

HUMORAL IMMUNE RESPONSES TO *PLASMODIUM VIVAX* SUBTELOMERIC TRANSMEMBRANE PROTEINS IN THAILAND

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Abstract. *Plasmodium vivax* subtelomeric transmembrane protein (PvSTP) is a homolog of *P. falciparum* SURFIN_{4.2}, a protein exposed on the parasite-infected erythrocyte (iE) surface, and is thus considered to be exposed on *P. vivax*-iE. Because antibodies targeting antigens located on the surface of *P. falciparum*-iE, such as *P. falciparum* erythrocyte membrane protein 1, play an important role in regulating the course of disease, we evaluated the presence of antibodies in *P. vivax*-infected patients against two PvSTP paralogs, PvSTP1 and PvSTP2. Recombinant proteins corresponding to cysteine-rich domain (CRD) of the PvSTP extracellular region and the cytoplasmic region (CYT) were generated and used for the enzyme-linked immunosorbent assay. Plasma samples ($n = 70$) reacted positively with recombinant PvSTP1-CRD (40%), PvSTP1-CYT (31%), PvSTP2-CRD (27%), and PvSTP2-CYT (56%), suggesting that PvSTP1 and -2 are naturally immunogenic. Specific response against either PvSTP1 or PvSTP2 indicates the existence of specific antibodies for either PvSTP1 or -2.

Keywords: *P. vivax*, PvSTP, ELISA, antibody response

INTRODUCTION

Plasmodium vivax accounts for more

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than 50% of all malaria cases outside of Africa. The parasite causes extensive morbidity in endemic countries, and its socio-economic impact, although large, is somewhat neglected (Mueller *et al*, 2009). However, there is mounting evidence that *P. vivax* can lead to severe malaria associated with, for example, pulmonary edema, anemia, and sometimes death (Kochar *et al*, 2005; Kumar *et al*, 2007). One of the factors associated with the

severity of falciparum malaria is rosette formation, which is defined as two or more uninfected erythrocytes adhering to a parasite-infected erythrocyte (iE) (David *et al*, 1988; Handunnetti *et al*, 1989; Carlson *et al*, 1990; Kaul *et al*, 1991). Rosette formation is also seen in *P. vivax*, predominantly at the trophozoite and schizont stages (Udomsanpetch *et al*, 1995; Chotivanich *et al*, 1998). This phenomenon is also seen in other malaria spp, including the human malaria parasites *P. ovale* and *P. malariae* (Lowe *et al*, 1998), the primate malaria parasites *P. fragile* and *P. coatneyi*, and the rodent malaria parasite, *P. chabaudi* (Udomsangpetch *et al*, 1991; Mackinnon *et al*, 2002). In *P. falciparum*, the major ligand involved in rosette formation is believed to be *P. falciparum*-erythrocyte membrane protein 1 (PfEMP1), which is located on the surface of the parasite-iE; however, homologs of PfEMP1 are not found in *P. vivax* (Rowe *et al*, 1997).

In *P. falciparum*, a protein termed PfSURFIN_{4.2} is expressed on both the surface of the merozoite and the iE (Winter *et al*, 2005). PfSURFIN_{4.2} is a type I transmembrane protein, encoded by one of the members of the surface-associated interspersed (*surf*) multigene family. *P. vivax* possesses a SURFIN ortholog, termed *P. vivax* subtelomeric transmembrane protein 1 (PvSTP1) (del Portillo *et al*, 2001). The N-terminal extracellular regions of both PfSURFIN_{4.2} and PvSTP1 are composed of a moderately conserved cysteine-rich domain (CRD) and a variable region (VAR), and the C-terminal cytoplasmic region (CYT) is composed of semi-conserved triple or single tryptophan-rich domains (WR), respectively (Winter *et al*, 2005). PvSTP1 has at least one paralog in the *P. vivax* genome, which we termed as PvSTP2

(PVX_090285) and collectively we called them PvSTP, hereafter. The extracellular region of PvSTP has homology with the members encoded by *P. vivax* interspersed repeat (*vir*) multigene family, which consists of around 350 members, some of which have been shown to be located on the parasite-iE surface (del Portillo *et al*, 2001). The orthologs of *vir* also are found in primate and rodent malaria parasites and are collectively termed *Plasmodium* interspersed repeat (*pir*) multigene family. The homology between PvSTP extracellular region and *pir* gene products is likely a result of gene expansion of the former to create the latter. Even if this is the case, the fact that PvSTP genes are still retained by the parasite suggests that they are still functional and may play important roles on the parasite-iE, such as in rosette formation or cytoadhesion.

