Title:

Plasmodium knowlesi thioredoxin peroxidase 1 binds to nucleic acids and has RNA chaperone activity

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Abstract

Malaria parasites are under oxidative attack throughout their life cycle in human body and mosquito vector. Therefore, *Plasmodium* antioxidant defenses are crucial for its survival and being considered as interesting target for antimalarial drug design. *Plasmodium knowlesi* has emerged recently from its simian host to human in Southeast Asia and has been recognized as the fifth *Plasmodium* species that can cause human malaria. In this study, we cloned and characterized thioredoxin peroxidase 1 from *P. knowlesi* (PkTPx-1). PkTPx-1 gene was cloned and recombinant protein was produced by heterologous over-expression in *Escherichia coli*. The recombinant protein was used for evaluation of enzymatic activity and polyclonal antibody production. Using the recombinant PkTPx-1 protein, its antioxidant activity was confirmed in a mixed function oxidation assay where PkTPx-1 prevented nicking of DNA by hydroxyl radicals. PkTPx-1 was able to bind to double strand DNA and RNA and had RNA chaperone activity in a nucleic acid melting assay indicating new function of PkTPx-1 other than antioxidant activity. Using specific polyclonal antibodies, it was indicated that PkTPx-1 is expressed in the cytoplasm of the parasite. Altogether, these results suggest that PkTPx-1 not only protect the parasite from the adverse effects of reactive oxygen species but also has RNA chaperone activity.

38 **Keywords**: *Plasmodium knowlesi*, thioredoxin peroxidase 1, Nucleic acid binding,

39 RNA chaperone

1. Introduction

All aerobic organisms are exposed to reactive oxygen species (ROS) such as superoxide anions, peroxynitrite, hydrogen peroxide and hydroxyl radicals which can damage their macromolecules (Rhee et al., 2001). Malaria parasites are highly sensitive to oxidative stress but live in a pro-oxidant rich environment containing oxygen and iron, the key perquisite for ROS production (Muller 2004). *Plasmodium* digests hemoglobin as the major source of amino acids into heme which is toxic for the parasite and mostly is sequestered into hemozoin (Becker et al., 2004). An appreciable amount of heme escapes from sequestration which constitutes the main source of ROS for the parasites and should be degraded by other means to prevent parasite death (Muller 2004). In order to maintain their redox homeostasis, malaria parasites are equipped with several antioxidant enzymes including superoxide dismutases and peroxiredoxins (Prxs) (Becker et al., 2004). Since catalase and genuine glutathione peroxidase do not exist in *Plasmodium*, Prxs play a vital role in the reduction of ROS (Kawazu et al., 2008; Kehr et al., 2010)

Prxs is a ubiquitous family of antioxidant enzymes that are present in organisms from all kingdoms (Rhee et al., 2001). The high expression level (Kawazu et al., 2008) and ubiquitous distribution (Kehr et al., 2010) make Prxs one of the first proteins to

encounter and detoxify hydrogen peroxides in the parasite. These enzymes exert their antioxidant role in the parasites through active site cysteine (Cys), where hydrogen peroxide, peroxinitrite and a wide range of organic hydroperoxides are reduced into water and alcohol (Wood et al., 2003). The redox-active Cys in the active site of the enzyme is conserved and based on the number of Cys involved in catalysis, Prxs are divided into three categories: 1-Cys, typical and atypical 2-Cys (Chae et al., 1999). Apart from the antioxidant activity, Prxs are known as an important mediator in cell signaling though reduction of hydrogen peroxides (Wood et al., 2003) and molecular chaperones (Jang et al., 2004; Castro et al., 2011; Rhee and Woo 2011). The structural flexibility, changing from oligomer to dimer or *vice versa* and phosphorylation of Prxs are the factors contributing to the functional diversity of Prxs (Wood et al., 2003; Richard et al., 2011). Whether inhibitors targeting parasite Prxs will lead to the parasite's death, that might prove the important role of Prxs in malaria parasites, remains to be determined.

P. knowlesi like *P. falciparum* can cause severe and fatal disease and is being considered as an emerging human malaria parasite (Kantele and Jokiranta 2011). Better understanding of basic biology of *P. knowlesi* is crucial for the control of this disease. In the present study, we characterized *P. knowlesi* thioredoxin peroxidase 1 (PkTPx-1), a

member of Prx family. Using recombinant protein, it was shown that PkTPx-1 was able to bind to nucleic acids and may acts as RNA chaperone which are novel functions other than known antioxidant activity for TPx-1.

2. Materials and Methods

2.1. Cloning of *P. knowlesi* TPx-1 gene

A set of primers was designed (Supplementary Table S1) using PlasmoDB database version 10 (PlasmoDB, 2013) to amplify the complete sequence of the gene coding for TPx-1 of *P. knowlesi* (PkTPx-1). The PkTPx-1 gene was amplified from genomic DNA of *P. knowlesi* H strain, ATCC No. 30158 which was extracted by phenol-chloroform technique from the parasite cell pellet. The PCR was done as previously described (Hakimi et al., 2013).

