

A Member Of The Novel Fikk Family Of Plasmodium Falciparum Putative Protein Kinases Exhibits Diacylglycerol Kinase Activity And Is Exported To The Host Erythrocyte

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**A MEMBER OF THE NOVEL FIKK FAMILY OF *PLASMODIUM*
FALCIPARUM PUTATIVE PROTEIN KINASES EXHIBITS
DIACYLGLYCEROL KINASE ACTIVITY AND IS EXPORTED TO THE HOST
ERYTHROCYTE**

by

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B.S. University of Central Florida, 1996

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Microbiology and Molecular Biology
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

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ABSTRACT

Plasmodium falciparum is one of four species known to cause malaria in humans and is the species that is associated with the most virulent form of the disease. Malaria causes nearly two million deaths each year, many of these occurring among children in underdeveloped countries of the world. One reason for this is the prevalence of drug resistant strains of malaria that mitigate the efficacy of existing drugs. Hence, the identification of a new generation of pharmacological agents for malaria is extremely urgent.

The recent identification of a group of novel protein kinases within the *Plasmodium falciparum* genome has provided researchers with a basis for what many hope to be new potential drug targets for malaria. Identified within the *Plasmodium* genome and a few select apicomplexans, these novel proteins have been predicted to be protein kinases based solely on certain sequence features shared with other eukaryotic protein kinases (ePKs). However, to date, no significant studies to determine the function of these novel kinases have been performed. Termed FIKKs, these proteins all possess a non-conserved N-terminal sequence that contains a *Plasmodium* export element (Pexel) which may target the proteins for export from the parasite and a conserved C-terminal catalytic domain containing a FIKK sequence common to all twenty members of this family.

We analyzed the localization of one of the FIKK proteins, FIKK11, encoded by the PF11_0510 locus, during intraerythrocyte differentiation of *P. falciparum* by Western blot analysis and indirect immunofluorescence assay. Western blot analysis demonstrated that FIKK 11 is expressed within the parasite at all stages of its erythrocytic life cycle with its highest expression occurring during the schizont stage.

Immunofluorescence assays showed that this protein is exported from the *Plasmodium* parasite into the host erythrocyte cytosol which is consistent with studies on other *Plasmodium* proteins that also have the Pexel motif.

To determine the enzymatic activity of FIKK11, we overexpressed the recombinant protein in *E. coli* and then purified it. However, no protein kinase activity was detected using several commonly used protein kinase substrates including histone H1, myelin basic protein, or dephosphorylated casein. We also did not detect any kinase activity of the native enzyme using pull-down assays of the *Plasmodium falciparum* cell extract against those same substrates. In addition, kinase substrate peptide array analysis of FIKK11 showed no evidence of protein kinase activity either for FIKK11. Interestingly, however, we were able to detect some kinase activity using the recombinant protein alone with no substrate.

The lack of the glycine triad within subdomain I of these FIKK kinases as compared with most traditional eukaryotic protein kinases may explain why we were unable to find any interactions between FIKK11 and other commonly protein kinase substrates. Of interest was the observation that the protein reproducibly exhibited what appeared to be an autophosphorylation activity when using the standard protein kinase assay. Further analyses, however, showed that FIKK11 actually possesses diacylglycerol kinase activity utilizing 1-Stearoyl-2-arachidonoyl-*sn*-glycerol as a substrate. This is the first evidence of diacylglycerol kinase activity in *Plasmodium falciparum*. Because FIKK11 is exported into the host cell and is localized on the erythrocyte membrane, its enzymatic activity may potentially have relevance in the pathophysiology of the disease.

This thesis is dedicated to my family and my friends that have always believed in me and supported me through the completion of this Master's degree.

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The completion of this work has involved many people. I would first like to thank my mentor and advisor, Debopam Chakrabarti, Ph.D. Without his continual support and motivation over the past few years, the completion of this work would likely never have come to fruition. I thank you for your advice and acknowledgement of my successes and my errors. Your ability to always examine my work with a critical eye was crucial in helping me to achieve my goals.

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I would also like to thank Melissa Schreiber, C. Shane Massey, and Mike Taylor for all the good times at UCF. Without them, it is quite likely I would have lost my mind completely.

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CHAPTER ONE: INTRODUCTION

Background

The genus, *Plasmodium*, is the causative agent of malaria, a disease that threatens approximately 40% of the world's population (Breman, 2001). The majority of the cases of malaria are found in the tropical and subtropical regions of the world and this disease results in 300 to 500 million clinical cases per year with one to three million of those cases leading to death (Breman, 2001). Of the known species of *Plasmodium*, four have been identified as being directly responsible for infection of the human host: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Among these four species, *Plasmodium falciparum* is responsible for the majority of the clinical cases of malaria and is, consequently, the most lethal.

The word malaria comes from the Italian term *mal'aria* that translates into "bad air". Its name comes from the belief among ancient cultures that the fevers common among the disease state were caused by breathing bad air within certain areas that had become inundated with standing, stagnant water. In 1716, the Italian physician Giovanni Maria Lancisi saw black pigmentation of brain and spleen samples from patients suffering from malarial symptoms. The first observation of the parasite itself, however, came in 1847 when the German scientist Heinrich Meckel observed black-pigmented granules in the blood and spleen of a deceased, diseased patient. It wasn't until 1879 that Afanasiev

suggested that the disease state itself was actually caused by the granules. In 1880, Louis Alphonse Laveran identified the parasite in human erythrocytes and described both trophozoite and schizont stages of the parasite. By 1897, the combined work of Patrick Manson and Ronald Ross confirmed that the parasite was transmitted to its human host via the mosquito vector.

The first actual treatment for malaria came in 1820 with the discovery of quinine, an alkaloid from the bark of the *Cinchona ledgeriana* tree. As the supply of quinine became limited during World War II, and soldiers were exposed to potential malaria on tropical and subtropical battlefields, there was an increase in research for synthetic antimalarial drugs. This prompted the discovery of chloroquine in 1934 (Greenwood, 1995; Schmidt and Roberts, 1989). Chloroquine has remained one of the most commonly prescribed treatments for malaria, partially because of its low production costs. The last several decades, however, have seen an alarming increase in the number of chloroquine-resistant cases throughout the world. The causes for this increase have been attributed to incorrect drug dosing, patient noncompliance with the duration of their treatment, poor drug quality and misdiagnosis (Bloland, 2001). Because most other antimalarial drugs are chemically related to chloroquine, many show drug-resistance to strains of malaria within a few years of release. This rise in resistance to antimalarial drugs makes the search for new, novel antimalarial agents more critical than ever. By having a better understanding of just how cellular functions within the *Plasmodium* parasite are regulated, this search for a consistent, effective treatment will have a better chance of reaching fruition.

Plasmodium falciparum Life Cycle

The life cycle of the malaria parasite is quite complex as can be seen in fig. 1. The Anopheles mosquito acts as the vector for the infection of a human host with *Plasmodium falciparum*. Infection of the human host begins when the mosquito delivers sporozoites into the bloodstream which then travel to the liver and establish an infection inside the host's hepatocytes. Here they undergo multiple rounds of multiplication called exo-erythrocytic schizogony that results in the formation of thousands of merozoites. These merozoites are then freed and infect the host's erythrocytes where they undergo additional schizogony resulting in the actual pathogenesis of the malaria parasite. Some of the merozoites may arrest their cell cycle and develop into male or female gametocytes that may then infect the mosquito vector should a subsequent bite occur. Once inside the mosquito, these gametocytes become gametes that then fuse to form a zygote. The zygote undergoes sporogony leading to the production of sporozoites. These sporozoites accumulate in the salivary glands of the where they await infection of a new human host.

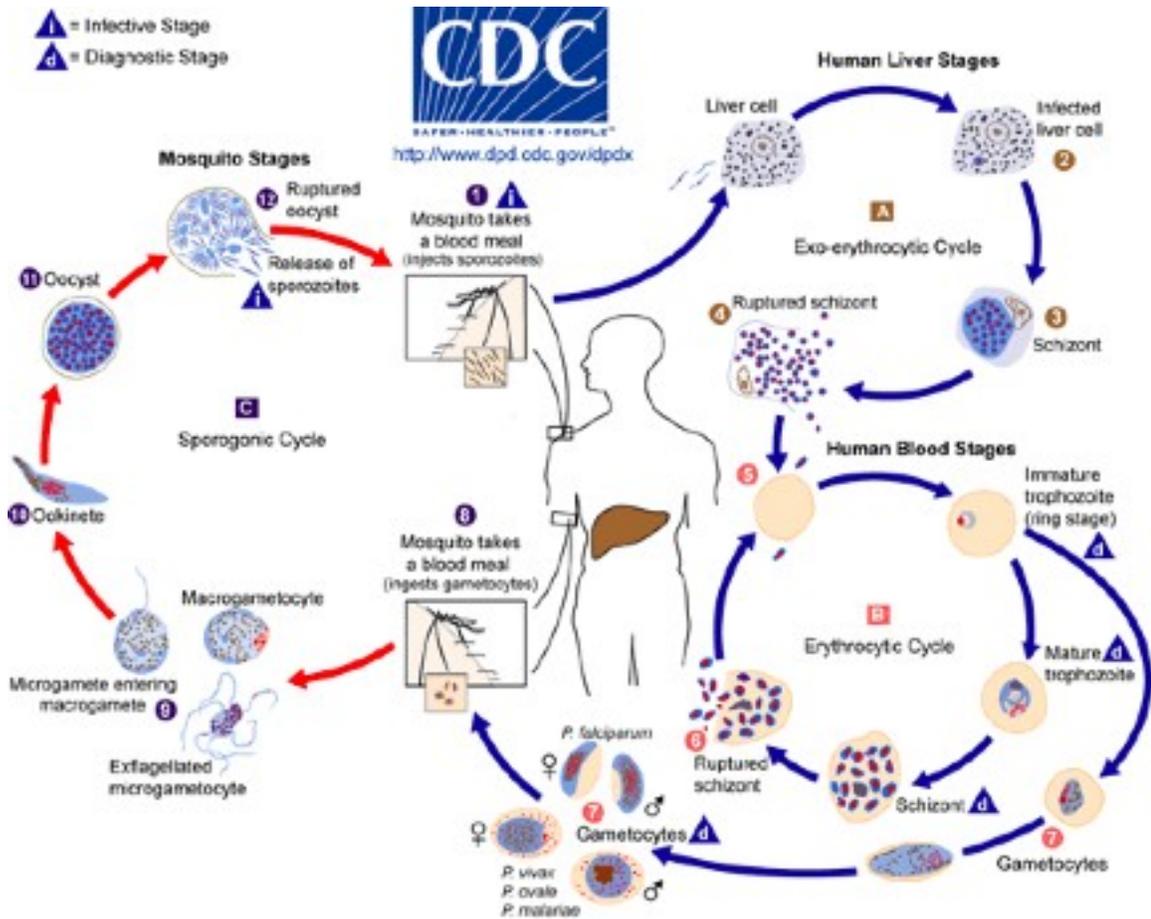


Figure 1. Life Cycle of the *Plasmodium falciparum* Parasite

Image borrowed from http://www.cdc.gov/malaria/images/graphs/malaria_LifeCycle.gif

A Novel Set of *Plasmodium falciparum* Kinases

With the increase in resistance of malaria to chloroquine and other chemotherapeutic agents, researchers are exploring new drug targets as potential agents in the fight against malaria. An interesting trend is the examination of protein kinases as chemotherapeutic agents. A well-documented example of this is the drug Gleevec produced by Novartis. This protein kinase inhibitor is used as a treatment for various forms of cancer since protein kinases are an important family of proteins that regulate diverse cellular activities.

Sequencing of the *P. falciparum* genome (Gardner et al., 2002) has led to the identification of the complete repertoire of parasite protein kinases based on homology searches (Ward et al., 2004). These searches, however, have only identified 65 protein kinases within the malaria genome which seems quite low compared to *Saccharomyces cerevisiae* which has a similar number of total genes but encodes nearly twice the number of protein kinases. The implication is that malaria protein kinases may possess more complex and novel cellular functions when compared to other eukaryotic kinomes. Of the *Plasmodium falciparum* protein kinases identified, the presence of a novel family of 20 putative protein kinases is particularly noteworthy (Schneider and Mercereau-Puijalon, 2005; Ward et al., 2004). This family of kinases is termed FIKK for the presence of a conserved amino acid sequence motif: phenylalanine-isoleucine-lysine-lysine (Ward et al., 2004). All family members contain a non-conserved N-terminal domain and a conserved C-terminal kinase domain (Fig. 2). Although FIKK

orthologues have been identified in other Plasmodium species and certain apicomplexans (*Toxoplasma gondii* and *Cryptosporidium parvum*), only *P. falciparum* and *P. reichenowi* exhibit extensive gene amplification with twenty distinct kinases found in the former and six found in the latter.

FIKKs as Potential Protein Kinases

Sequence comparisons of conventional eukaryotic protein kinases and the FIKK kinases show that the FIKKs possess many the conserved residues believed to be important for catalytic activity with the exception of the glycine triad in subdomain I. Although this sequence (GxGxxG) helps to anchor ATP or GTP in the catalytic cleft, the phosphotransfer ability of a kinase is not entirely dependent on it. A third of human protein kinases do not contain the third glycine residue of the triad and the first residue is absent in about 11% of the kinases (Kostich et al., 2002). *S. pombe* MIK1 tyrosine kinase that phosphorylates p34cdc2 lacks all three residues of the triad (Lee et al., 1994). *P. falciparum* PfPK7 kinase contains only the second glycine residue of the triad (Dorin et al., 2005). Though catalytically inactive proteins that maintain a kinase fold are not uncommon, these “pseudokinases” lack selective pressure to maintain residues directly involved in catalysis and possess amino acid substitutions in one or more of the conserved catalytic residues analogous to Lys72, Asp166, or Asp184 (mammalian PKA numbering).

