. From this, it is clear that NBD94 can assume three different structural states based on the presence of
ATP, ADP or no nucleotide consistent with the idea that NBD94 functions as ATP/ADP sensor inside the Py235 complex. Importantly, the observation that addition of ATP/ADP modulates the direct binding of Py235 to the erythrocyte strongly suggests that the conformational changes induced by these nucleotides regulate accessibility of the binding domain in the full length protein.

The ability of Py235 to sense ATP adds another level of complexity to the already complex process of host cell recognition and invasion by the merozoite. Previous studies have indicated that intracellular ATP is important for merozoites to establish an infection inside the erythrocyte (32-35) possibly playing a role in the phosphorylation of the erythrocyte cytoskeleton (68). Within this context, it would be of benefit to the merozoite to detect, whether an erythrocyte contained sufficient ATP, to provide a suitable environment before committing to invasion. Secretion of ATP by erythrocytes occurs under normal physiological conditions but is significantly enhanced by mechanical deformation (29) as well as other stimuli like hypoxia and hypercapnia (69). This secretion of ATP from the erythrocyte would lead to the establishment of an ATP gradient that could be sensed by the merozoite. The very small difference in the binding affinities by NBD94 for ATP as compared to ADP indicates that small changes in the ratios of ATP/ADP such as would be expected to be observed at the surface of the erythrocyte would trigger the conformational change observed in the study reported here. Such structural changes could serve as an activation step of Py235 that directly leads to Py235 binding to its erythrocyte receptor, thereby enabling the invasion process to proceed.

In summary, the data presented demonstrate for the first time, that besides the already characterized signal peptide, erythrocyte binding properties and transmembrane domain of the reticulocyte binding protein Py235, this large multidomain protein also contains a nucleotide binding domain. The preference of MgATP over MgADP binding, associated with specific structural alterations in the C-terminal domain of NBD94 implies its role as a mediator, which might facilitate the linkage of nucleotide signaling and Py235 binding to the blood cell. The finding of a nucleotide binding domain inside Py235 will be a stimulus for studying the detailed function(s) and interactions of the ensemble of domains within this important class of invasion proteins.

**REFERENCES**

FOOTNOTES

We thank Dr. Shahid Khan for introducing J. Ramalingam to the SMART program and stimulating discussions and Dr. Sze S. K. for mass spectrometry analysis. We are grateful to Prof. H.-J. Schäfer (Johannes-Gutenberg University Mainz, Germany) for the generous gift of the analogues 8-N$_3$-3’-biotinyl-ATP. This research was supported by A*STAR BMRC (06/1/22/19/456) and ARC (MLC03/03).

FIGURE LEGENDS

FIGURE 1: (A) Schematic representation of RBLs as revealed by bioinformatics analyses (see Experimental Procedures). (B), The primary sequence (791 amino acids) of NBD94 with the 8-N$_3$-3’-biotinyl-ATP binding peptide as revealed by MALDI-TOF and as highlighted in black shading. The gray shading indicates the amino acids which are deleted in the truncated form NBD$_{1-550}$.

FIGURE 2: Far-UV CD spectra of NBD94 in the absence (—) and presence of 2mM Mg-ATP (····) and Mg-ADP (– ·– ·), respectively. (Inset), SDS-gel shows a sample of the purified NBD94.

FIGURE 3: Photoaffinity labeling of NBD94 by 8-N$_3$-3’-biotinyl-ATP. (A), an SDS-PAGE of NBD94, and an immunoblot of the same sample labeled with Mg-8-N$_3$-3’-biotinyl-ATP in the absence of nucleotide inhibitors (lane 3) as well as in the presence of 4 mM Mg$^{2+}$-AMP-PNP (lane 4), 4 mM Mg$^{2+}$-ADP (lane 5) and 4 mM Mg$^{2+}$-ATP (lane 6). Lane 1 and 2 are light and dark controls in the presence and absence of photolabel ATP, respectively. The label was detected using a streptavidin/POD conjugate according to Schäfer et al., 2001. (B), MALDI-TOF mass spectrometry of peptides obtained by in-gel trypsin digestion of 8-N$_3$-3’-biotinyl-ATP labeled NBD94 (upper spectrum) and of NBD94 only. The peak labeled by an (*) represents the peptide labeled with 8-N$_3$-3’-biotinyl-ATP.