2.2. Expression and purification of recombinant PkTPx-1 protein

The *Escherichia coli* cells harboring PkTPx-1/pET-28a plasmid were grown at 37°C until the optical density at 600 nm reached 0.6. Expression of the recombinant PkTPx-1 protein (rPkTPx-1) was induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) for 4 h at 37°C. rPkTPx-1 was purified using Ni-NTA agarose beads (Qiagen

Inc., Valencia, CA) as described previously (Hakimi et al., 2012)

2.3. Antioxidant activity of rPkTPx-1

The antioxidant activity of rPkTPx-1 was assessed by thiol mixed-function oxidation (MFO) assay as described (Sauri et al., 1995). The reaction mixture (50 µl) containing 40 µM $FeCl_3$, 10 mM dithiothreitol (DTT), 20 mM EDTA, 25 mM HEPES, pH 7.0 was pre-incubated with or without rPkTPx-1 (10–400 µg/ml) at 37°C for 1 h. 500 ng of pBluescript plasmid DNA (Stratagene, La Jolla, CA) was added and the reaction mixture was further incubated for 3 h at 37°C. Nicking of the supercoiled plasmids by the MFO assay was evaluated by 0.8% agarose gel electrophoresis.

2.4. Evaluation of DNA and RNA-binding activity

Different concentrations of rPkTPx-1 and recombinant *P. knowlesi* 1-Cys-Prx (rPf1-Cys-Prx) were incubated with 500 ng of pBluescript plasmid DNA for 30 min room temperature. For DNase protection assay, DNase (Takara, Otsu, Japan) was added to the mixture and incubated for another 30 min at 37°C. The DNA-binding activity and DNase protection of recombinant proteins were evaluated by 0.8% agarose gel electrophoresis and further stained with ethidium bromide.

112 In order to evaluate RNA-binding activity, pBluescript plasmid was digested
113 with *Sal* I and 32 bp biotin labeled ssRNA was produced using the plasmid fragments as
114 template with T7 RNA polymerase (New England BioLabs) in the presence of 2.5 mM
115 Biotin-16-UTP, 5 mM UTP, and 7.5 mM ATP, GTP and CTP. Binding reactions were
116 performed with 1-5 μg of recombinant proteins, 0.3 ng of biotinylated RNA, 2 μg tRNA
117 and 1× RNA electrophoretic mobility shift assay (REMSA) binding buffer (Thermo
118 Scientific) in a 20 μl final volume. The reactions were incubated in room temperature
119 for 30 min and further separated by 4% non-denaturing polyacrylamide gel
120 electrophoresis in 4°C. The separated reactants were transferred onto nitrocellulose
121 membrane and biotinylated RNA was detected with streptavidin-horseradish peroxidase
122 conjugate using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific)
123 according to the manufacturer's instructions.
124
125 2.5. Evaluation of RNA chaperone activity of PkTPx-1
126 *In vitro* nucleic acid melting assay using molecular beacon was conducted to
127 evaluate the RNA chaperone activity of PkTPx-1. The molecular beacon was a 78-
128 nucleotide hairpin molecule (Kim et al., 2012) labeled with 6-FAM as flourophore and
129 TAMRA as quencher (Sigma Aldrich). The recombinant proteins were dialyzed in 20

mM Tris-Hcl pH: 7.5 and fluorescence measurement was performed in 96-well plate using Fluoroskan Ascent (Thermo Scientific), with excitation and emission at 485 and 518 nm, respectively.

2.6. Indirect immunofluorescence microscopy

In order to show the cellular localization of PkTPx-1, indirect immunofluorescence microscopy was done using thin smears of *P. knowlesi*-infected RBCs from an experimentally infected male monkey, *Macaca fuscata* and mouse serum against rPkTPx-1, prepared as previously described (Hakimi et al., 2012). The smears were examined by confocal laser-scanning microscopy (TCS-SP5, Leica Microsystem, Wetzlar, Germany). The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation at the Dokkyo Medical University (Permission number 0656) and the Obihiro University of Agriculture and Veterinary Medicine (Permission number 25-74).

3. Results and Discussion

PkTPx-1 was cloned and recombinant PkTPx-1 protein (rPkTPx-1) was produced and purified with molecular weight of 21.9 kDa (Fig. 1). The pairwise amino