The invariant lysine in subdomain II that is involved in the orientation of α and β phosphate groups of ATP appears in the FIKK signature motif of this novel family of kinases. Presence of motifs close to HRDLKPxN (HLDxxPxN) and APE (PPE) in subdomains VIB and VIII, respectively, suggests that FIKK family members may function as kinases. The aspartic acid in the HRDLKPxN consensus sequence acts as a general base during catalysis to facilitate phosphate

transfer. The aspartic acid of the conserved DFG motif (Dxx in FIKKs) coordinates the divalent cation that bridges with the β - and γ -phosphates of the purine nucleotide. The invariant arginine residue in subdomain XI that stabilizes the large carboxy-terminal lobe is present in FIKKs. Because each of these residues is absolutely conserved among all members of the FIKK family (Fig. 3), it is expected that they are likely to function as protein kinases. The FIKK family exhibits 38-64% identity among the paralogs within the presumed catalytic domain (Schneider and Mercereau-Puijalon, 2005).

In spite of significant conservation of residues that are important for catalytic activity, the absence of the defined glycine triad calls into question their roles as authentic protein kinases. Therefore it is important to confirm that these proteins are in fact functional proteins.

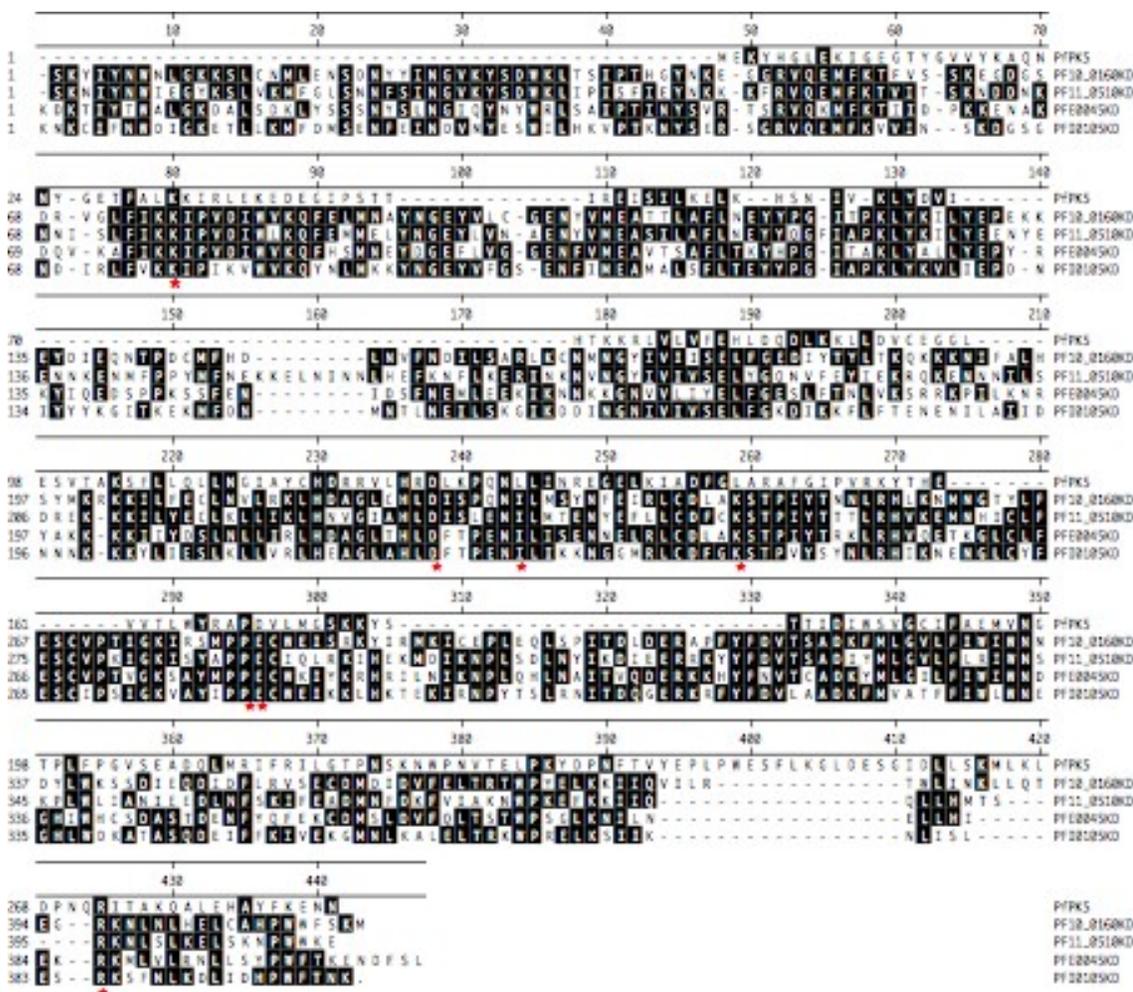


Figure 3. Sequence Alignment of Members of the FIKK Kinase Family with PfPK5

The Sequence alignment was done by CLUSTALW algorithm. Conserved residues in eukaryotic protein kinases are indicated by a red asterisk.

The N-terminal domain is not conserved among the paralogs and this region contains a stretch of hydrophobic residues corresponding to a predicted trans-membrane or signal sequence domain (Schneider and Mercereau-Puijalon, 2005). A recently described host-targeting signal motif, RxSRILAE_{xxx} (Hiller et al., 2004) is present in six of the FIKK paralogs while the Plasmodium export element RxLx(D, E, Q) (Marti et al., 2004) can be detected in all FIKK paralogs downstream of the signal sequence. Because HT/Pexel motifs have been shown to mediate export of proteins beyond the parasitophorous vacuole into the erythrocyte cytoplasm (Hiller et al., 2004; Marti et al., 2004), it is expected that the FIKK kinases are also trafficked to the erythrocyte.

Goal of the Work Within this Thesis

The distant relationship between the FIKK kinases and the more typical eukaryotic protein kinases generates optimism that it may be possible to engineer selective inhibitors targeting this novel family of kinases. However, clear physiological and/or cell biological data are needed to implicate FIKK kinases as an attractive target for drug therapy. Therefore, we decided to characterize FIKK11, a member of the FIKK family. The goal was two-fold: first, to determine whether selected members of the FIKK kinase family are indeed expressed in vivo within the Plasmodium falciparum parasite and whether the proteins are exported into the host erythrocyte as is implicated by the presence of an HT/Pexel motif within these proteins. Secondly, to identify the potential

function of selected members of the FIKK kinase family by assessing whether they are truly protein kinases as has been speculated.

CHAPTER TWO: METHODOLOGY

Cell Culture

Plasmodium falciparum strain 3D7 was maintained in 100mm tissue culture dishes using human A+ red blood cells. The culture was incubated at 37°C with a 5% carbon dioxide/95% air mixture. The culture medium used was RP10S consisting of RPMI-1640 (MOD.) supplemented with 25mM HEPES, 0.2% dextrose, 0.15% hypoxanthine, 25µg/ml gentamycin, and 10% human serum or 0.5% Albumax. Collection of the culture for parasite isolation involves resuspending the cells and transferring them to a 50ml conical tube. Release of the parasite from the red blood cell was achieved by treating the suspension with 0.1% saponin followed by incubating the mixture at room temperature for five minutes. Afterwards, the mixture was centrifuged at 4000rpm for seven minutes at 4°C to separate the parasites from the red blood cell lysate. The supernatant was then removed and the pellet was resuspended in 1X PBS. The pellet was washed once with PBS and was subsequently stored at -80°C until needed. In order to perform stage-specific analyses on the parasite, the cell culture was synchronized by treatment with 5% D-sorbitol for 15 minutes at 37°C. The D-sorbitol treatment preferentially selects in the parasites in the ring stage (Lambros and Vanderberg, 1979). The D-sorbitol was thoroughly removed from the culture using several washes with RPMI-1640 and the culture was resuspended in RP10S media as described above and plated again onto tissue culture plates. The culture

was then incubated as above until the desired stage was reached and then harvested as above at either six or eight hour intervals to assure that ring, trophozoite, and schizont stages were equally represented.

Plasmodium RNA Extraction

The asynchronous *Plasmodium falciparum* 3D7 culture was harvested as previously mentioned. After the final PBS wash, the pellet was treated with 3ml RNAagents Denaturing Solution (Promega) and stored at -80°C until needed. The pellet was later thawed on ice and 600µl 2M sodium acetate (pH 4) was added. This mixture was then treated with 6ml Phenol:Chlorophorm:Isoamyl Alcohol and incubated on ice for fifteen minutes. The entire mixture was then transferred to a DEPC-treated centrifuge tube and centrifuged at 10000g for twenty minutes at 4°C. The upper, aqueous phase was transferred to a clean DEPC-treated tube and an equal volume of isopropanol was added and allowed to incubate at -20°C overnight. The mixture was centrifuged as above and the supernatant removed from the RNA pellet. 2.5ml Denaturing Solution was added and the pellet was resuspended. Next, 2.5ml isopropanol was added and incubated at -20°C overnight. The mixture was centrifuged again as above and the supernatant was removed. The pellet was then washed with 1ml ice-cold 75% ethanol and centrifuged as above. The supernatant was discarded and the pellet was air-dried one hour. The RNA pellet was resuspended in 100µl nuclease-free water and transferred to RNase-free, screw-capped microcentrifuge tubes and stored at -

80°C until needed. The purity and integrity of the RNA sample was verified by running on a 1% formaldehyde denaturing gel for three hours at 75V using 1X MOPS buffer before it was accepted for use in RT-PCR.

cDNA Synthesis by Reverse Transcription

RNA obtained from the asynchronous *Plasmodium falciparum* 3D7 isolates as outlined above was used to synthesize cDNA for cloning. PlasmoDB was searched for valid sequences for PF11_0510 (FIKK 11) and PF10_0160 (FIKK 10.1), two putative *Plasmodium falciparum* protein kinases belonging to the FIKK kinase family. Based on these sequences, full length and fragment genes containing only the C-terminal kinase domain were then analyzed and the appropriate primers designed to obtain the desired sequence. Invitrogen synthesized the primers for cloning the open reading frames into the pET43.1 Ek/LIC vector. The RT-PCR reaction was carried out using the ProStar RT-PCR Kit (Stratagene). Table 1 lists the primers used cloning into pET43.1 and Table 2 shows the actual RT-PCR conditions used for cloning.

Table 1. Primer Sequences Used for Cloning into the pET43 Vector

Primers were designed using PlasmDB to determine the consensus sequence for primer creation. The oligo-(DT) primers were ordered through Invitrogen.

Construct	Length	Direction	Primer Sequence	T _m
FIKK10.1	1.227 kb	Forward	GAGGAGAAGCCCGGTTTACATTTTAGAAAACCACCA	50
		Reverse	GACGACGACAAGATGTGGAATTTAGGTAAAAAATC	50
FIKK11	1.218 kb	Forward	GACGACGACAAGATGTGGATAGAGGGTTATAAAT	50
		Reverse	GAGGAGAAGCCCGGTTTATTCTTCCACCATGG	50

Table 2. PCR Conditions for Amplification of FIKK10.1 and FIKK11

Protein	Template	5 Cycles						35 Cycles						Elongation	
		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (min)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
FIKK10.1	1µg RT	95	30	50	30	65	6	95	30	58	30	65	6	65	10
FIKK11	1µg RT	95	30	50	30	65	6	95	30	58	30	65	6	65	10

cDNA Cloning

After RT-PCR, 5 μ l of the PCR sample was run on a 0.8% agarose gel to verify the amplification. The PCR products were purified using the QIAquick Purification Kit (Qiagen) and the purified samples were run again on a 0.8% agarose gel to verify the recovery of the product and to determine the concentration of the samples. Approximately 0.2 μ mol of purified PCR product was then treated with T4 DNA Polymerase mix consisting of T4 DNA Polymerase, 25mM dATP, 100mM DTT, and 10X T4 DNA Polymerase Buffer for thirty minutes at room temperature. The product was cloned into the pET43.1 Ek/LIC vector utilizing the LIC sequence and then transformed into NovaBlue supercompetent *Escherichia coli* cells by heat-shock treatment as per the manufacturer's protocol (Novagen). SOC media was added to the heat-shocked cells and the mixture incubated at 37°C for ninety minutes with agitation before being plated onto LB agar plates containing Kanamycin (50 μ g/ml). The plates were incubated overnight at 37°C. The following day, isolated colonies were chosen for inoculation into 5ml LB broth containing Kanamycin (50 μ g/ml) and incubated at 37°C overnight with agitation. The cultures were then used to isolate plasmid DNA using the QIAquick Spin Miniprep Kit (Qiagen).

DNA Sequencing

The isolated, digested inserts that corresponded to the correct size for the given gene were further verified by sequencing performed at the University of Florida DNA Sequencing Core facility using the T7 primer for forward sequences and the SP6 primer for reverse sequences. The sequences were then analyzed and verified using the DNASTAR SeqMan software.

Protein Expression

The isolated plasmids were transformed into BL21 Codon+ DE3 RIPL supercompetent *Escherichia coli* cells by heat-shock treatment and plated onto LB agar with Kanamycin (50µg/ml). The plates were incubated overnight at 37°C and colonies selected the next day for inoculation into 5ml LB with Kanamycin (50µg/ml). These cultures were incubated overnight at 37°C with agitation. The following day, the cultures were used to inoculate 195ml LB with Kanamycin (50µg/ml) and incubated at 37°C until the OD₆₀₀ reached 0.6. At that time 1mM IPTG was added to induce expression of the desired protein and incubated at 27°C for another 4 hours. Afterwards, the cultures were spun down at 6000rpm for twenty minutes, the supernatant removed, and the pellet resuspended in 10ml TALON Wash Buffer (50mM Sodium Phosphate pH 7, 300mM NaCl, and protease inhibitors (Roche Mini Complete EDTA-free)).

TALON Affinity Purification

The resuspended pellet in TALON wash buffer was thawed on ice and incubated for thirty minutes in the presence of 75 μ l Lysozyme to digest the cell walls. The cell suspension was sonicated for seven cycles of twenty seconds (to release the proteins. There was a rest of forty seconds between sonication cycles. The sonicated lysate was centrifuged at 13000rpm for twenty minutes and the clarified supernatant was added to pre-washed TALON beads (BD Bioscience). The column was washed using 1X Wash Buffer (50mM Sodium Phosphate, 300mM NaCl, pH7) and non-bound proteins removed using 1X Extraction Buffer (50mM Sodium Phosphate, 300mM NaCl, pH8).. The protein of interest was eluted from the column using 1X Elution Buffer (50mM Sodium Phosphate, 300mM NaCl, 150mM Imidazole, pH7). After elution, the protein sample was stored at -80°C until needed.