The development of a vaccine against *P. vivax* is urgent. Efforts have focused on, for example, molecules expressed on the merozoite surface (apical membrane antigen-1, AMA1; merozoite surface protein 1, MSP1; and Duffy binding protein, DBP), on sporozoite surface (circumsporozoite protein, CSP), and on oocyst/ookinete surface (Pvs25) (Arévalo-Herrera *et al*, 2010). However, studies of the molecules expressed on *P. vivax*-iE have been limited: only one study was conducted for VIR, a protein product of *vir*, in northern Brazil (Oliveira *et al*, 2006). It is important to evaluate the natural immune response among individuals in vivax malaria endemic areas, to identify potential vaccine target antigens. Therefore, we evaluated the naturally acquired antibody responses against PvSTP1 and -2, using plasma obtained from *P. vivax*-infected patients in the western border of Thailand.

Table 1
Characteristics of the study population.

	Age (years)	Body temperature (°C)	Parasite density (parasites/μl)	Hemoglobin concentration (g/dl)
Median	22	37	8,000	13.8
Range	(13-45)	(35-40)	(40-44,000)	(10.9-17)
Number	24	23	70	22

MATERIALS AND METHODS

Study site and sample collection

Seventy patients, who visited the outpatient clinic of Mae Sot Hospital, Tak Province, Thailand, and were positive for *P. vivax* by microscopic examination of blood smears, were recruited for this study. Patients were treated with chloroquine (2,500 mg) for 3 days and primaquine (15 mg) for 14 days (15 mg of primaquine was given for 8 times in patients with G6PD deficiency). Informed consent was obtained from all adult participants and from parents or legal guardians of minors. Subsequent to obtaining informed consent, 10 ml of blood was drawn into a heparinized tube and centrifuged at 800g for 5 minutes and plasma was collected. Parasite density was determined (Shute, 1988) and *P. vivax* infection was reconfirmed by polymerase chain reaction (PCR)-based diagnosis (Snounou, 1996). Plasma were also collected from 11 volunteers in Thailand who had no history of malaria infection and used as negative control. Only 24 patients allowed collection of the clinical history (age, body temperature, hemoglobin concentration, and past history of malaria infection) by interview and medical records (Table 1). This study was approved by Ethical Review Committee of Mahidol University, No. MUTM.2006-020.

Recombinant protein preparation

The recombinant proteins were produced as described previously (Tsuboi *et al*, 2008). In brief, a DNA linker (5'-AG-ATATCCCCGGGAGGCCTAAGCT-3' and 5'-TAGGCCTCCCGGGGATATC-TAGCT-3') containing *EcoRV*, *SmaI* and *StuI* sites was inserted into the *SacI* site of pEU-E01-HisGST(TEV)-N2, an expression plasmid encoding N-terminal hexa-His-tag and glutathione S transferase (GST)-tag followed by a tobacco etch virus (TEV) protease cleavage site, designed specifically for a wheat germ cell-free protein expression system (CellFree Sciences, Matsuyama, Japan), to yield pEU-HGtevK_ES. A DNA fragment corresponding to nucleotide positions (nt) 4 - 642 of the PvSTP1 gene was PCR-amplified from genomic (g)DNA of *P. vivax* (isolated from a Japanese patient who visited Myanmar in 2005) and ligated into the *EcoRV* site of pEU-HGtevK_ES, to produce a plasmid expressing recombinant protein consisting of 6xHis-GST followed by the N-terminal CRD of PvSTP1 (H-GST-vSTP1-CRD). In a similar manner, DNA fragments corresponding to nt 1608 - 2675 of PvSTP1 (encoding putative cytoplasmic region, CYT), nt 4 - 645 of PvSTP2 (CRD), and nt 1932 - 3203 of PvSTP2 (CYT) were PCR-amplified from gDNA of *P. vivax* Salvador I strain (Sal-I), and were subsequently ligated into *EcoRV* site of pEU-HGtevK_ES,

Table 2
Oligonucleotide used for recombinant protein production.