148 acid sequence alignment using gene and protein analysis software (GENETYX
149 Corporation, Tokyo, Japan) showed 84.6% and 94.4% identity when PkTPx-1 was
150 compared with orthologous protein in *P. falciparum* (PfTPx-1) and *P. vivax* (PvTPx-1),
151 respectively (Fig. 2). Moreover, it was 43.5% identical to its closest human orthologue,
152 Prx1. As shown in Fig. 2, PkTPx-1 has two conserved cysteine (Cys), the active site of
153 the enzyme at positions 50 and 170 with the conserved amino acid sequence of FVCP
154 and DVCP, respectively (Wood et al., 2003), proving that it is a typical 2-Cys type Prx.
155 The protein produced a dimer under non-reducing condition which is particularly a
156 homodimer resulting from the inter-subunit disulfide bond (Fig. 1). All Prxs contain a
157 conserved Cys at the N-terminus called peroxidatic Cys (Cys_P) (Rhee et al., 2001).
158 During catalysis, Cys_P is oxidized by ROS to sulfenic acid (Cys-S-OH) (Hall et al.,
159 2009). PkTPx-1, like all other typical 2-Cys Prxs, contains a C-terminal Cys referred as
160 resolving Cys (Cys_R) and reacts with adjacent sulfenic acid subsequently forming an
161 intermolecular disulfide bond (Wood et al., 2002). This bond further could be reduced
162 by a reducing agent such as thioredoxin or glutathione.

163 The antioxidant activity of PkTPx-1 was evaluated by MFO assay. In this assay,
164 $FeCl_3$ and DTT generate hydroxyl radicals that cleave DNA (Sauri et al., 1995). In the
165 absence of rPkTPx-1, the hydroxyl radicals in the system resulted in the nicking of the

166 super-coiled plasmid DNA, changing the running behavior of the DNA in the agarose
167 gel electrophoresis (Fig. 3). However, adding 25µg/ml and the higher concentrations of
168 rPkTPx-1in the reaction prevented nicking of the DNA, confirming the antioxidant
169 activity of PkTPx-1. Interestingly, PkTPx-1 showed DNA-binding activity in the MFO
170 assay by shifting the mobility of DNA which was previously seen by PvTPx-1 (Hakimi
171 et al., 2012) suggesting this biding activity is a common character of TPx-1 among
172 these malaria parasites. The DNA-binding activity was further evaluated in comparison
173 with *P. knowlesi* 1-Cys-Prx (Pk1-Cys-Prx) which is another cytoplasmic Prx (Hakimi et
174 al., 2013). As shown in Fig. 4, the DNA-binding of PkTPx-1 was concentration-
175 dependent and shifted the mobility of pBluescript DNA in the gel electrophoresis
176 whereas the Pk1-Cys-Prx did not bind to the DNA. Moreover, PkTPx-1 protected DNA
177 from degradation by DNase (Fig. 4). The DNA-binding activity and protection of DNA
178 from DNase was also seen in human Prx 1 which is also a typical 2-Cys Prx (Kim et al.,
179 2012). It is quite interesting to further study the benefits of this DNA-binding activity
180 for the parasite.

181 To further evaluate nucleic acid-binding activity of PkTPx-1, RNA-binding
182 activity was assayed by performing REMSA using biotinylated ssRNA. Adding
183 rPkTPx-1 shifted the mobility of RNA indicating the RNA-PkTPx-1 interaction,

whereas no change was observed when rPk1-Cys-Prx was added (Fig. 5). To investigate the benefits of this nucleic acid-binding for the parasite and possible RNA-chaperone activity, we examined helix-destabilizing activity in nucleic acid melting assay. In this assay, a molecular beacon was used with stem-loop structure and a fluorophore and a quencher at the ends. Because of the proximity of fluorophore and quencher, the fluorescence is efficiently quenched when two parts of the molecular beacon remained annealed to each other. When a protein binds and melts the stem-loop structure, fluorescence is produced. Proteins with RNA chaperone activity assist RNA molecules with folding or opening up misfolded RNA structures under a process without ATP consumption (Semrad 2011). This system has been used to evaluate the RNA chaperone activity of several proteins (Phadtare and Severinov 2005; Kim et al., 2012). Adding rPkTPx-1 to the molecular beacon, a marked increase in fluorescence intensity occurred, indicating rPkTPx-1 melted the secondary structure of molecular beacon while adding rPk1-Cys-Prx did not increase fluorescence intensity (Fig. 5). Similar to PkTPx-1, PfTPx-1 and PvTPx-1 had nucleic acid-binding and RNA chaperone activity (data not shown), suggesting that these functions are common characteristics of *Plasmodium* TPx-1. Recently it was shown that PfTPx-1 gene-disrupted parasites (knock out) are hypersensitive to heat stress, suggesting a possible chaperone activity for this protein

(Kimura et al., 2013). Molecular chaperone activity of 2-Cys Prx is shown before in yeast where cytosolic yeast cPrx I and II have peroxidase function in lower molecular weight (MW) forms, while the chaperone function predominates in higher MW complexes (Jang et al., 2004). PfTPx-1 forms decamers consisting of pentamers of homodimers (Akerman and Muller 2003) which resemble heat shock proteins with molecular chaperone activity (Kim et al., 1998). To the best of our knowledge, this is the first report of nucleic acid-binding and RNA chaperone activity of *Plasmodium* TPx-1.