Protein Kinase Assay

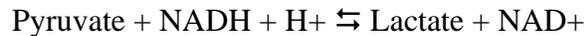
To determine whether the purified proteins exhibited any protein kinase activity, the proteins were assayed for protein kinase activity in a reaction containing 0.1 μ Ci of γ^{32} P-ATP, 50 μ M “cold” ATP and the 5X kinase buffer mixture (250mM Tris-HCl (pH 7.4), 50mM MgCl₂, 10mM DTT, 200mM β -Glycerophosphate, and 0.5mM Sodium Orthovanadate). Following incubation for 5 minutes, 0.5 μ g of the FIKK 11 protein was added and the reaction was allowed

to incubate for one additional hour at 30° C. At the end of this incubation period, the reaction was stopped by addition of 0.5M EDTA. 10µl of the reaction mixture was spotted onto Whatmann P81 filter discs and allowed to dry. The unbound ³²P was removed with 3 washes with 1% phosphoric acid. The radioactivity present in the samples was measured using the Beckman scintillation counter. The remaining reaction mixture was run on an SDS-PAGE gel and stained to verify that the protein of interest was present. Additional kinase assays were done in which the incubation time of protein in the presence of radioactive ATP, buffer pH, and buffer ionic cofactors were varied.

Glycerol Kinase Assay

To determine whether FIKK 11 had any glycerol kinase activity, the protein was incubated in the presence of glycerol and ATP using the oxidation of NADH to determine whether the glycerol was utilized by the kinase. 2.5mM ATP was incubated for five minutes in the presence of a reaction mixture (0.05M Triethanolamine-HCl (pH 7.0), 2.5mM MgCl₂, 20mM KCl, 0.1mM β-Mercaptoethanol, 0.2mM Phospho(enol)pyruvate, 0.2mM NADH, 2mM Glycerol, 5U/ml Pyruvate Kinase, and 5U/ml Lactate Dehydrogenase). Then 100ng of FIKK 11 or a commercially available E. coli glycerol kinase was added and the activity measured over the course of eight minutes using a Hewlett-Packard 8453 spectrophotometer to determine the decrease in absorbance of NADH at 340nm.

The reaction velocity is measured in a coupled system with pyruvate kinase and lactate dehydrogenase as follows:



One unit of glycerol kinase results in the oxidation of one μmol of NADH per minute at 25°C and pH 8.9 and is measured using a spectrophotometer at 340nm over the course of 5-8 minutes.

Diacylglycerol Kinase Assay

To analyze whether FIKK 11 had any diacylglycerol kinase activity, the protein was incubated in the presence of radioactive ^{32}P ATP and a commercially available diacylglycerol as described below and analyzed by thin layer chromatography and phosphoimaging to determine activity. $250 \mu\text{M}$ Cardiolipin and $500 \mu\text{M}$ 1-Stearoyl-2-arachidonoyl-sn-glycerol or 1,2-Dioleoyl-sn-glycerol was dissolved in 1mM Sodium Desoxycholate. $1\mu\text{g}$ FIKK 11 or commercially available E. coli diacylglycerol kinase was added to the lipid mixture along with a reaction mixture (40mM Bis-Tris (pH 7.5), 5mM MgCl_2 , 0.1mM EDTA, 1mM Spermine, 0.5mM DTT, and 0.02% Triton X-100) and incubated at room temperature for five minutes. In addition, $30\mu\text{M}$ calcium was added in some reactions to see if it had any impact on kinase activity. Then, 1mM ATP (with $5\mu\text{Ci}$ radioactive ^{32}P ATP) was added and the reaction allowed to incubate at

room temperature for thirty minutes. The reaction was stopped by adding 750 μ l chloroform/methanol (1:2) with 1% HCl. Next, 1ml of chloroform/methanol (1:1) and 500 μ l 1M KCl with 0.2M phosphoric acid was added and the samples vortexed and centrifuged at 2000rpm for five minutes. The lower phase was transferred to a new reaction tube and the samples dried under a nitrogen stream. The samples were then resuspended in 50 μ l chloroform/methanol (2:1) and applied to TLC silica plates (EMD Chemicals). The plates were run using a chloroform/acetone/methanol/acetic acid/water (40:15:14:12:8) mixture. The TLC Silica plate was then exposed BioMax MS film at -80°C before being developed. Finally, the TLC plates were exposed to a phosphoimager screen before being scanned and analyzed using ImageQuant TL software.

Plasmodium falciparum Cell-free Extract

Synchronized *Plasmodium falciparum* 3D7 cells were collected from intraerythrocytic cultures as previously described and stored at -80°C until needed. The pellet was then resuspended in a Lysis Buffer (20mM Tris-HCl (pH8.0), 10mM EDTA, 800mM NaCl, 3% Triton X-100, 10mM NaF, and 10mM β -Glycerophosphate) with protease inhibitors (Roche Mini Complete EDTA-free). Lysis of the cells was done by sonication and the cell lysate was clarified by centrifugation at 14000rpm for twenty minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and the protein concentration was determined by Bradford analysis (Bradford, 1979) using Protein Assay Reagent

(BioRad) and varying concentrations of BSA (Sigma) as the protein standard based on the expected sample concentration.

Generation of Polyclonal Antibodies

Initial Western blot analyses of the FIKK proteins were performed using purified antibodies obtained from Sigma Genosys. The FIKK11 antibodies were raised against the DELKDNIHSKNIYNC peptide. The peptides were emulsified with Freund's adjuvant and injected into rabbits (Freund, 1956; Freund and McDermott, 1942). The animals were subjected to six injections before obtaining the final bleed. For additional Western blot and immunofluorescence analyses, purified chicken antibodies were obtained from Dr. Christian Doerig, a collaborator in Glasgow, Scotland. The FIKK11 antibody was raised against the RFTKEKSDVRNSDEF peptide corresponding to the C-terminal portion of the protein. The secondary rabbit- α -chicken antibody was obtained from commercial sources (Jackson ImmunoResearch Labs).

SDS-PAGE and Transfer of Proteins to Nitrocellulose

Samples were resolved on SDS-PAGE as described by Ornstein and Davis (Davis, 1964 and Ornstein, 1964). The samples were denatured by the addition of 4X SDS-PAGE Gel Loading Buffer (250mM Tris (pH 6.8), 8% SDS, 40% Glycerol, and 20% β -Mercaptoethanol) to the protein samples and boiling the

mixture for five minutes. The samples were resolved via a discontinuous buffer system consisting of a stacking gel made with Tris-HCl (pH 6.8) on top of a resolving gel made with Tris-HCl (pH 8.8), 10% Acrylamide, and a Tris-Glycine buffer (pH 8.3). Pre-stained molecular weight marker (BioRad) was used as the standard and the samples were run on the gel for ninety minutes at 150V. The gel was removed and the proteins were electrophoretically transferred to nitrocellulose membrane (Burnette, 1981 and Towbin, et al., 1979). The transfer was performed for one hour at 100V.

Immunoblot Analysis

Immunoblot analysis was performed with polyclonal chicken and rabbit antibodies. The nitrocellulose membrane was incubated in blocking solution (5% milk in TBS-T) overnight at 4°C. The primary antibody was diluted (Table 3) with 5% milk in TBS-T before incubation on the membrane for two hours at room temperature. Unbound antibodies were removed by three 10 minute washes with 5% milk in TBS-T. The membrane was then incubated in the presence of diluted (Table 3) secondary antibody for thirty minutes at room temperature. The membrane was then washed three times in TBS-T for 5 minutes each wash. Next, the membrane was incubated in the dark in the presence of SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and subsequently exposed to film (Classic Blue Sensitive, MidWest).

Table 3. Antibody Dilutions for Western Blot Analysis

Antibody Against:	Rabbit Antibody	Secondary Anti-Rabbit
FIKK11	1:3000	1:5000
Antibody Against:	Chicken Antibody	Secondary Anti-Chicken
FIKK11	1:10000	1:30000

Indirect Immunofluorescence Assay

Synchronous *Plasmodium falciparum* 3D7 was harvested from culture plates and placed into a 15ml or 50ml conical tube depending on the amount of culture collected. 500 μ l was transferred to a clean microcentrifuge tube and the cells washed with 1X PBS. The cell suspension was centrifuged at 4000rpm for one minute at 4°C and the supernatant was removed before being fixed in a 4% Paraformaldehyde / 0.00075% Glutaraldehyde solution for thirty minutes. The cells were then permeabilized by incubating them in 0.05% Triton X-100 for ten minutes followed by a ten minute incubation in 0.1mg/ml NaBH₄ to remove any free aldehyde groups. Next, the cells were blocked in 3% BSA for one hour at room temperature and incubated in the presence of primary antibody diluted (Table 4) in 3% BSA overnight at 4°C. Three washes in 3% BSA removed any unbound primary antibody and the cells were incubated for one hour in the presence of secondary antibody diluted (Table 4) in 3% BSA in the dark at room temperature. After a final PBS wash, 2 μ l of cells and 2 μ l of ToPro in Antifade Agent were pooled onto a 12mm round coverslip pre-coated with 1% Poly(ethyleneimine) solution. The mixture was incubated for twenty minutes at room temperature and then the coverslips inverted onto a clean microscope slide for viewing.

Table 4. Antibody Dilutions for Immunofluorescence Assays

Antibody Against	Chicken Antibody	Secondary Anti-Chicken
FIKK11	1:1000	1:1000

CHAPTER THREE: RESULTS

Immunoblot Analysis of Stage-specific *Plasmodium* Extracts

Microarray analysis data obtained from PlasmoDB suggests that FIKK11 is mostly expressed in the schizont stage of the malaria life cycle with lower expression occurring in the ring and trophozoite stages. However, no experimental evidence has yet been shown to support the expression of FIKK family members at the protein level using *Plasmodium falciparum* cultures. To verify the expression of FIKK11 by the parasite *in vivo*, we examined synchronized *Plasmodium* cell-free extracts to ensure that each stage of the parasite life cycle was represented. Fig. 4 shows a Gel Code Blue analysis of malarial proteins as compared to uninfected red blood cells. To verify that FIKK11 is expressed *in vivo*, we performed a Western blot analysis using peptide antibodies raised specifically against its catalytic domain. Our Western blot data confirms that the expression of FIKK11 begins to increase in the late trophozoite/early schizont stage and reaches a peak accumulation in the late schizont stage of the parasite's life cycle (Fig. 5). Furthermore, there isn't any indication of FIKK11 protein expression within the uninfected red blood cells indicating a lack of cross-reactivity. This data confirms that FIKK11 is indeed expressed within *Plasmodium falciparum* intraerythrocytic stages and supports the microarray data showing its preferential expression in the schizont-stage.

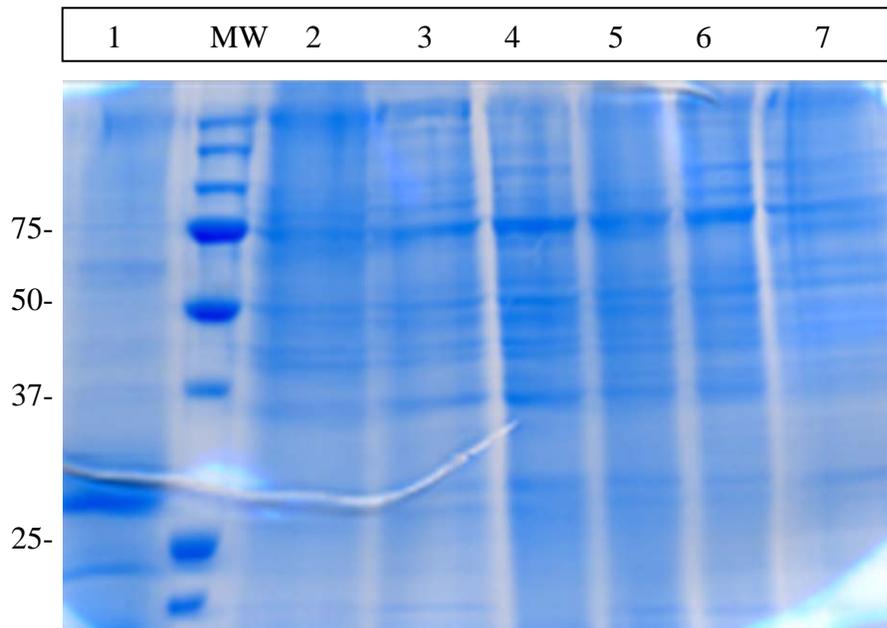


Figure 4. Gel Analysis of Uninfected Erythrocytes and Stage-Specific Malarial Extracts

Stage-specific extracts were harvested from synchronized *Plasmodium falciparum* 3D7. 25ug of each sample were loaded onto 12.5% SDS-PAGE. The gel was then stained using Gel Code Blue to resolve the proteins. Lane 1, Uninfected Red Blood Cells; MW, Molecular Weight Marker; Lane 2, Ring; Lane 3, early Trophozoite; Lane 4, Trophozoite; Lane 5, early Schizont; Lane 6, Schizont; Lane 7, late Schizont.