Protein	Region	Sequence ^a
PvSTP1	CRD	gggTCATTTCAAAATCAATTTAAATTAG gggTCGAATATGTGCTGGAATATG
	CYT	gggACCATTATAGATATACACTTGG gggCTAAATTACATTAATGTTTTGCTT
PvSTP2	CRD	gggGCATACGCAAAAATAATTAATTTTG gggCAAACCTATAGGGAAGATGATG
	CYT	gggGGCTCAAAAACGATTGTAGAG gggTTACCCGTTGTATGTTGCATAA

^aTriple guanine (g) residues were added to the 5' end of each primer to prevent deletions of the 5' and 3' end of the PCR products during the ligation step, in which both EcoRV enzyme and T4 DNA ligase were present at the same time in the tube (Ghoneim *et al*, 2007).

to express the recombinant proteins, H-GST-vSTP1-CYT, H-GST-vSTP2-CRD, and H-GST-vSTP2-CYT, respectively (nt positions are based on Sal-I gDNA sequence). Oligonucleotides, based upon Sal-I sequence, used in the PCR amplification are summarized in Table 2. Recombinant His-GST-fused proteins were absorbed onto a glutathione-Sepharose 4B column, subjected to the TEV protease cleavage to remove N-terminal His-GST region, and eluted with phosphate buffered saline (PBS) to obtain recombinant (r) proteins rPvSTP1-CRD, rPvSTP1-CYT, rPvSTP2-CRD, and rPvSTP2-CYT, respectively (Figs 1 and 2). As a negative control, recombinant His-GST (rGST) was expressed from pEU-HG(tev)N02.

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottom 96-well microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were

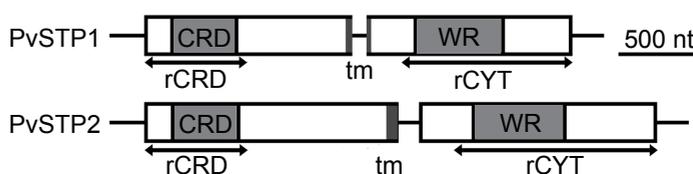


Fig 1—Schematic representation of the exon-intron regions for the genes encoding for PvSTP1 and -2 and domains of the encoded proteins. CRD, tm, and WR indicates cysteine-rich domain, transmembrane region, and tryptophan-rich domain, respectively. Regions used to generate recombinant proteins are indicated by lines with arrow under each scheme. rCRD and rCYT indicates recombinant proteins for CRD and putative cytoplasmic region, respectively.

coated with 50 µl of 1 µg/ml each recombinant protein in carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), each well was blocked with 200 µl of blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) for 30 minutes at 37°C. Fifty microliter aliquots of plasma-containing blocking buffer (1:50 dilution) were added into each well, each plasma sample in triplicate at a single plate, and incubated for 2 hours at 37°C.

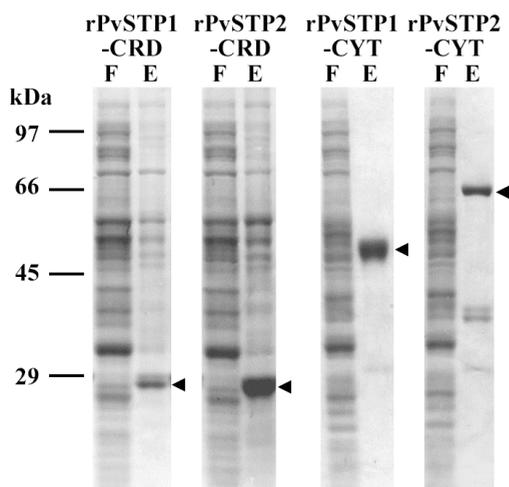


Fig 2—SDS-PAGE analysis of the recombinant PvSTP1 and PvSTP2 proteins. Recombinant proteins were separated on SDS-12.5% polyacrylamide gel-electrophoresis under reducing condition and stained with Coomassie brilliant blue. F and E indicates flow through of the GST-affinity column containing unbound proteins and eluate from the column after TEV protease cleavage, respectively (Tsuboi *et al*, 2008). Arrowheads indicate purified recombinant proteins; CRD, cysteine-rich domain; CYT, cytoplasmic region.