Immunofluorescence microscopy was performed to determine the cellular localization of PkTPx-1. Figure 6 shows a schizont and two trophozoite of *P. knowlesi* where the nuclei are stained with Hoechst. The presence of green fluorescence around nucleus indicates the cytoplasmic expression of PkTPx-1. Similar expression patterns were seen in PfTPx-1 and PvTPx-1 (Hakimi et al., 2012; Yano et al., 2005).

Taken together, TPx-1 not only acts as antioxidant enzyme to protect *P. knowlesi* from oxidative stress but also was able to bind to nucleic acids and had RNA-chaperone activity which are novel functions for *Plasmodium* TPx-1. Given that nucleic acid-binding and RNA chaperone activity of PkTPx-1 was not seen in Pk1-Cys-Prx and PkTPx-1 has no nucleic acid-binding motif, these novel functions might be related to

220 unique characteristic of eukaryotic typical 2-Cys-Prx such as dimer and higher
221 molecular weight oligomer formation which should be studied is future.
222
223 **Acknowledgements**
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297 **Figure legends**

298 **Fig. 1.** Recombinant PkTPx-1 protein (rPkTPx-1) expression and purification verified
299 by SDS-PAGE. (A) rPkTPx-1 was expressed in *E. coli*. M, Protein marker. Lane 1 is *E.*
300 *coli* culture before adding IPTG, lane 2 is 4h after adding IPTG and lane 3 is purified
301 rPkTPx-1. (B) Lane 1 is rPkTPx-1 under reducing condition (with 2-mercapto ethanol)
302 and lane 2 is rPkTPx-1 under non-reducing condition (without 2-mercapto ethanol).

303

304 **Fig. 2.** Deduced aminoacid sequence alignment of PkTPx-1, PfTPx-1 and PvTPx-1.
305 Asterisks indicate the conserved cysteine residues and the identical residues among the
306 three sequences are boxed.

307

308 **Fig. 3.** Mixed-function oxidation (MFO) assay to evaluate rPkTPx-1 antioxidant
309 activity. Prevention of nicking the plasmid DNA was evaluated by MFO assay. M, 1
310 kbp DNA marker; lane 1, pBluescript plasmid DNA and $FeCl_3$; lane 2, pBluescript
311 plasmid DNA and DTT; lane 3, pBluescript plasmid DNA, $FeCl_3$ and DTT; lanes 4-8,
312 pBluescript plasmid DNA, $FeCl_3$, DTT and 400, 200, 100, 25 and 10µg/ml of rPkTPx-1,
313 respectively. The nicked form (NF), supercoiled form (SF) and rPkTPx-1-DNA
314 complex are indicated on the right.

315

316 **Fig. 4.** DNA-binding activity of rPkTPx-1. (A) rPkTPx-1 and rPk1-Cys-Prx were
317 incubated with pBluescript plasmid DNA and the rPrx-DNA complexes were evaluated
318 using agarose gel shift assay. (B) DNA protection activity of rPkTPx-1. The mixture of
319 recombinant proteins and DNA was treated with DNase and analyzed by gel shift assay.

320

321 **Fig.5.** RNA-binding and RNA chaperone activity of rPkTPx-1. (A) RNA-binding
322 activity of PkTPx-1 evaluated by RNA electrophoretic mobility shift assay. (B) Nucleic
323 acid melting activity of rPkTPx-1. rPkTPx-1, rPk1-Cys-Prx or buffer alone (control)
324 was added to the reaction in presence of molecular beacon and fluorescence was
325 monitored. The excitation and emission wavelength were 485 and 518 nm, respectively.

326

327 **Fig. 6.** Indirect immunofluorescence microscopy to confirm PkTPx-1 expression in the
328 parasite cell. Figure shows a schizont with several merozoites and two trophozoites of *P.*
329 *knowlesi*. Cytoplasmic expression of PkTPx-1 in intra-erythrocytic *P. knowlesi* parasite
330 was observed by indirect immunofluorescence microscopy using the rPkTPx-1-
331 immunized mouse sera. (GF: green color). The parasite nuclei were stained with
332 Hoechst 33342 (Hoechst: blue color). Bars 2.5 µm.