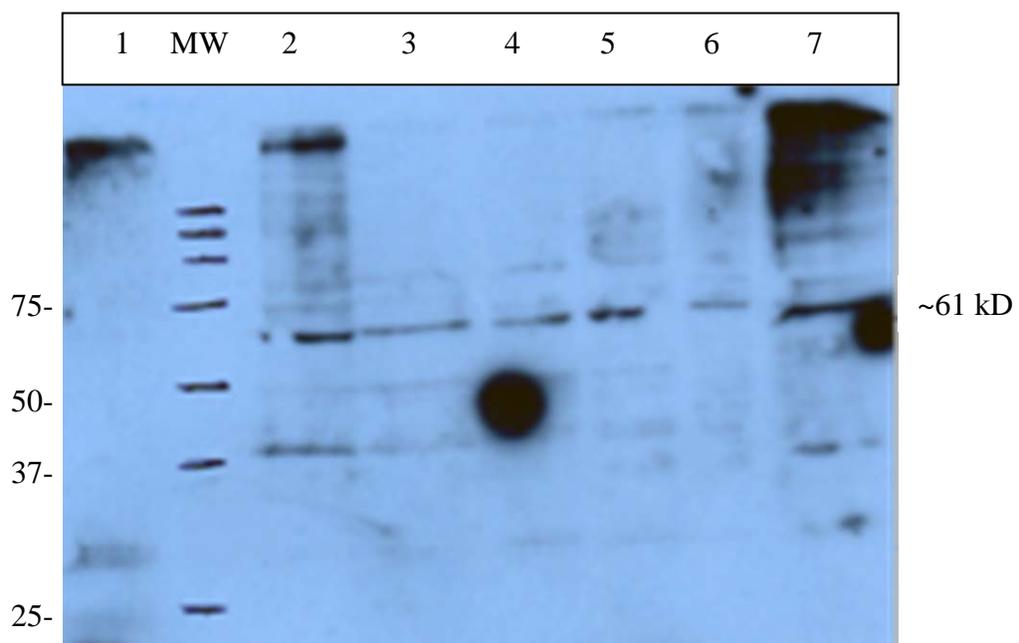


Figure 5. FIKK11 is Expressed Within All Stages of the Malaria Lifecycle

Stage-specific extracts were harvested from synchronized *Plasmodium falciparum* 3D7. 25ug of each sample were loaded onto 12% SDS-PAGE and the transferred to a nitrocellulose membrane. Chicken-raised FIKK11 antibodies were used as the primary antibody and commercially available anti-chicken antibody was used as the secondary. The membrane was exposed to film and developed to resolve the image. Lane 1, Uninfected Red Blood Cells; MW, Molecular Weight Marker; Lane 2, Ring; Lane 3, early Trophozoite; Lane 4, Trophozoite; Lane 5, early Schizont; Lane 6, Schizont; Lane 7, late Schizont.

Subcellular Localization of FIKK11 Using Immunofluorescence Imaging

Because of the presence of a double Host Targeting/Plasmodium Export Element (HT/Pexel) in FIKK11, we were interested to analyze whether FIKK11 was indeed exported from the malarial parasite *in vivo* to a location within the periphery of the parasite membrane or to the host erythrocyte. Using *Plasmodium falciparum*-infected erythrocytes, we performed an immunofluorescence assay to ascertain the localization of the protein. Fig. 6 shows the Differential Interference Contrast (DIC) image and the immunofluorescence image of parasitized erythrocytes. As can be seen from fig. 6, FIKK11 is localized in the erythrocyte plasma membrane, parasitophorous vacuolar membrane, and unknown distinct foci during the late ring/early trophozoite stages and not in the erythrocyte or parasite cytosol. Our result is consistent with the fact that malarial proteins containing the HT/Pexel motifs are targeted for export from the parasite (Hiller et al., 2004; Marti et al., 2003). It has also been demonstrated that exported parasite proteins often localize within intraerythrocytic structures called Maurer's Clefts (Stanley et al., 1989; Hinterberg et al., 1994). These will often appear as punctate dots when viewed using an indirect immunofluorescence assay (Nunes et al., 2006). Fig. 6 clearly shows a punctate appearance of FIKK11 suggesting a possible association between it and the Maurer's Clefts. Additionally, the fluorescence seen strongly suggests an association with the infected erythrocyte membrane and possibly the parasitophorous vacuolar membrane.

To verify that that FIKK11 expression was found within all stages of the parasite life cycle beginning with the ring stage and increasing to Schizont stages of the parasitic infection as is suggested by the immunoblot analysis, the immunofluorescence assays were done with cells predominantly at those specific stages of the cell cycle. Fig. 7 clearly demonstrates that the level of FIKK11 expression does increase in vivo at the Schizont stage of the parasite. This further verifies the known microarray data for this protein as well as verifies the Western blot analysis shown by our lab. Interestingly, fig. 7 shows the localization of FIKK11 is restricted exclusively to the parasitophorous vacuolar membrane at the Schizont stage. This suggests a stage-dependent dynamics of FIKK11 localization in the intra-erythrocytic stages.

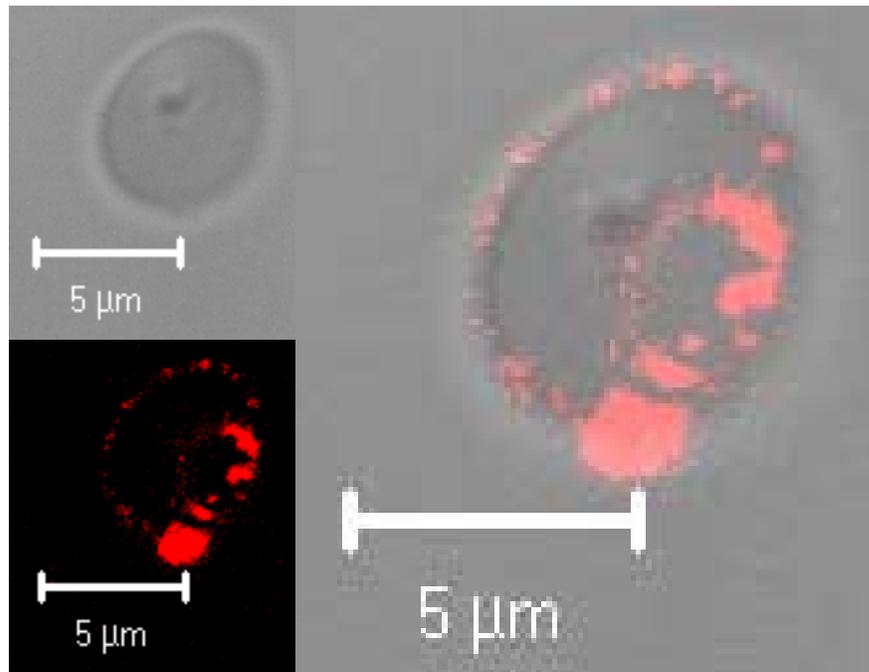


Figure 6. FIKK11 is Localized within the Erythrocyte Membrane and Parasitophorous Vacuolar Membrane in the Early Trophozoite Stage of the Malaria Lifecycle

Immunofluorescence was determined by fixing and permeabilizing cells prior to the addition of a primary antibody raised against FIKK11 and the addition of Alexa Fluor 555 secondary antibody. This figure represents a cell as visualized using the Axiovert 100M confocal microscope. The upper left panel of each shows the DIC image of the cell only. The lower left panel shows the immunofluorescence image of the FIKK11. The larger panel represents the overlay of the DIC and immunofluorescence image.

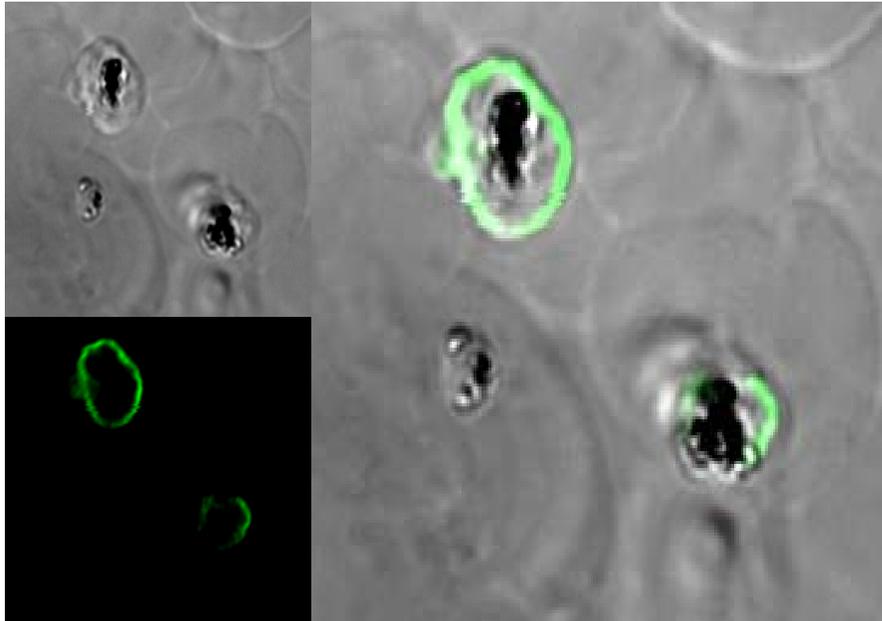


Figure 7. FIKK11 is Localized within the Parasitophorous Vacuolar Membrane at the Schizont Stage of the Malaria Lifecycle

Immunofluorescence was determined by fixing and permeabilizing cells prior to the addition of a primary antibody raised against FIKK11 and the addition of Alexa Fluor 488 secondary antibody. This figure represents a cell as visualized using the Axiovert 100M confocal microscope. The upper left panel of each shows the DIC image of the cell only. The lower left panel shows the immunofluorescence image of the FIKK11. The larger panel represents the overlay of the DIC and immunofluorescence image.

Cloning, Overexpression, and Purification of Malarial FIKK Kinases

Information gathered from the PlasmoDB database provided open-reading frame sequences for many of the FIKK proteins. We chose FIKK10.1 and FIKK11 as candidates to study the catalytic properties of FIKK proteins (Fig. 8). To establish whether these FIKKs function as protein kinases and for subsequent functional analysis of these members of the FIKK kinase family, we amplified the kinase domains of FIKK10.1 and FIKK11 by RT-PCR using mRNA isolated from asynchronous 3D7 *Plasmodium falciparum* parasites (fig. 9 and fig. 10). We chose to amplify the kinase domains only because our initial attempts to express full-length proteins containing the N-terminal secretory domain resulted in mostly insoluble proteins. The amplification of the kinase domains, however, should be sufficient to establish whether or not FIKK kinases are catalytically active. The resulting amplified kinase domain sequences were verified by DNA sequencing and were cloned into the T7 RNA polymerase-driven pET43.1 Ek/LIC vector. Inserts for the open reading frames were verified after being released from the pET43.1 vector by digestion with XbaI and XhoI (Fig. 11 and fig. 12). The increase in size of the released inserts is due to the presence of the genes encoding the Nus-His tag. Cloning into this vector was accomplished by a ligation-independent method that uses the 3' → 5' exonuclease activity of T4 DNA polymerase to generate single-stranded nucleotide overhangs in the insert which are complementary to a complementary overhang in the vector. Following annealing of the vector and insert, the reaction mixture was transformed into E.

coli Novablue supercompetent cells. This method is extremely efficient and most of the resulting colonies contained the insert product.

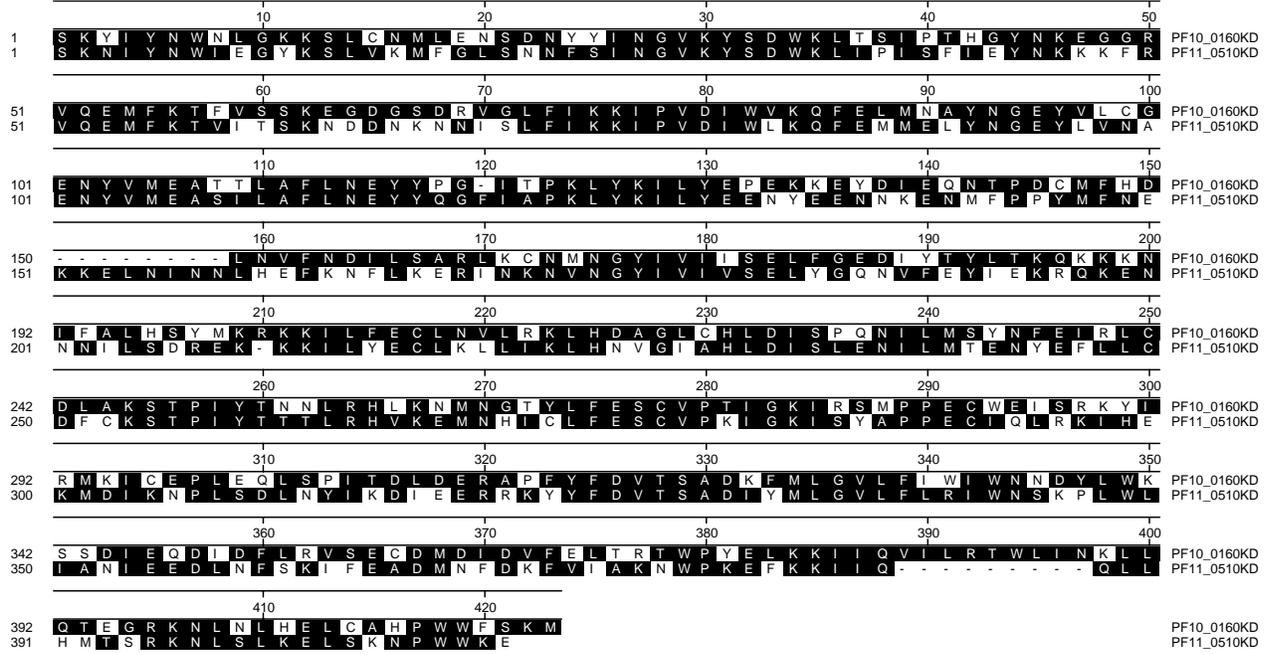


Figure 8. Comparative Sizes of the FIKK10.1 and FIKK11 Kinase Domain Sequences

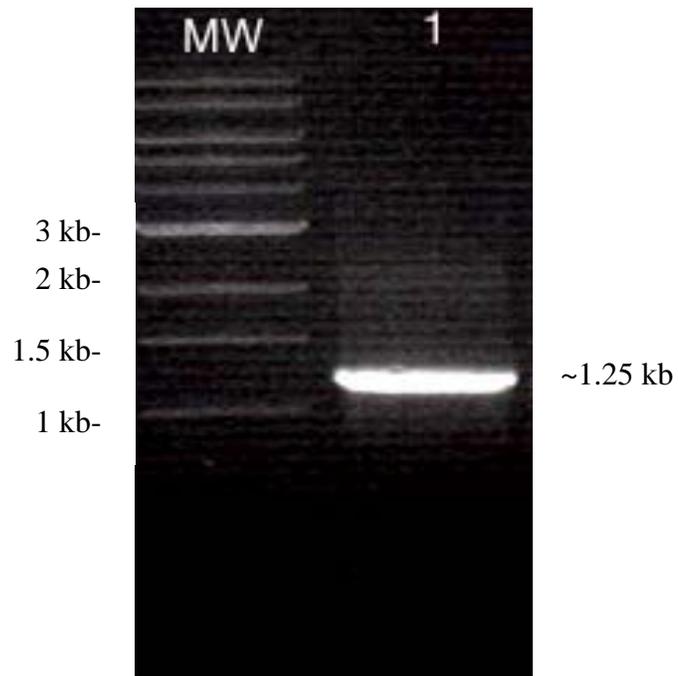


Figure 9. PCR Amplification of FIKK10.1 Kinase Domain

The Kinase domain of FIKK10.1 was amplified by RT-PCR. The PCR product was resolved on 0.8% agarose gels using 1X TAE buffer.