After washing with PBS-T, 50 μ l aliquots of horseradish peroxidase-conjugated goat anti-human IgG (H+L; 1:10,000 dilution; Promega, Madison, WI) were added into each well and the plates were incubated for 1.5 hours at room temperature. After further washing, 50 μ l aliquots of ABTS [2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma, St Louis, MO] were added into each well and the plates were incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 50 μ l/well of 1 M NaF solution (Wako, Osaka, Japan), and absorbance measured using an automated microplate reader (iMark™; Bio-Rad, Hercules, CA) at 405 nm.

Data analysis

The adjusted absorbance value of each sample was obtained by dividing the mean optical density (OD) value of each individual sample by the mean OD value for the 11 malaria non-exposed individuals (baseline). Samples with adjusted absorbance values greater than the baseline absorbance +5 standard deviation (SD) were considered to be positive for antibodies to the antigen tested. Statistical analyses were performed using PASW Statistics 18 software version 11.5 (SPSS, New York, NY). The differences of adjusted absorbance values to each antigen between vivax patients and malaria non-exposed people were assessed by Mann-Whitney *U* test. The correlations between responses to each antigen were analyzed by Spearman's rank test. The correlations of parasite density with adjusted absorbance value to each antigen were analyzed by Spearman's rank test. *P*-values ≤ 0.05 are considered statistically significant.

RESULTS

Antibody responses to rPvSTP1 and -2

The antibody responses against rPvSTP were determined by ELISA for 70 samples collected from *P. vivax*-infected patients. The comparative antibody responses to PvSTP1 and -2 between *P. vivax* patients and malaria non-exposed controls to each PvSTP region were significantly different ($p < 0.01$, Mann-Whitney *U* test) (Fig 3). The percentage of plasma that recognized any of the rPvSTP1 and -2 proteins was 54% (38/70) and 64% (45/70), respectively. The percent positive rates were 40% for rPvSTP1-CRD (28/70), 31% for rPvSTP1-CYT (22/70), 27% for rPvSTP2-CRD (19/70), and 56% for rPvSTP2-CYT (39/70) (Fig 4). All plasma