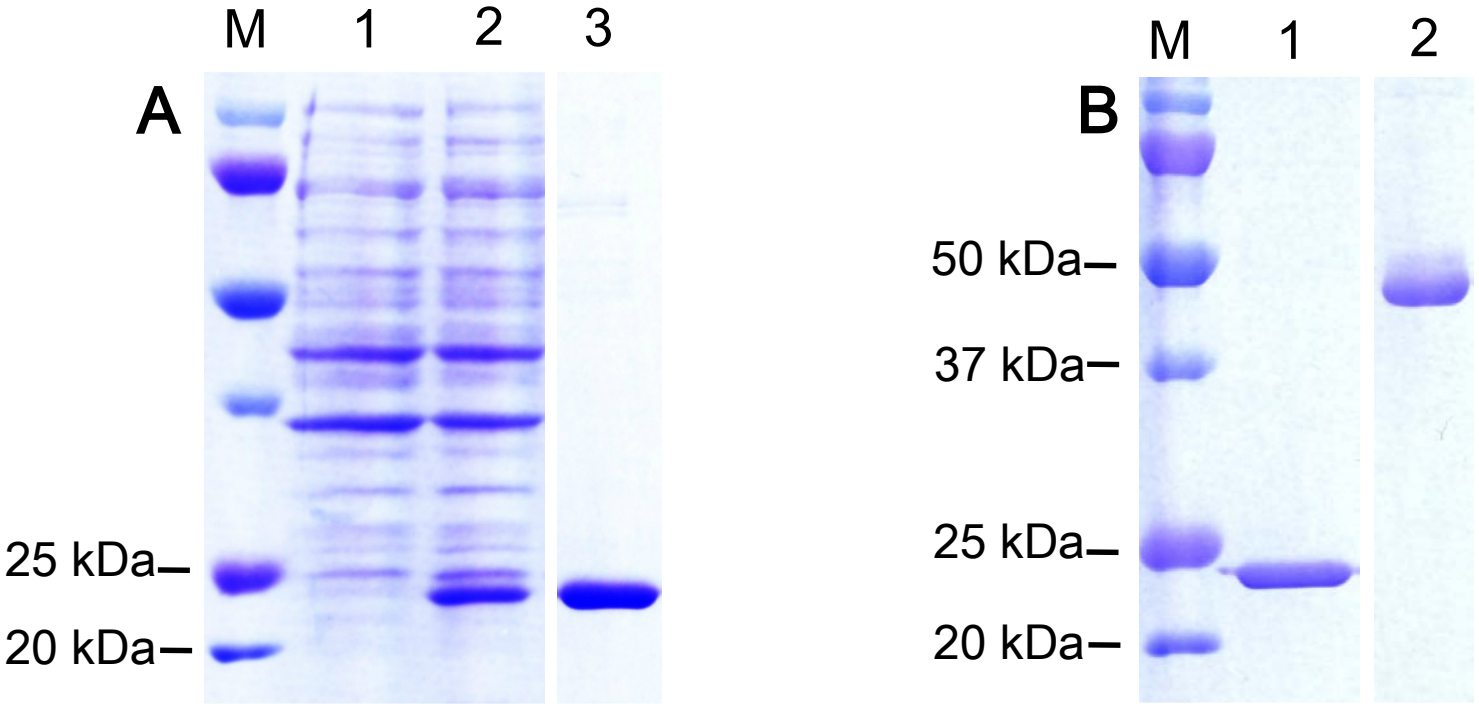
333

334 **Supplementary information**

335 **Table S1.** Primers for amplification of *P. knowlesi* TPx-1 (PkTPx-1). Start and stop

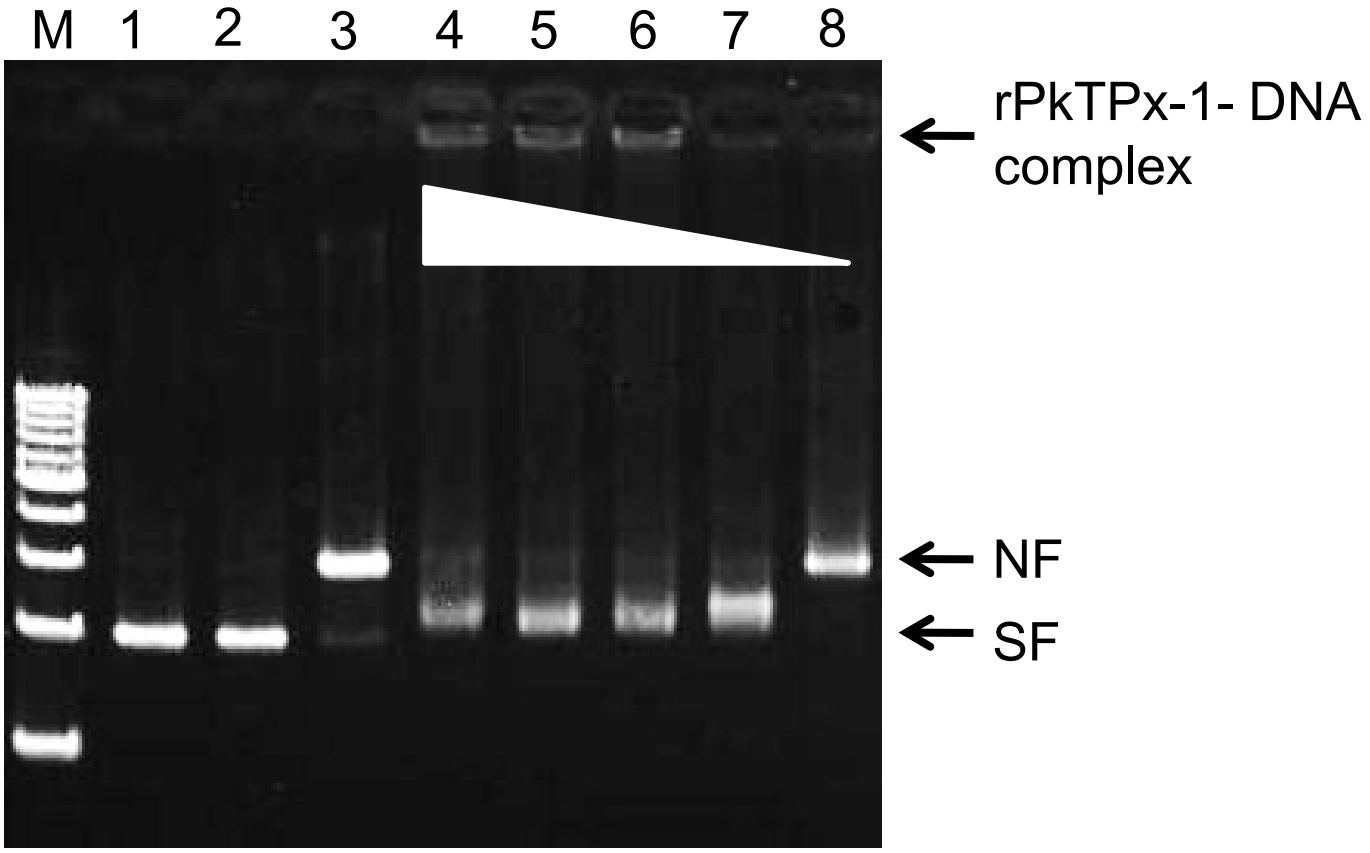
336 codons are underlined and restriction sites are italicized.