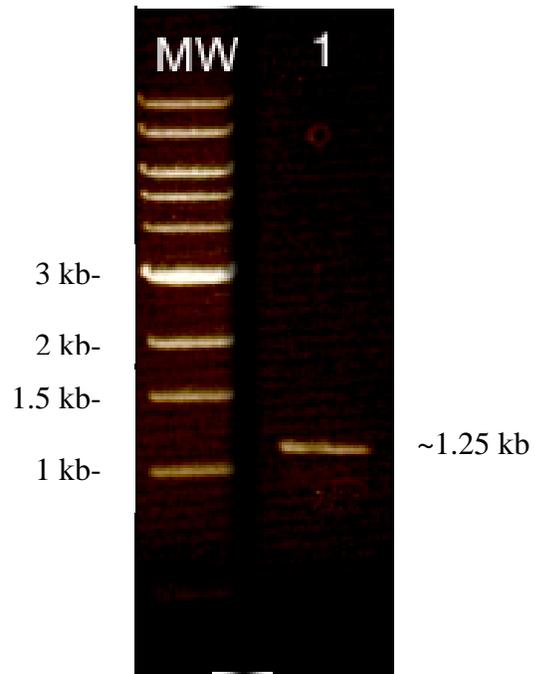


Figure 10. PCR Amplification of FIKK11 Kinase Domain

The Kinase domains of FIKK11 were amplified by RT-PCR. The PCR product was resolved on 0.8% agarose gels using 1X TAE buffer.

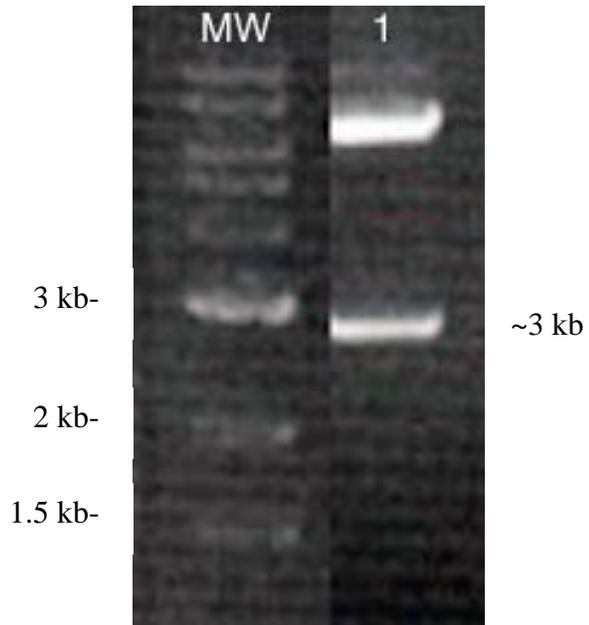


Figure 11. Verification of FIKK10.1 Kinase Domain Cloning into pET43 by Restriction Enzyme Digestion

The PCR products were then cloned into the pET43LIC vector using a ligation-independent cloning approach. Expression constructs were digested with XbaI and XhoI to release the Nus-tagged insert and the restriction digest was resolved on 0.8% agarose gels.

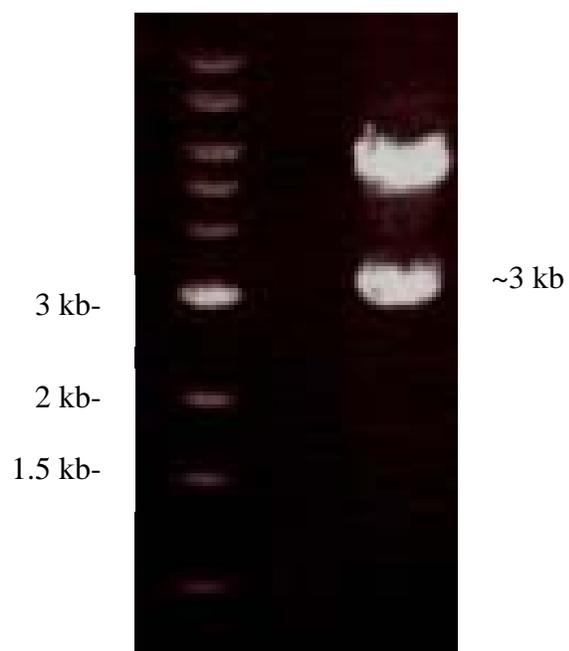


Figure 12. Verification of FIKK11 Kinase Domain Cloning into pET43 by Restriction Enzyme Digestion

The PCR product was then cloned into the pET43LIC vector using a ligation-independent cloning approach. Expression constructs were digested with XbaI and XhoI to release the Nus-tagged insert and the restriction digest was resolved on 0.8% agarose gels.

Cells harboring the pET43.1-FIKK11 plasmid were induced with IPTG and the cultures were then examined to determine the solubility of the proteins from different fractions. As was expected, SDS-PAGE analysis of induced soluble and insoluble fractions showed the Nus-FIKK fusion proteins to be present in the soluble fraction in sufficient amounts for subsequent purification (Fig. 13).

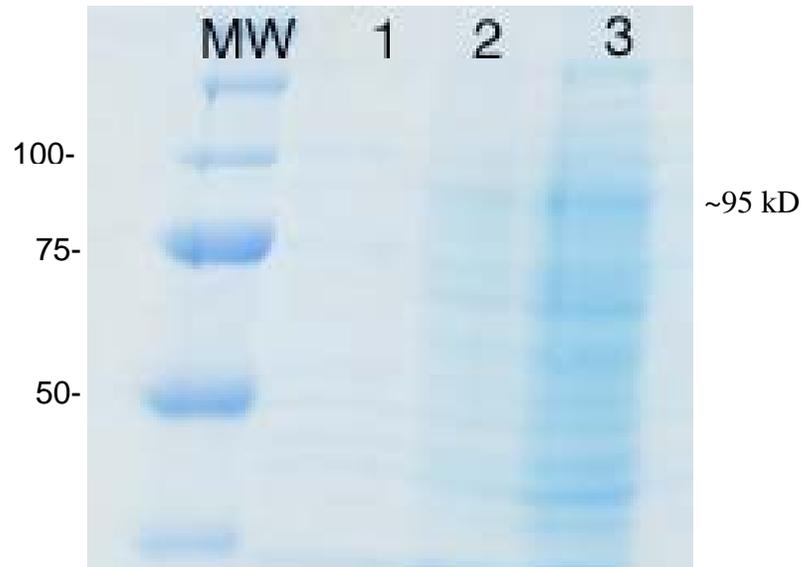


Figure 13. Induction of FIKK11 to Verify the Solubility of the Nus-tagged Fusion Construct

BL21 (DE3) Codon+ *E. coli* cells were transformed with the FIKK11 recombinant protein and induced with 1mM IPTG at 27°C overnight. The cells were then spun down and resuspended in a lysis buffer and sonicated for 6 cycles. The soluble and insoluble fractions were separated and the samples run on SDS-PAGE gel. The final gel was stained with Gel Code Blue. MW, molecular weight marker; Lane 1, uninduced; Lane 2, soluble fraction; Lane 3, insoluble fraction.

The His-tagged recombinant FIKK fusion proteins were purified using a TALON cobalt affinity chromatography (Clontech) Clontech (Fig. 14). This method uses Immobilized Metal Affinity Chromatography (IMAC) for separating proteins through the interaction of a histidine amino acid tag with an immobilized cobalt metal ion. This can allow purification of proteins under native or denaturing conditions and allows for elution of the bound, His-tagged protein under relatively mild conditions. Fractions were examined to determine which contained more of the recombinant FIKK proteins relative to other proteins. Fractions enriched in recombinant FIKK were then concentrated and further purified using a SuperDex 75 size exclusion chromatography for further purification. Western blot analysis with His-tag antibodies confirmed the identity of the purified recombinant FIKK11 (Fig. 15). The recombinant FIKK10.1 kinase domain construct yielded similar results.

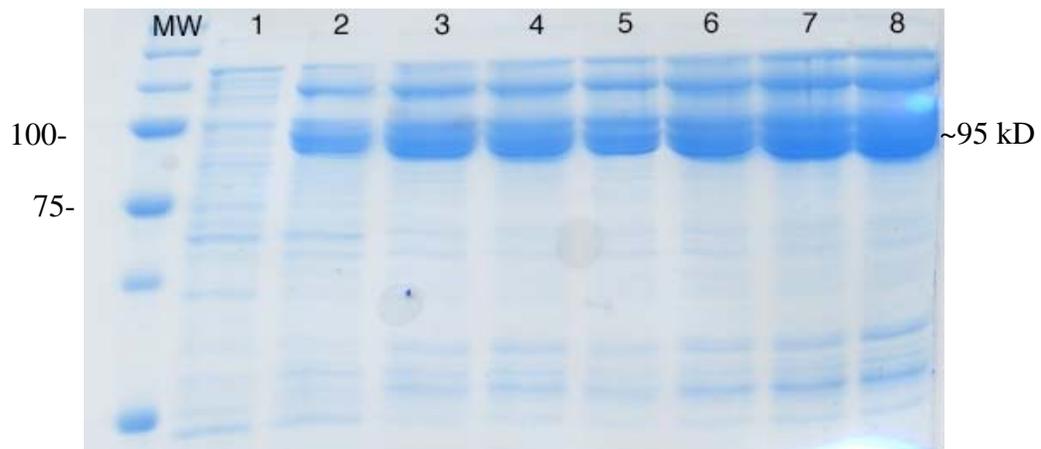


Figure 14. TALON Purification of FIKK11

Using a TALON cobalt affinity purification column, 500 μ l samples were collected and 20 μ l of each sample was run on SDS-PAGE gels. The gel was subsequently stained with Gel Code Blue for visualization. MW, molecular weight marker; Lanes 1-8, the eight fractions recovered during the TALON purification of FIKK11. Fractions 3-5 were retained and used for further enzymatic analysis.

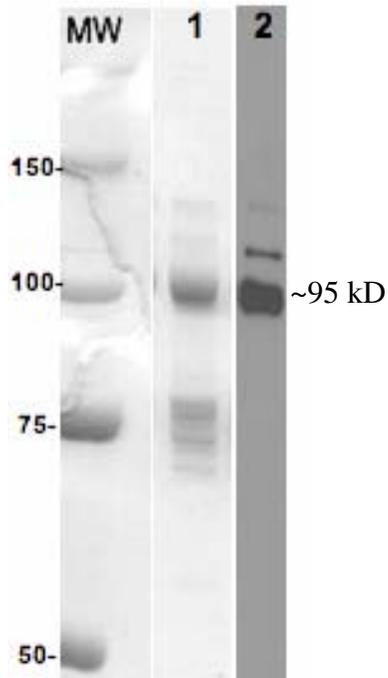


Figure 15. Verification of FIKK11 Recombinant Kinase Domain Purification by Western-blot Analysis

The recombinant protein expression was induced by 1mM IPTG at 27⁰C overnight. Purification of the recombinant protein was achieved by using a TALON cobalt affinity column (Clontech). The recombinant protein was resolved on 8% SDS-PAGE. Lane 1 is a stained gel of the purified protein; Lane 2 is a Western blot analysis of the recombinant protein using anti-His antibodies.

Characterization of Recombinant FIKK Protein Kinase Activity

The recombinant FIKK kinase domains of FIKK11 and FIKK10.1 were assayed for either autophosphorylation, histone H1, myelin basic protein, or dephosphorylated casein phosphorylation activity using a standard protein kinase assay with ATP as the phosphodonor. The resulting reaction mixture was either spotted on phosphocellulose filters and analyzed using a Beckman scintillation counter and/or run on SDS-PAGE gels for visualization by autoradiography. None of the potential substrates used in the assay showed any phosphorylation by the recombinant FIKK kinase domain proteins. The recombinant FIKK10.1 kinase domain also showed negligible autophosphorylation activity, however, the apparent autophosphorylation activity of the recombinant FIKK11 appeared to be quite robust (Fig. 16). Interestingly, a significant mobility shift of the FIKK11 protein following autophosphorylation was also observed (Fig. 17).

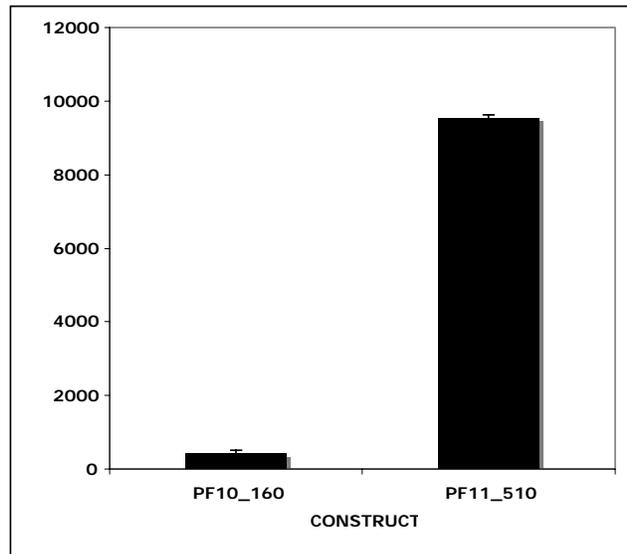


Figure 16. FIKK11 Recombinant Kinase Domain Shows Potential Autophosphorylation Activity

Kinase activity of recombinant proteins were assessed in a reaction containing 50 mM Tris HCl, pH7.5, 10 mM MgCl₂, 1 mM DTT, 40 mM b glycerophosphate, 100 μM sodium vanadate, 50 μM ATP, 1 μCi γ ³²P ATP, 0.5 μg enzyme in a 25 μl vol for 60 min at 30°C. 10 μl samples were spotted on P81 circles (Whatman) and ³²P incorporation was quantitated following phosphoric acid washes.