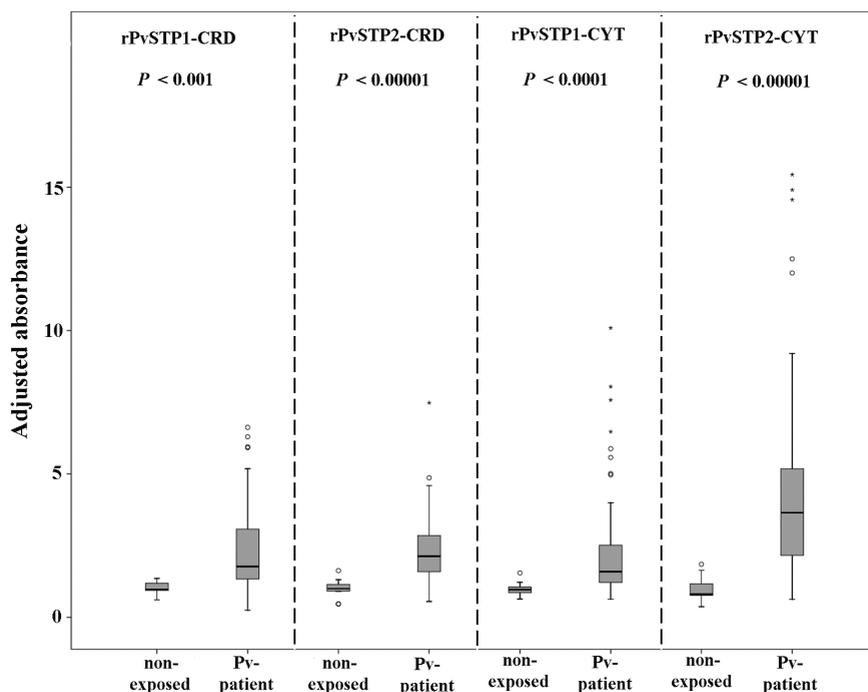


Fig 3—Antibody responses against rPvSTP1 and -2 of *P. vivax*-infected patients' plasma. Values represent adjusted absorbance for each antigen (rPvSTP1-CRD, rPvSTP2-CRD, rPvSTP1-CYT, and rPvSTP2-CYT). Box plots represent median values with 25th and 75th percentiles. Each bar marks the 10th and 90th percentiles. Outlier and extreme values are plotted as stars and circles, respectively. CRD and CYT indicates the cysteine-rich domain and cytoplasmic region, respectively. Differences are assessed by Mann-Whitney *U* test.

samples were negative for rGST (data not shown). Of the patients' plasma, 17% (12/70) and 19% (13/70) reacted positively to both CRD and CYT regions of PvSTP1 and -2, respectively. The adjusted absorbance values against each recombinant PvSTP proteins are shown in Fig 4.

Significant correlations of adjusted absorbance values were observed between anti-PvSTP1 and -2 in each antigen region (CRD: $R = 0.74$, $p < 0.01$; CYT: $R = 0.48$, $p < 0.01$) using Spearman's rank test, suggesting cross-reactivity between anti-PvSTP1 and -2, or a co-exposure of both proteins to host immunity. No significant correlation

was found between antibody response to rPvSTP and parasite density.

Specific antibody response to PvSTP1 and -2 in *P. vivax*-infected patients

To evaluate the degree of cross-reactivity between anti-PvSTP1 and -2, ELISA positive samples were grouped for each antigen region, according to their reactivity against PvSTP1 or -2 (Fig 5). Some patients were positive only for either PvSTP1 or -2, indicating the existence of specific antibodies for either PvSTP1 or -2. More samples were positive only for rPvSTP1-CRD (12 cases) than the samples only positive for rPvSTP2-CRD (3 cases),

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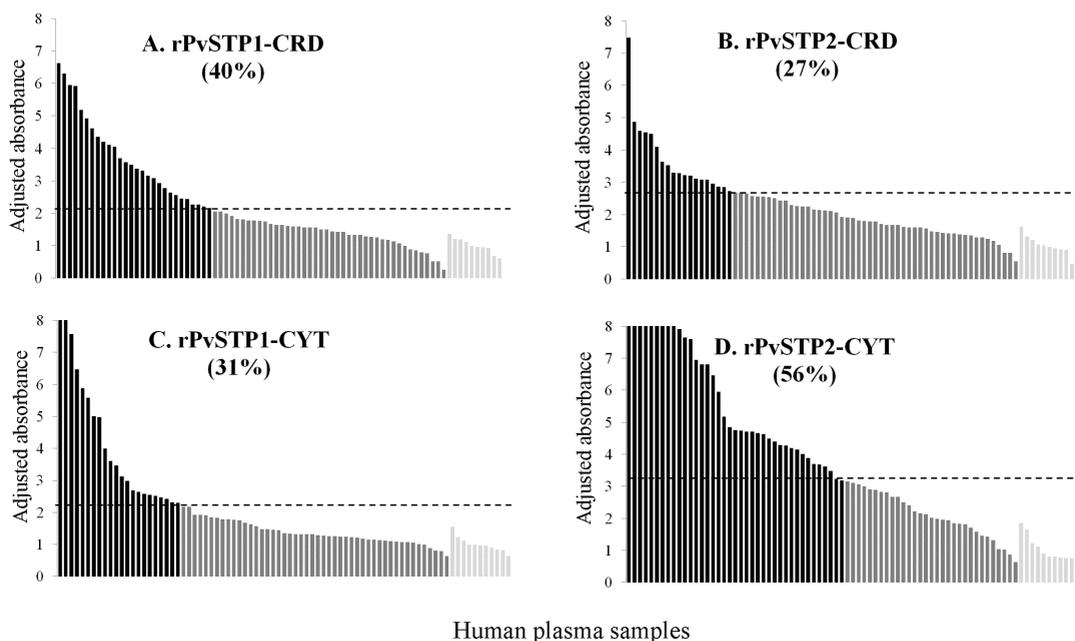