Hakimi et al. Figure 1

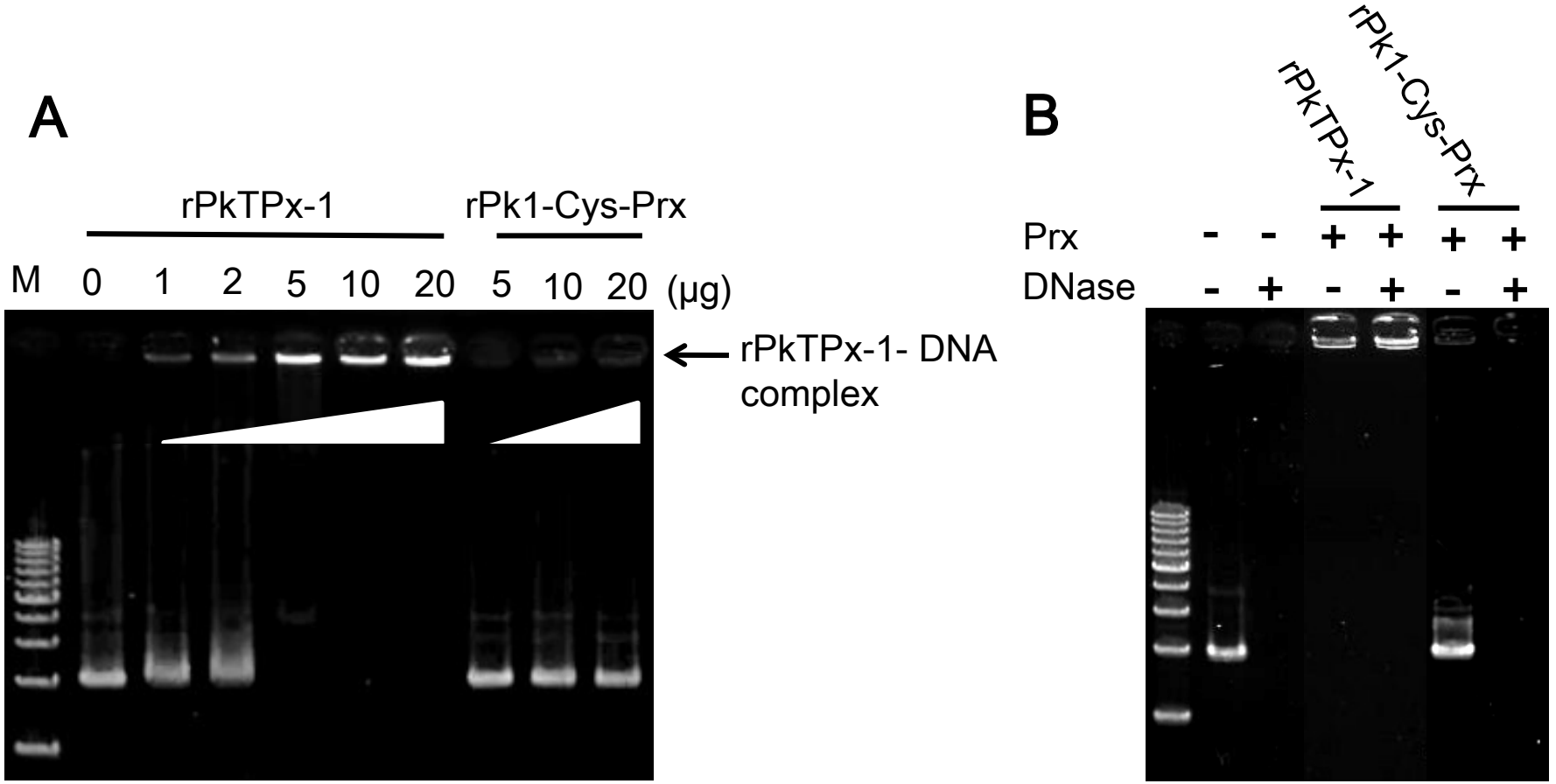


Hakimi et al. Figure 2

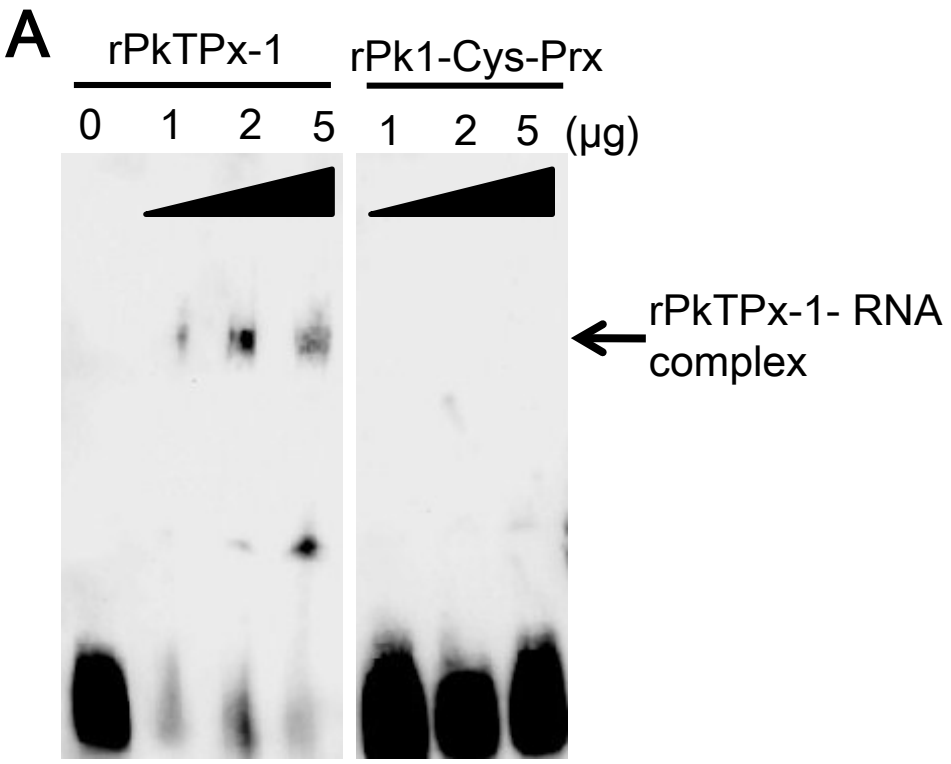
Hakimi et al. Figure 3