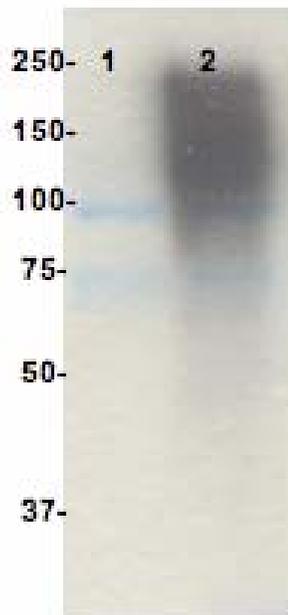


Figure 17. Autoradiography Analysis of FIKK11 Recombinant Kinase Domain Shows a Significant Mobility Shift

Kinase activity of recombinant proteins were assessed in a reaction containing 50 mM Tris HCl, pH7.5, 10 mM MgCl₂, 1 mM DTT, 40 mM β-glycerophosphate, 100 μM sodium vanadate, 50 μM ATP, 1 μCi $\gamma^{32}\text{P}$ ATP, 0.5 μg enzyme in a 25 μl vol for 60 min at 30°C. 20 μl reaction sample of FIKK11 was resolved on a 10% SDS-PAGE. The autoradiograph was exposed at -80°C for 3 hours using Transcreen HE and BioMax MS film. Lane 1, no enzyme; Lane 2, wild type protein.

To determine whether FIKK11 required a specific bivalent-metal cofactor to function properly, the recombinant FIKK11 protein was incubated in the presence of protein kinase reaction mixtures containing various bivalent cations. This assay showed that the recombinant FIKK11 kinase has an absolute requirement for a free bivalent-metal cofactor, with Mg^{2+} being the preferred cation. No significant catalytic activity was detected when Mn^{2+} , Ca^{2+} , or Zn^{2+} were substituted in the reaction mixture (Fig. 18).

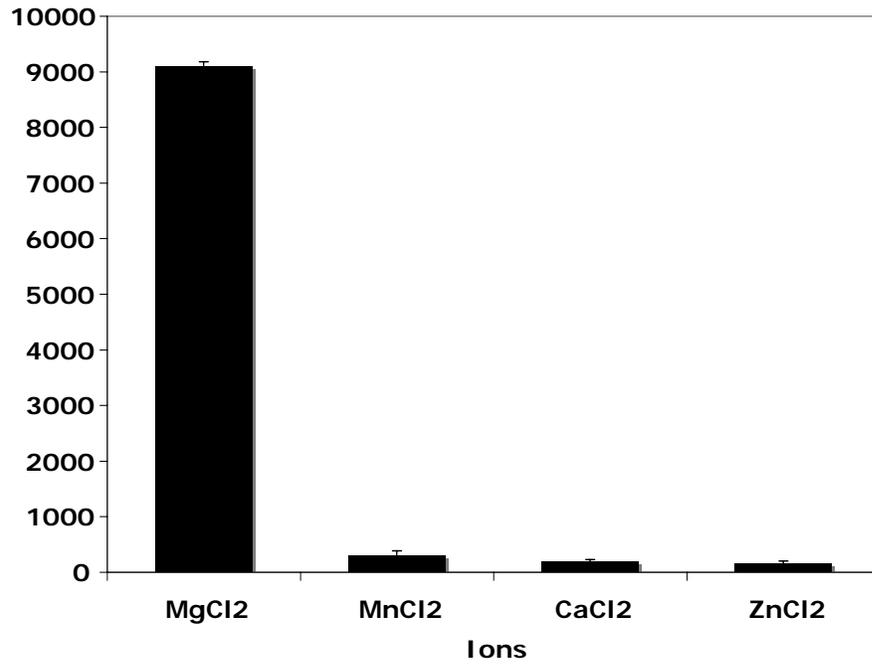


Figure 18. FIKK11 Activity is Dependent on Mg^{2+}

Purified recombinant enzyme was incubated in a kinase reaction mixture containing 10 mM of divalent cations.

We examined the dependence of phosphorylation activity with increasing enzyme concentration as well. Fig. 19 shows that the rate of the reaction is roughly proportional to the amount of FIKK11 recombinant enzyme present. About 6-7pmol of phosphate was incorporated per pmol of the enzyme.

To determine the reaction time for phosphorylation, kinase assays were continued for different durations of time ranging from one minute to one hour before stopping the reaction. As can be seen in fig. 20, recombinant FIKK11 activity was nearly linear with time beginning at 10min and showed no signs of leveling off after even one hour of incubation.

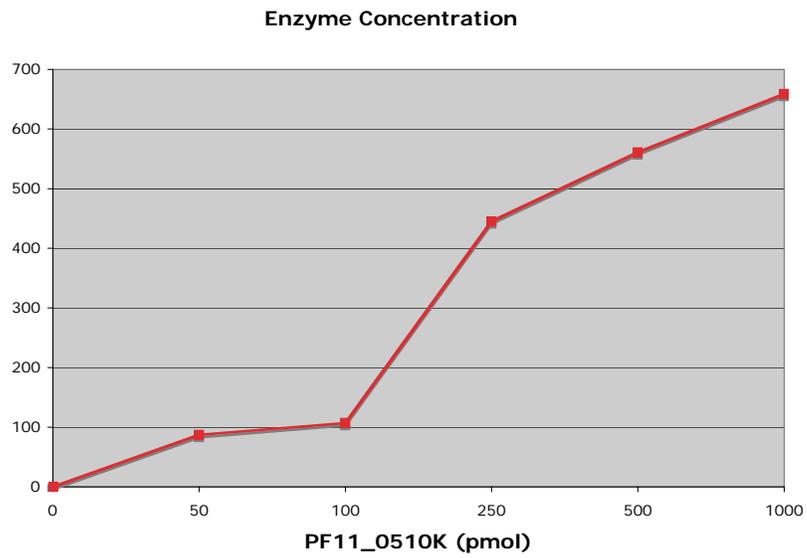


Figure 19. FIKK11 Activity Increases with Enzyme Concentration

0-10pmol of recombinant enzyme was incubated in a kinase reaction mixture for 1hr at 30°C.

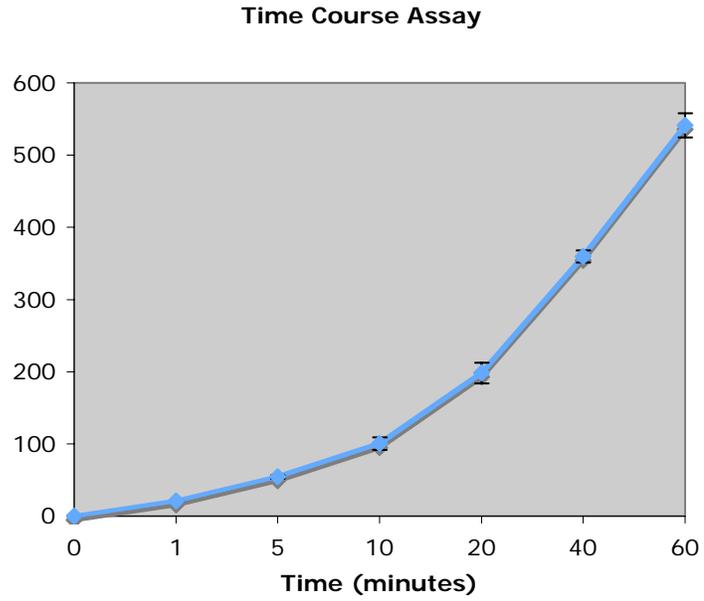


Figure 20. FIKK11 Activity Increases with Incubation Time

4pmol of recombinant enzyme was incubated at 30°C using a kinase assay system for 0-60 min.

Because kinases are often characterized by their sensitivity to specific diagnostic inhibitors, we tested the effect of a panel of inhibitors on the activity of recombinant FIKK11. We used the following inhibitors: (a) Ro-31-8220, a selective inhibitor for protein kinase C as well as MSK1, p90RSK, p70S6 kinase 1, and GSK3 (Davies et al., 2000; Deak et al., 1998), (b) KT 5720, a potent inhibitor of protein kinase A, (c) genistein, an inhibitor of protein tyrosine kinases (Hidaka and Kobayashi, 1993), and (d) staurosporine, a broad-spectrum inhibitor of protein kinases. Only genistein and Ro-31-8220 showed significant inhibition (Fig. 21). Genistein (4,5,7-trihydroxyisoflavone) gave an IC₅₀ of about 25 μM while Ro-31-8220 gave an IC₅₀ of about 15 μM. Interestingly, the broad-spectrum protein kinase inhibitor, staurosporine, which inhibits both Ser/Thr and Tyr kinase is totally ineffective against recombinant FIKK11 even at 50 μM. This suggests that, although recombinant FIKK11 is inhibited by a Tyr kinase inhibitor (genistein) and a PKC inhibitor (Ro-31-8220), it is refractory to staurosporine which is an effective inhibitor of both Tyr kinases and PKC. This raises the question of whether FIKK11 is a true protein kinase at all.

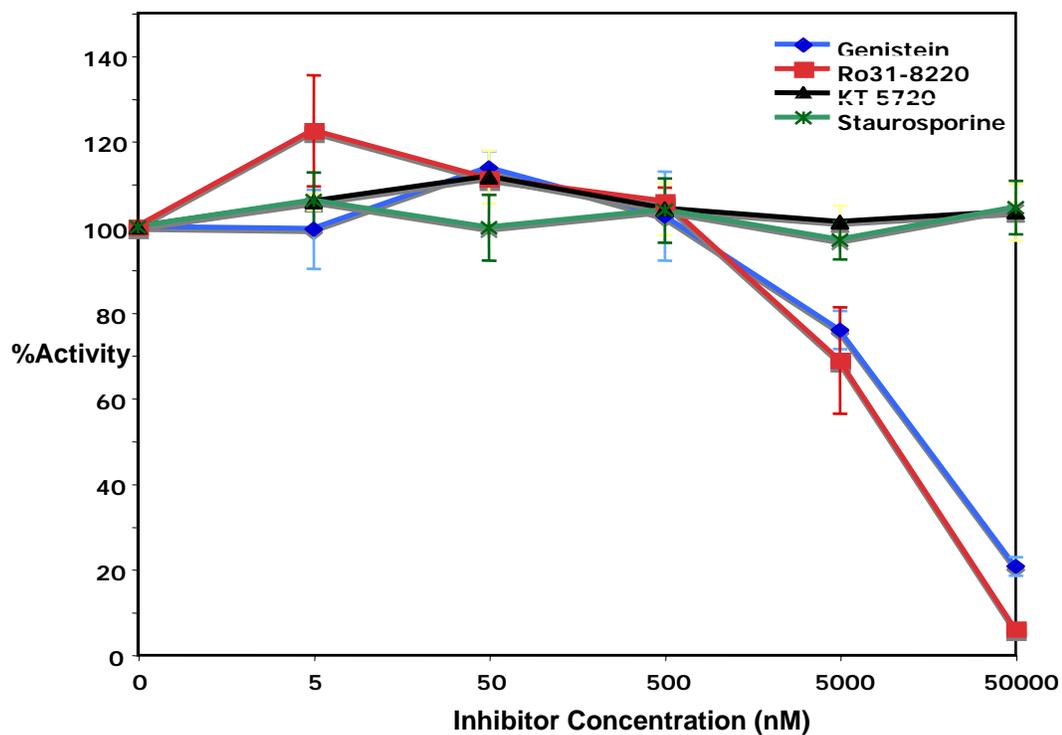


Figure 21. Effects of Protein Kinase Inhibitors on FIKK11 Activity

The inhibitors were added to a standard protein kinase assay at various concentrations (5 nM-50 μ M). Effects on protein phosphorylation were analyzed by binding to phosphocellulose paper circles (P81).

Determination of Plasmodium Protein Kinase Phosphorylation Specificity Using a Positional Scanning Peptide Library

Recently, Dr. Benjamin Turk of Yale University developed a rapid method to characterize eukaryotic protein kinase phosphorylation motifs using a combinatorial peptide library. This method systematically evaluates the preference of a given kinase for every amino acid at each of nine sites surrounding the phosphorylation site of a given substrate (Hutti et al., 2004). The method uses a library of roughly 200 distinct peptide mixtures each containing a central fixed phosphorylation site flanked on either side by degenerate positions. For each of the nine positions surrounding the phosphoacceptor site, twenty-two peptide mixtures are made in which each of the twenty unmodified proteogenic amino acids as well as phosphothreonine and phosphotyrosine are fixed. The entire collection consists of 198 peptides. These peptide substrates are arrayed in a spatially addressable manner in a 384-well plate and treated with the kinase of interest and radiolabeled ATP. Control reactions are also run in the absence of peptide to determine the background from kinase alone. At the end of the incubation time, aliquots of each reaction are spotted simultaneously onto a streptavidin membrane using a capillary pin-based liquid transfer device. The membrane is immersed in a quenching solution, washed extensively to remove unincorporated label, dried, and exposed to a phosphor screen. This allows the amount of radiolabel incorporated into the peptide to be quantified, providing an indication of which peptides are the most

efficient substrates for a particular kinase. This in turn indicates which amino acids at a particular position influence phosphorylation by that kinase. This method has been used to profile over 50 serine-threonine and tyrosine kinases. Fig. 22 shows a diagrammatical representation of how the positional scanning peptide library works.

Because of the conflicting results observed when studying the recombinant FIKK11 kinase, samples were sent to Dr. Turk's lab to be assayed for testing using the positional scanning peptide library. Peptide array substrate analysis was not able to identify a single interacting peptide substrate for FIKK11.