FIGURE 4: Fluorescence correlation spectroscopy of NBD94 and of fluorescent nucleotide-analogues in the presence of 2 mM Mg$^{2+}$. (A) Normalized autocorrelation functions of MgATP ATTO-647N in the absence and presence of different concentrations of the full length protein NBD94. (B) Normalized autocorrelation functions of MgATP ATTO-647N in the absence and presence of different concentrations of NBD94$_{1-550}$. (C) NBD94 (▲) in comparison with truncated NBD94$_{1-550}$ (□) with Mg-ATP-ATTO-647N. The percentage of bound nucleotides was analyzed using a two component binding scheme, [E]+[S]→ [ES]. Best fits yielding the binding constants are represented as fitted lines (see the text). (D) Concentration-dependent binding of NBD94 (▲) and NBD94$_{1-550}$ (△) to MgADP-ATTO-647N. (E) Normalized autocorrelation functions of Mg-ATP-ATTO-647N bound to NBD94 (40 μM) by increasing the quantity of the inhibitor NBD-Cl (from right to left 0 nM (black) of NBD-Cl as well as 0.1 μM (blue) and 0.5 μM (green) of the inhibitor). The autocorrelation function of unbound Mg-ATP-ATTO-647N is shown in red. The arrow indicates the drop of nucleotide-binding.

FIGURE 5: Nucleotide-dependent tyrosine fluorescence emission spectra of NBD94. The tyrosine fluorescence emission spectrum of NBD94 was measured with a 1:1 ratio of Mg$^{2+}$ to nucleotide at room temperature. The protein was diluted in 50 mM Tris/HCl, pH 7.5, 250 mM NaCl and 0.7 mM DTT and incubated with 2 mM Mg$^{2+}$GTP (curve x), 2 mM Mg$^{2+}$ATP (curve □) and 2 mM Mg$^{2+}$ADP (curve o) on ice for 5 min. Curve △, NBD94 in the absence of nucleotides. (B) Fluorescence titration of NBD94 with MgATP (○) and MgADP (+), respectively. Excitation was measured at 277 nm.

FIGURE 6: Chromatographic purification of NBD94$_{1-550}$. (A), ion-exchange chromatography using a DEAE column in Tris-HCl buffer. After sample injection, an isocratic elution of 5 column volumes with
buffer A (Tris/HCl, pH 7.5 and 1 mM DTT) and a flow rate of 3.0 ml/min was employed, followed by a
gradient program 0 to 40 % buffer B (Tris/HCl, pH 7.5, 1 M NaCl and 1 mM DTT; (---)). (B), 1 µl of the
collected and concentrated sample of the main peaks was applied on an SDS-gel, stained with Coomassie
Blue G250.

FIGURE 7: Far-UV CD spectra of NBD94_{1-550} in the absence (——) and presence of 2mM Mg-ATP (····)
and -ADP (– ·– ·), respectively.

FIGURE 8: Erythrocyte binding assay of dialyzed *P. yoelii* YM parasite culture supernatant with or
without nucleotide-treatment were analyzed by western blotting using mouse MAb 25.77. An
approximately 260 kDa protein of supernatant was detected by MAb 25.77 (lane 4); supernatant treated
with 3 mM Mg^{2+}ADP bound to erythrocytes (lane 1); supernatant treated with 3 mM Mg^{2+}ATP bound to
erthrocytes (lane 2); untreated supernatant bound to erythrocytes (lane 3). Molecular sizes are indicated
on the left (in kDa). Relative intensities of the bands in lane 1, 2 and 3 under the nucleotide conditions
described above were determined by scanning the blot shown in (A) with an HP (ScanJet 4c) flat-bed
scanner. The intensity of each band was digitized after background subtraction and calculated by the
program AIDA 2.40 (advanced image analyzer; Raytest) according to (70).
### TABLE I. SMART program predicted nucleotide-binding domains within the protein sequences of RBLs in *P. yoelii*, *P. falciparum* and *P. vivax*

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*Peptide labeled with 8-N<sub>3</sub>-3'-biotinyl-ATP
Figure 1; Ramalingam et al.
Figure 2: Ramalingam et al.
Figure 3: Ramalingam et al.
Figure 4A-D: Ramalingam et al.
Figure 4E; Ramalingam et al.
Figure 5A & B; Ramalingam et al.
Figure 6A-B: Ramalingam et al.
Figure 7; Ramalingam et al.
Figure 8A-B; Ramalingam et al.