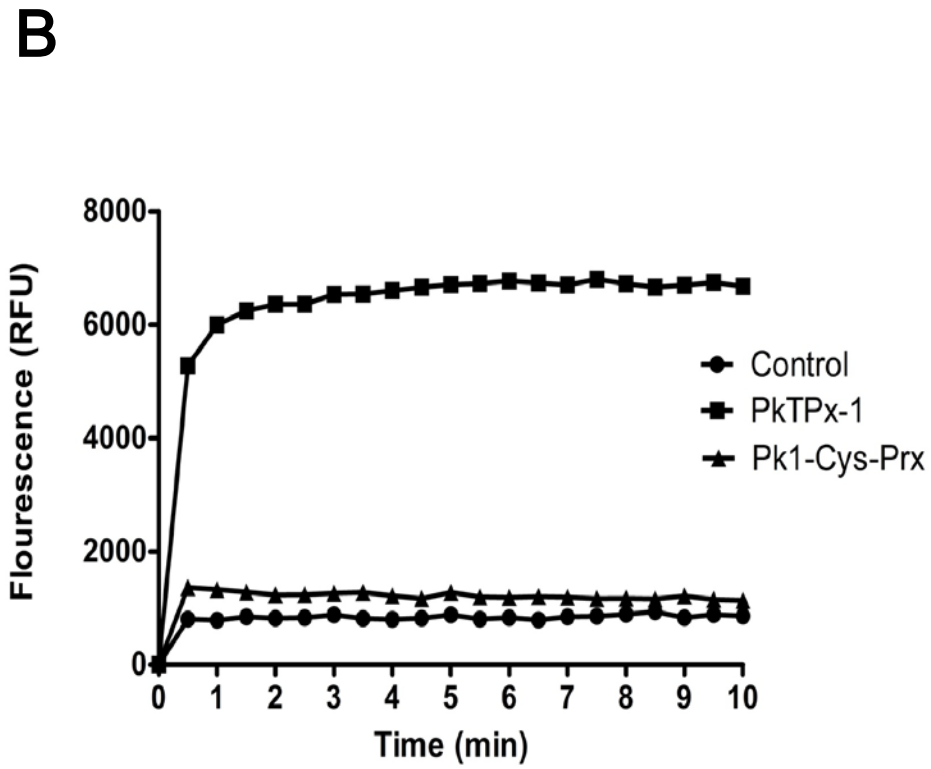


Hakimi et al. Figure 4

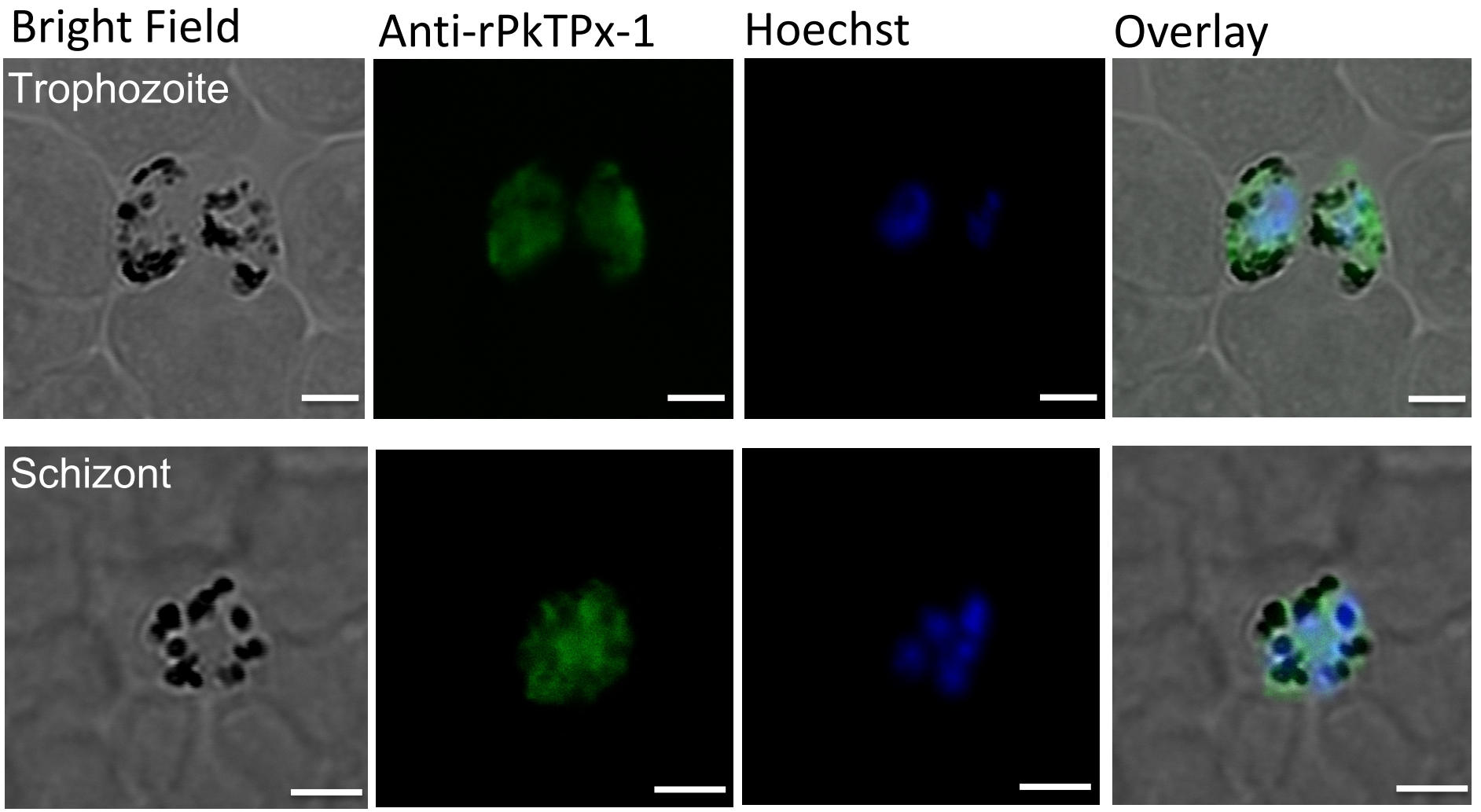


Hakimi et al. Figure 5





Hakimi et al. Figure 6



Hakimi et al. Supplementary table S1

Gene	PlasmoDB ID	Primers
PkTPx-1	PKH_126740	F: 5'- *CATATG*GTGACATACGTAGGAAAAGAAGC -3' (*Nde* I)
		R: 5'- *GAATTC*TTATAATGTGGACAAATATTGCGC-3‘ (*EcoR* I)