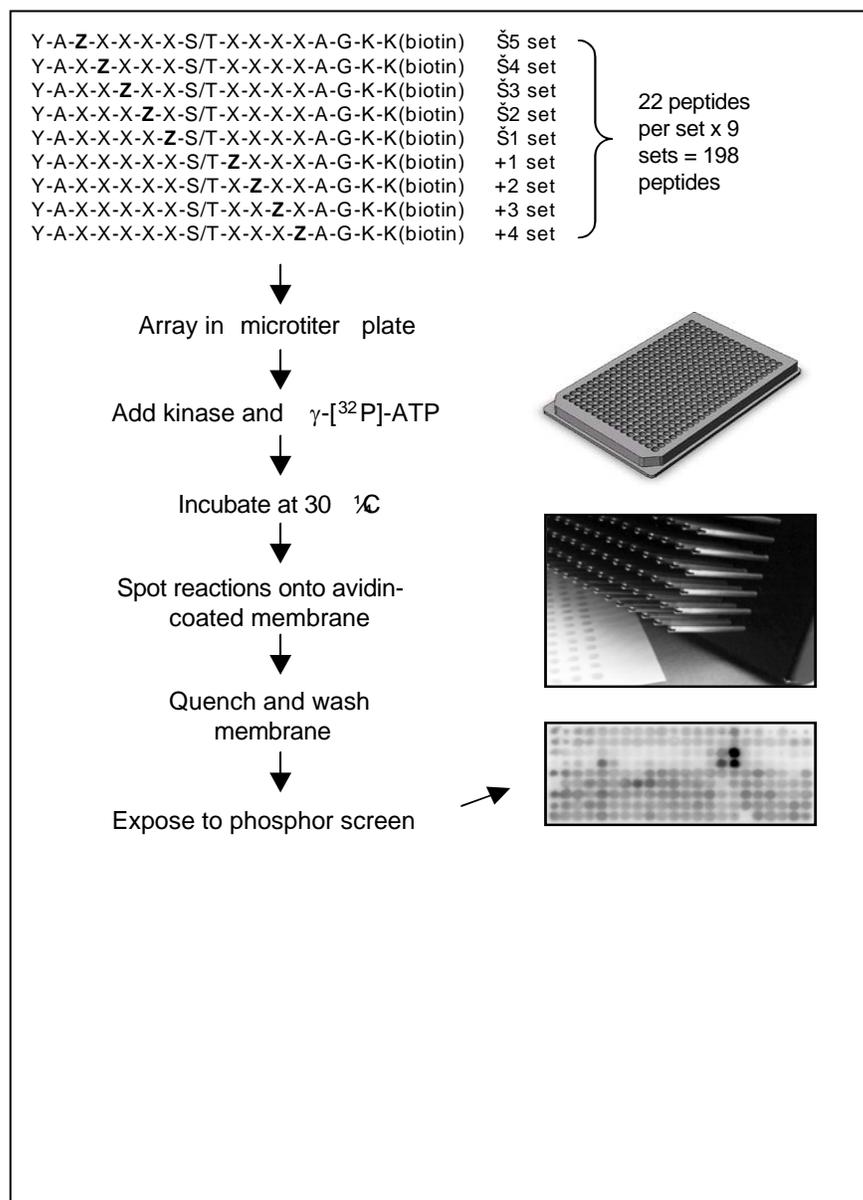


Figure 22. Peptide Array Method for Determining Protein Kinase Phosphorylation Motifs

For each of 9 positions surrounding the phosphorylation site, 22 peptides are synthesized in which Z is fixed as one of the 20 unmodified proteogenic amino acids, phosphothreonine or phosphotyrosine. X positions are equimolar mixtures of all residues (excluding serine, threonine and cysteine).

Analysis of Protein Kinase Assay Components

Since no interacting substrates were identified against recombinant FIKK11, we began to suspect that our enzyme was phosphorylating some component within our reaction mixture. To examine this possibility, reaction mixtures were made in which one of the selected components was eliminated and the protein kinase assay carried out as before in the presence of radiolabelled ATP. Only the reaction mix lacking MgCl₂ prevented the phosphorylation previously seen from taking place (Fig. 23). This is expected since we had earlier shown that MgCl₂ is the preferred bivalent metal cation for this enzyme.

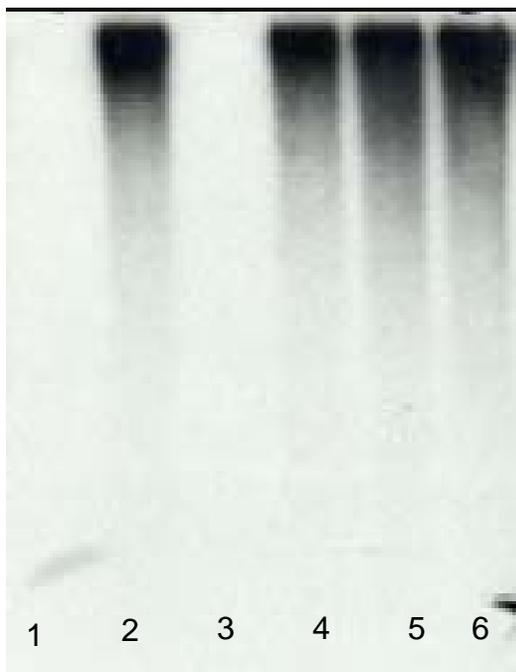


Figure 23. Removal of MgCl_2 from the Reaction Mixture Eliminated the Activity of Recombinant FIKK11

Standard reaction mixes were made including several that lacked one of the key components of the mixture. The reaction was carried out as before and the resulting samples run on an SDS-PAGE gel and exposed to BioMax MS film overnight to determine the results. Lane 1, no enzyme; Lane 2, enzyme and standard reaction mix, Lane 3, reaction mix lacking MgCl_2 ; Lane 4 reaction mix lacking DTT; Lane 5, reaction mix lacking β -glycerophosphate, Lane 6, reaction mix lacking sodium vanadate.

Glycerol had been used during purification of our protein to enhance the stability of the recombinant FIKK11, a common practice during the purification of proteins. Therefore, we decided to investigate the contribution of glycerol on actual kinase activity to see if residual glycerol within the recombinant protein samples is phosphorylated which is giving us the impression of FIKK11 autophosphorylation. To this end, a sample of recombinant FIKK11 protein was purified without glycerol. When assayed using the protein kinase assay system, the recombinant protein purified without glycerol showed no phosphorylation compared with the recombinant protein purified with glycerol (Fig. 24). This result may be due to the fact that the protein is no longer active since the stabilizing nature of the glycerol had been removed during its purification. However, another possibility is that glycerol may actually be the substrate for the recombinant FIKK11 protein.

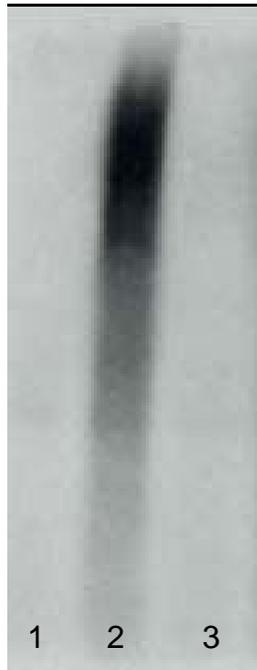


Figure 24. Removal of Glycerol During Protein Purification Eliminated Recombinant FIKK11 Activity

Standard reaction mixes were used on recombinant FIKK proteins purified either with or without the addition of glycerol and the reaction was carried out as before and the resulting samples run on an SDS-PAGE gel and exposed to BioMax MS film overnight to determine the results. Lane 1, no enzyme; Lane 2, enzyme purified with glycerol; Lane 3, enzyme purified without glycerol.

Characterization of Recombinant FIKK11 Glycerol Kinase Activity

Based on the observation that recombinant FIKK11 kinase showed no activity at all when purified in the absence of glycerol, it is possible that the enzyme is not a true protein kinase. This is even more likely given the fact that FIKK11 lacks the traditional glycine triad common to most eukaryotic protein kinases. To determine whether the recombinant FIKK11 kinase might have an activity other than that of a protein kinase, the enzyme was assayed using glycerol as a potential substrate. The recombinant FIKK11 kinase, however, showed no significant glycerol kinase activity as compared to a commercially available glycerol kinase (Fig. 25).

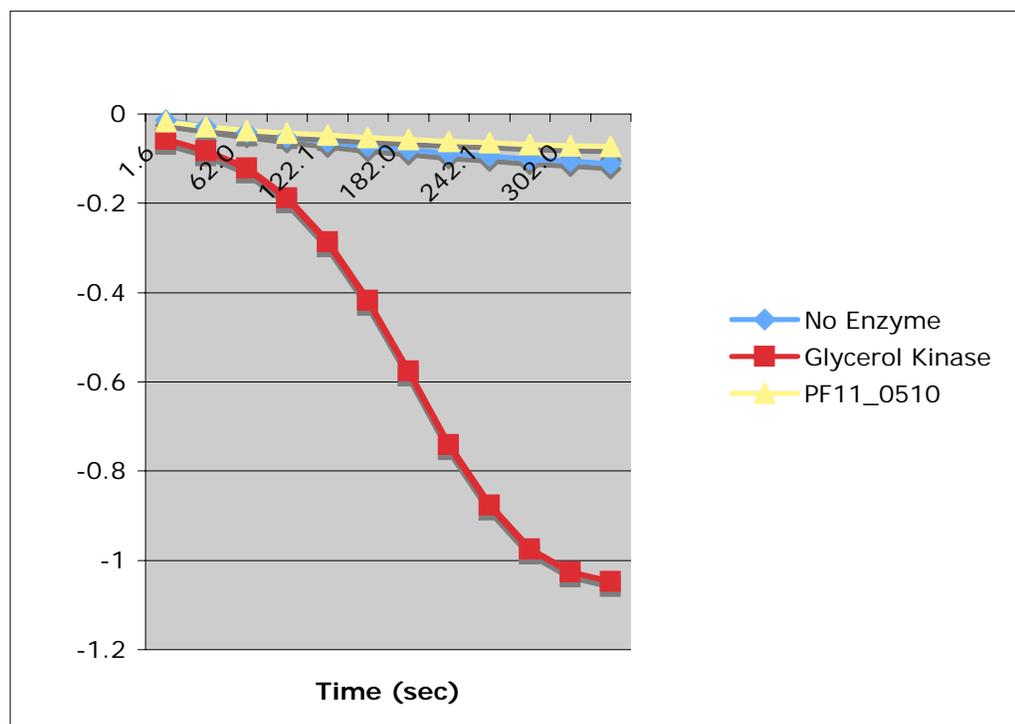


Figure 25. FIKK11 Exhibits no Glycerol Kinase Activity

Glycerol kinase activity of FIKK11 was compared to a commercially available glycerol kinase using a coupled reaction to measure the oxidation of NADH. 2.5mM ATP was incubated for five minutes in the presence of a reaction mixture (0.05M Triethanolamine-HCl (pH 7.0), 2.5mM MgCl₂, 20mM KCl, 0.1mM β-Mercaptoethanol, 0.2mM Phospho(enol)pyruvate, 0.2mM NADH, 2mM Glycerol, 5U/ml Pyruvate Kinase, and 5U/ml Lactate Dehydrogenase). 100ng of FIKK 11 or a commercially available E. coli glycerol kinase was added and the activity measured over the course of 8 minutes using a Hewlett-Packard 8453 spectrophotometer to determine the decrease in absorbance of NADH at 340nm.

Characterization of Recombinant FIKK11 Diacylglycerol Kinase Activity

Since our data showed conclusively that FIKK11 was not a glycerol kinase, we tested the possibility whether the protein has any diacylglycerol kinase activity. To determine whether the recombinant FIKK11 kinase has diacylglycerol activity, the enzyme was assayed using 1-Stearoyl-2-arachidonoyl-sn-glycerol as a potential substrate. As evident in fig. 26, film autoradiography analysis of the TLC plate shows that FIKK11 does exhibit diacylglycerol kinase activity when compared to a commercially available DGK and BSA and no enzyme used as negative controls. Additionally, quantification by phosphoimage analysis of the radiolabelled spots shows that the recombinant FIKK11 kinase has significant activity as a diacylglycerol kinase against 1,2-SAG as a potential substrate. However, the activity is significantly less than that seen using a commercially available diacylglycerol kinase (Fig. 27).

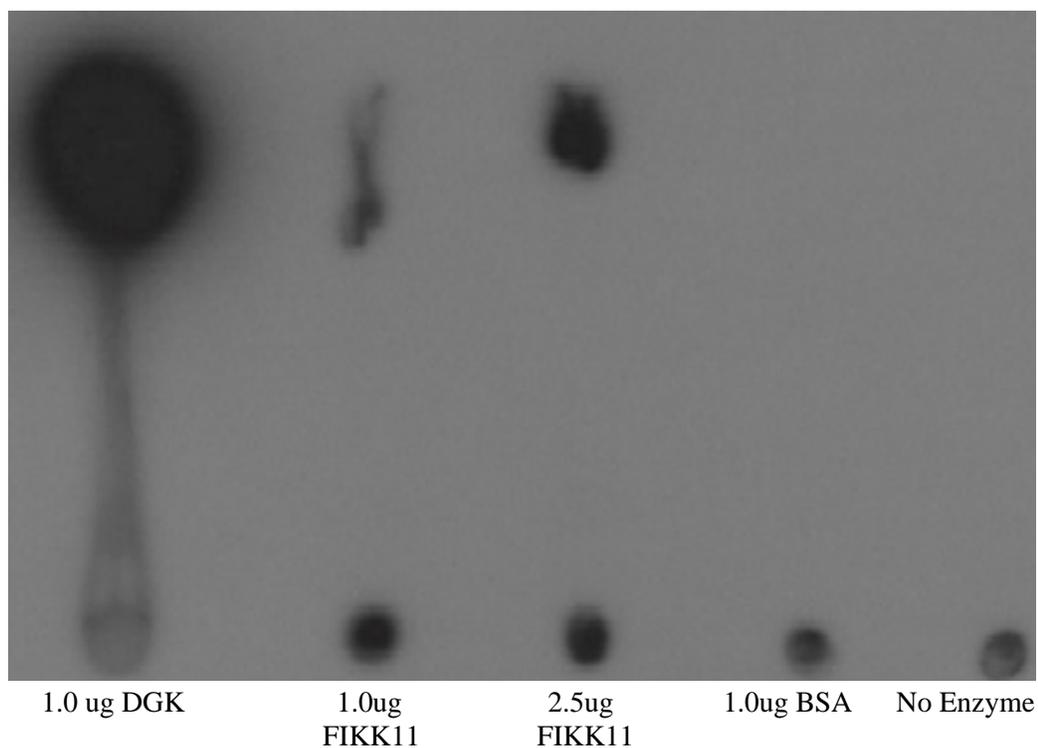


Figure 26. Recombinant FIKK11 Exhibits Diacylglycerol Kinase Activity

DGK activity of recombinant proteins were assessed in a reaction containing 40mM Bis-Tris (pH 7.5), 5mM MgCl₂, 0.1mM EDTA, 1mM Spermine, 0.5mM DTT, 0.02% Triton X-100, 1mM Sodium Desoxycholate, 250μM Cardiolipin, 500μM 1,2-SAG, 1mM ATP (with 5μCi $\gamma^{32}\text{P}$ ATP) and 1μg enzyme for 30 minutes at room temperature. The reactions were spotted on TLC and then exposed at -80°C for 30 minutes using Transcreen HE and BioMax MS film.