Fig 4—Individual antibody responses to recombinant proteins for PvSTP1 and PvSTP2. (A) rPvSTP1-CRD, (B) rPvSTP2-CRD, (C) rPvSTP1-CYT, and (D) rPvSTP2-CYT. Antibody responses against recombinant proteins are expressed as adjusted absorbance values for *P. vivax*-infected patient plasma in black and gray ($n = 70$) and for malaria non-exposed individuals ($n = 11$) as white. Dashed lines indicate positive cut-off values for each antigen, obtained by calculation of mean value + 5 standard deviation of adjusted absorbance values from the 11 malaria non-exposed individuals in Thailand. Positive rates are shown in parentheses. CRD and CYT, indicates the cysteine-rich domain and cytoplasmic region, respectively.

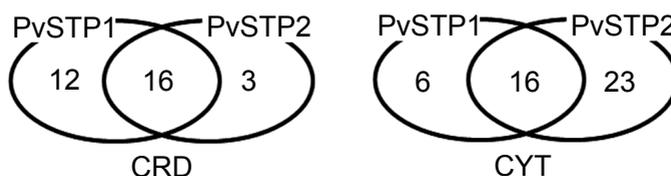


Fig 5—Specific response to rPvSTP1 and -2 in *P. vivax*-infected patients. Numbers indicate individuals positive for antibodies specific to rPvSTP1 or rPvSTP2. CRD and CYT indicates the cysteine-rich domain and cytoplasmic region, respectively.

suggesting that the extracellular region of PvSTP1 was more immunogenic than PvSTP2, or patients were exposed to the extracellular region of PvSTP1 more frequently than to that of PvSTP2. On the

other hand, more samples were positive only for rPvSTP2-CYT (23 cases) than positive only for rPvSTP1-CYT (6 cases), suggesting the PvSTP2 cytoplasmic region was more immunogenic than that of

PvSTP1, or that the PvSTP2 cytoplasmic region was more frequently exposed to the patient's immune system than the PvSTP1 cytoplasmic region.

DISCUSSION

This is the first report showing evidence that PvSTP1 and -2 are immunogenic in natural infection. Our results suggest that both PvSTP1 and -2 elicit specific antibodies. Proteins on the surface of malaria iEs have been a major focus of malaria research because of their role in pathogenesis and their potential as targets for immunity and for vaccine development. In *P. vivax*, however, only VIR family proteins have been studied (del Portillo *et al*, 2001). In a malaria-endemic area in Brazil, 49.0% of vivax malaria patients possessed antibodies (IgG or IgM) against at least one of 4 distinct types of VIR protein; however, IgG response against each VIR type proteins was lower (2.0 - 17.5%) (Oliveira *et al*, 2006). In the same study, the positive rate of IgG antibodies against two *P. vivax* merozoite proteins, PvAMA1 and PvMSP1-19kD region, were found to be 57% and 90.5%, respectively. Thus, it was concluded that the prevalence of antibodies against each VIR was much lower than those against PvAMA1 and PvMSP1-19kDa. In this study, we evaluated antibody responses (IgG or IgM) against PvSTP1 and -2 in plasma of *P. vivax*-infected patients living in endemic area of Thailand and found that 44% of patients produced antibodies against the extracellular region of rPvSTP1 or -2 (40% for rPvSTP1 and 27% for rPvSTP2) a percentage similar to that previously recorded for the VIR family proteins (Oliveira *et al*, 2006).

Initially, we expected that the extracellular CRD region, would be more

immunogenic than the cytoplasmic CYT region, but our results showed that this was not the case for PvSTP2, in which the CYT region was more immunogenic than the CRD region. A possible explanation is that the recombinant PvSTP2 cytoplasmic region used in this study may contain epitope(s) that induce high levels of antibody production.

In summary, the presence of antibodies in Thai *P. vivax*-infected patients to recombinant proteins corresponding to *P. vivax* subtelomeric transmembrane protein indicate that PvSTP1 and -2 are naturally immunogenic. The characteristics of these proteins need to be further investigated.

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