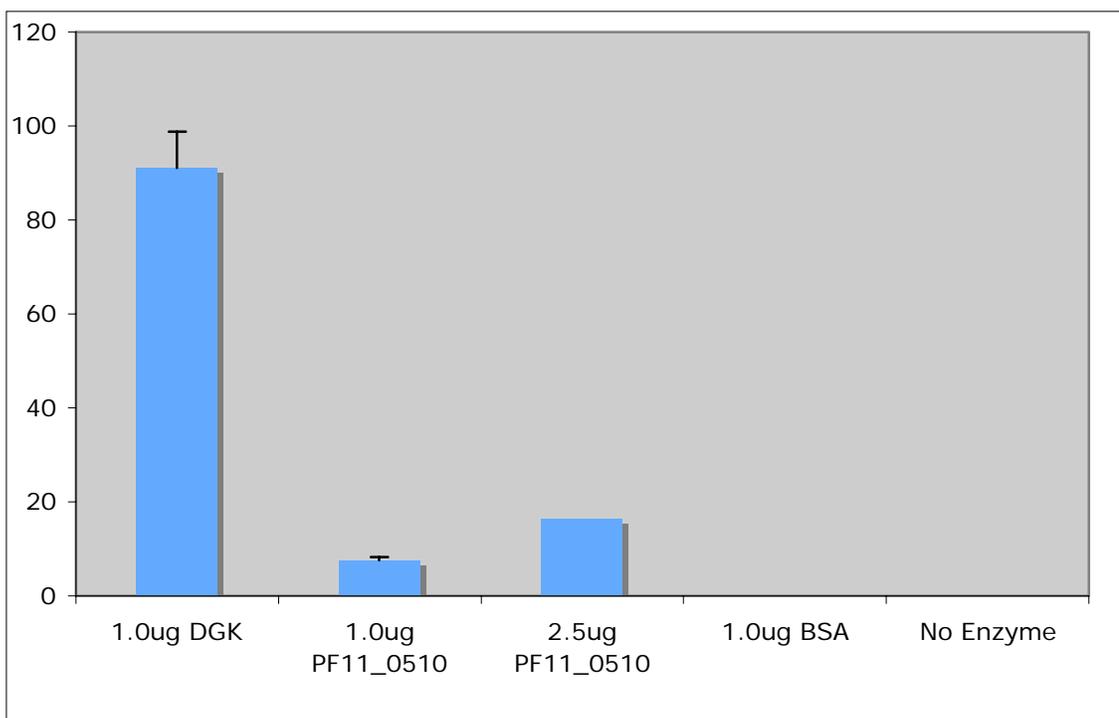


Figure 27. DGK Activity of Recombinant FIKK11 as Measured by Phosphoimaging

DGK activity of recombinant proteins were assessed in a reaction containing 40mM Bis-Tris (pH 7.5), 5mM MgCl₂, 0.1mM EDTA, 1mM Spermine, 0.5mM DTT, 0.02% Triton X-100, 1mM Sodium Desoxycholate, 250μM Cardiolipin, 500μM 1,2-SAG, 1mM ATP (with 5μCi $\gamma^{32}\text{P}$ ATP) and 1μg enzyme for 30 minutes at room temperature. The percent activity for each reaction was measured on a phosphoimager after overnight exposure to a phosphoimager cassette.

CHAPTER FOUR: DISCUSSION

The identification of a novel family of FIKK kinases within the *Plasmodium falciparum* genome is a source of excitement for their potential as targets for malaria therapy. However, little is actually known about the function of these proteins. To this end, we decided to focus on the characterization of several of these kinases in order to better understand their role and potential use as drug targets. Our studies focused on two objectives: (1) are these proteins actually expressed by the malaria parasite and are they exported from the malaria parasite into the erythrocyte cytoplasm or plasma membrane as is suggested by their HT/Pexel motif and (2) are these proteins true protein kinases as is suggested by sequence analysis?

Our first goal was to determine whether these proteins were in fact expressed *in vivo* as has been suggested based on microarray studies. Immunoblot analysis using mono-specific, affinity-purified antibodies raised against FIKK11 confirms a stage-dependent expression of the protein in parasites with a peak expression in the schizont stage. This result correlates well with the microarray analysis gathered from Plasmo DB.

Since FIKK11 was shown to be present *in vivo*, we sought to determine the localization of the protein within the parasite or host erythrocyte. Recent evidence regarding the HT/Pexel motif found in malarial proteins suggests that this motif is important in the export of those proteins into the erythrocyte cytosol (Hiller et al., 2004; Marti et al., 2003). If the sequence seen in the FIKK11

protein is a true HT/Pexel motif, then the same should be true for it. A recent study that examined the localization of FIKK4.1 and FIKK12 (Nunes et al., 2007) was able to show that those proteins were indeed exported from the parasite to a location associated with the erythrocytes membrane. That same study, however, also showed that FIKK9.2 was not exported beyond the parasite cell membrane suggesting that not all of these FIKK proteins behave in the same manner. Using an immunofluorescence assay, FIKK11 was clearly identified outside of the parasite, within the host erythrocyte which is consistent with previous observations for proteins containing the HT/Pexel motif. The distribution of fluorescence within the infected erythrocytes strongly suggests an association with the infected erythrocyte membrane and the parasitophorous vacuolar membrane. It should be noted that significant localization of the FIKK11 protein was detected on the erythrocyte membrane during the trophozoite stage of the parasite life cycle. This may provide a basis for its role in antigenicity, something characterized with the R45 protein (FIKK4.2) (Schneider and Mercereau-Puijalon, 2005). Additionally, the localization of FIKK11 appears to change with the intra-erythrocytic maturation of the parasite and the expression of FIKK11 also clearly increases with age of the parasite confirming our immunoblot data. Interestingly though, the expression of FIKK11 in the schizont stage appears to be confined only to the parasitophorous vacuolar membrane. This suggests a dynamic expression of the protein based on the parasite developmental stages.

Although no direct evidence exists that the FIKK proteins alone are truly protein kinases, we chose members of this family to create constructs for

recombinant fusion proteins. It has been suggested that members of this family are true protein kinases (Nunes et al., 2007), however the studies performed were done using an *in vivo* pulldown followed by an *in vitro* kinase assay. The problem with this approach is that the kinase activity detected in their studies could have come from a contaminating or associated kinase rather than the FIKK4.1 or FIKK12. Additionally, their studies only looked at FIKK4.1 and FIKK12, so the assumption that all FIKK kinases are indeed protein kinases lacks support. Our initial protein kinase assays were performed with purified recombinant protein and showed no phosphorylation of several standard protein kinase substrates. However, there was significant activity using the recombinant FIKK protein alone suggesting that there may be autophosphorylation. However, the significant mobility shift and general smearing of the protein seen in the SDS-PAGE gel strongly suggests an alternative explanation may be responsible for this phenomena. It is possible that the presence of a pronounced mobility shift might be due to the presence of something else co-purifying with the FIKK11.

To further examine the potential of FIKK11 as a protein kinase, inhibition assays were performed on the recombinant FIKK11 protein but the results were equally confusing. Our data showed that only Ro-31-8220, a protein kinase C inhibitor, and genistein, a protein tyrosine kinase inhibitor, had any inhibitory affect on the recombinant FIKK protein. However, staurosporine, which is a broad-spectrum protein kinase inhibitor, had no affect on the activity of recombinant FIKK11. KT 5720, an inhibitor of protein kinase A, also had no affect on the recombinant FIKK protein. These discrepancies, along with the

smearing seen earlier, raise the possibility that FIKK11 is not a protein kinase after all.

To confirm whether any protein kinase peptide substrate could be identified for FIKK11, we sent the recombinant FIKK11 protein to Dr. Benjamin Turk's laboratory at Yale University for use in a phosphorylation assay using a combinatorial peptide library. This method can quantify the amount of radiolabel incorporated into a peptide and can indicate which amino acids at a particular position influence phosphorylation. His lab has successfully profiled over 50 serine-threonine and tyrosine kinases. Unfortunately, no peptide sequences were identified to be substrates for FIKK11. The data collected also ruled out the possibility that an autophosphorylation event may be occurring with FIKK11.

The inability of Dr. Turk's laboratory to identify a single interacting substrate from the peptide substrate arrays phosphorylated by eukaryotic protein kinases caused us to consider other possibilities for FIKK11. Because of the general kinase activity observed, it became apparent that the phosphorylation previously observed was likely due to something within our protein kinase reaction mixture that was reacting with the recombinant protein.

The use of glycerol in the purification of proteins is a common practice to increase the stability of the protein that has been extracted. However, it might be possible that residual glycerol from the purification remains in the protein sample and the glycerol is the actual substrate for the recombinant FIKK11 protein. To test this, additional recombinant FIKK11 was expressed and purified without the use of glycerol. The recombinant FIKK proteins were then assayed using the

same protein kinase assay system as before. Interestingly, the removal of glycerol from the purification procedure caused the cessation of phosphorylation that had been observed.

Based on observations that recombinant FIKK11 kinase showed no activity at all when purified in the absence of glycerol, we began focusing on FIKK11 as a glycerol kinase. This seemed likely given the fact that FIKK11 lacks the traditional glycine triad common to most eukaryotic protein kinases. To this end, the recombinant FIKK11 protein was measured for glycerol kinase activity. Glycerol kinases catalyze the transfer of a phosphoryl group from Mg-ATP to glycerol to generate glycerol 3-phosphate and Mg-ADP (Lin, 1976). Using a standard glycerol kinase assay which measures the oxidation of NADH through a coupled reaction involving pyruvate kinase and lactate dehydrogenase in intermediate steps, the activity of recombinant FIKK11 could be compared to a commercially available glycerol kinase to assess its potential as a true glycerol kinase. Unfortunately, spectrophotometric analysis of the recombinant protein showed no glycerol kinase activity when compared to the positive control.

Having ruled out the possibility for FIKK11 to be a glycerol kinase, we examined its potential role as a diacylglycerol kinase. Diacylglycerol kinases are responsible for the phosphorylation of the second messenger diacylglycerol to phosphatidic acid (Los et al., 2004). This results in a recycling of phosphatidylinositol 4,5-bisphosphate that serves as an intermediate in protein kinase C-mediated signal transduction (Sakane et al., 1991; Goto and Kondo, 1993). This suggests that diacylglycerol kinases may serve to inhibit the activity

of PKC by attenuating diacylglycerol levels (Shindo et al., 2003). The majority of work regarding diacylglycerol kinases, however, has been done using mammalian systems in which five different classes have been identified all of which possess two or three cystein-rich domains labeled C1A, C1B, and C1C and a conserved catalytic domain (Hurley et al., 1997). Unfortunately, there is relatively little information available regarding the actual function of the many different isozymes and their conserved domains (van Blitterswijk and Houssa, 2000; van Blitterswijk and Houssa, 1999; Topham and Prescott, 2002). The amount of variability within these classes makes finding a concrete common structure difficult. Many of these contain an EF-hand motif responsible for calcium binding. This EF-hand motif and the typical cystein-rich domains are not present in the FIKK11 kinase which may explain its preference for magnesium as a cofactor. Indeed, recent studies involving plant diacylglycerol kinases have also shown members that prefer magnesium instead of calcium (Gomez-Merino et al., 2004).

Due to the fact that diacylglycerol kinases tend to be more heterogeneous in their composition displaying a wide array of structures, it is possible that the recombinant FIKK11 is actually acting as a diacylglycerol kinase despite not displaying the structure seen among the more widely studied mammalian diacylglycerol kinases. Using a diacylglycerol kinase assay system, the recombinant protein was measured against several diacylglycerol substrates and its activity compared to a commercially available diacylglycerol kinase. Interestingly, the recombinant FIKK11 showed a fairly significant amount of

activity with 1-stearoyl-2-arachidonoyl-sn-glycerol as a substrate when compared to the same activity seen using negative controls, although it was substantially less than the activity seen with the commercial diacylglycerol kinase. Based on these results, coupled with the results from the protein kinase and glycerol kinase assays, led to the conclusion that the recombinant FIKK11 kinase actually possesses diacylglycerol kinase activity and not protein kinase activity as has been recently speculated. The implications of these results are quite exciting considering this would provide yet another potential target for signaling between the host erythrocyte and the parasite and provides a new focus for future studies considering no work to date has been done looking at the role of diacylglycerol kinases within *Plasmodium falciparum*.

The study of this novel group of FIKK proteins within the *Plasmodium falciparum* genome focused on one protein in particular, FIKK11. To characterize its potential function, the recombinant protein was cloned, expressed, and subjected to a series of kinase assays. Although previous analysis has suggested that this family may function as protein kinases, at least the recombinant FIKK11 studied here appears to instead function as a diacylglycerol kinase. The HT/Pexel motif of the protein does indeed appear to target the protein for export from the parasite into the erythrocyte cytosol and beyond.

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