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# *In vivo* formation of *Plasmodium falciparum* ribosomal stalk — A unique mode of assembly without stable heterodimeric intermediates ☆

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ABSTRACT

*Background:* The ribosomal stalk composed of P-proteins constitutes a structure on the large ribosomal particle responsible for recruitment of translation factors and stimulation of factor-dependent GTP hydrolysis during translation. The main components of the stalk are P-proteins, which form a pentamer. Despite the conserved basic function of the stalk, the P-proteins do not form a uniform entity, displaying heterogeneity in the primary structure across the eukaryotic lineage. The P-proteins from protozoan parasites are among the most evolutionarily divergent stalk proteins.
*Methods:* We have assembled P-stalk complex of *Plasmodium falciparum in vivo* in bacterial system using tricistronic expression cassette and provided its characteristics by biochemical and biophysical methods.
*Results:* All three individual P-proteins, namely uL10/P0, P1 and P2, are indispensable for acquisition of a stable structure of the P stalk complex and the pentameric uL10/P0-$(P1\text{-}P2)_2$ form represents the most favorable architecture for parasite P-proteins.
*Conclusion:* The formation of *P. falciparum* P-stalk is driven by trilateral interaction between individual elements which represents unique mode of assembling, without stable P1–P2 heterodimeric intermediate.
*General significance:* On the basis of our mass-spectrometry analysis supported by the bacterial two-hybrid assay and biophysical analyses, a unique pathway of the parasite stalk assembling has been proposed. We suggest that the absence of P1/P2 heterodimer, and the formation of a stable pentamer in the presence of all three proteins, indicate a one-step formation to be the main pathway for the vital ribosomal stalk assembly, whereas the P2 homo-oligomer may represent an off-pathway product with physiologically important nonribosomal role.


## 1. Introduction

Ribosome represents a highly conserved entity among all domains of life in respect to its basic structure and function. The evolutional development of this translational machine in eukaryotes is usually attributed to regulatory regions, leaving the core structure intact. The changes are associated with extension of already existing ribosomal proteins and addition of new proteins accompanied with expansion of rRNA segments, forming a unique intertwined RNA-protein layer [1]. This development is very pronounced on the 60S ribosomal subunit, especially within the P and L1 stalks [2]. The P-stalk belongs to the GTPase Associated Center (GAC) responsible for binding and activation of translational factors [3–5]. This structure consists of P-proteins, which form a pentameric complex composed of a single conserved uL10 protein — according to new nomenclature, the former name P0 [6], and two eukaryotic specific P1–P2 heterodimers, in short uL10-$(P1\text{–}P2)_2$ [7–9]. P1–P2 heterodimers are bound to the well-defined, specific α-helical regions located on the eukaryote specific C-terminal domain of the uL10 protein [10,11]. Among ribosomal components, the P-proteins constitute a significantly heterogeneous group of proteins. P1 and P2 proteins existing in higher eukaryotes display further expansion into a group of four (P1A, P1B, P2A and P2B) in the yeast cells and five (additional P3 protein) in plants [4,5]. Moreover, as it was shown recently, the yeast P1-P2 heterodimers play unequal roles in respect to interaction with external protein factors, underscoring that P-proteins within the stalk structure underwent

*Abbreviations:* NAI, naturally acquired immunity; GAC, GTPase Associated Center; SEC, Size-exclusion chromatography; CD, circular dichroism; BACTH, bacterial two-hybrid system; MS, mass spectrometry; MW, molecular weight; Da, daltons.

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structural and functional differentiation [12]. It is considered that the primary function of the stalk is recruitment of translational GTPases and stimulation of factor-dependent GTP hydrolysis [13–15]. However, it is postulated that the P-stalk, especially fluctuating composition of the P-proteins, may also regulate expression of genetic information at the translational level [16,17], therefore placing the stalk as a regulatory element on the 60S ribosomal subunit.

Despite the role directly connected with translation, ribosomal P-stalk proteins were defined as moonlighting proteins [18] exhibiting extra-ribosomal functions involved in several cellular processes such as DNA repair [19], mRNA redistribution [20], tumorigenesis [21,22] and apoptosis [23]. The P-proteins have also been recognized as an antigen and specific pathogenic factor in systemic lupus erythematosus [24], an *Alternaria alternata*, *Cladosporium herbarum*, and *Aspergillus fumigates* allergen [25], and an antigen of *Trypanosoma cruzi* infection [26]. A very interesting feature of P-proteins is their presence on the surface of yeast, mammalian, and protozoan cells [23,27,28]. In particular, uL10/P0 has been localized on the *Plasmodium* sp. cell wall at all major life cycle stages [27], whereas the P2 homo-oligomeric complex is exported to the surface of infected red blood cells at an early stage of the parasite development [29]. The existence of individual stalk proteins during the parasite life cycle indicates that their molecular behavior departs from the general view of P-proteins from other species, showing their uniqueness in this respect.

In this work, we provide an insight into the complex architecture of the *Plasmodium falciparum* P-proteins, showing an intricate interplay among its particular elements. In the course of the study, we have reconstructed *in vivo* the whole *Plasmodium* stalk, using an approach based on the polycistronic expression system in *Escherichia coli*. Our analysis has demonstrated that the pentameric organization of the *Plasmodium* stalk proteins represents the most stable form. We have shown that unlike in other tested eukaryotes, stalk formation is the result of simultaneous trilateral interactions between P1, P2, and uL10/P0 with no sign of heterodimeric intermediates, underscoring the fact that all the three elements are indispensable for stable structure acquisition. P2-homo-oligomers may represent biologically relevant off-pathway species. The study revealed the unique path of *P. falciparum* ribosomal stalk assembling, giving foundation for the explanation of unusual biological phenomena attributed to parasite P-stalk proteins.

## 2. Experimental procedures

### 2.1. Genetic manipulations

DNA fragments carrying genes for the full length uL10/P0, P1, and P2 proteins were synthesized based on the sequences deposited in the *P. falciparum* 3D7 genome database (www.genedb.org/Homepage/Pfalciparum) under accession numbers PF11_0313, PF11_0043, and PFC0400w for the uL10/P0, P1, and P2 proteins, respectively [30]. For all genetic manipulations, the DNA containing the genes of interest was PCR amplified using specific primers. For expression of individual proteins, amplified DNA fragments were introduced into the pT7-7 vector using specific restriction sites EcoRI/BamHI. In all the cases, the DNA sequence encoding the 6xHis-tag was introduced at the 5′-end of the genes, which resulted in fusion of the tag at the N-terminal part of the protein. The bicistronic expression cassette was constructed using the pT7-7 vector, where DNA carrying genes for the P1 and P2 proteins was PCR amplified and introduced into the vector, using specific restriction EcoRI/BamHI sites for P1 and BamH1/SalI for P2. The autonomous ribosome-binding site (RBS) and 7-nucleotide long DNA spacer sequence were introduced at the non-coding region between two genes to ensure efficient expression of the P2 protein. The 6× His-tag was located either on the N-termini of P1 or the C-termini of the P2 protein.

The tricistronic expression cassette was constructed by consecutive sub-cloning of the genes for the ΔuL10/ΔP0 fragment (amino acids 197–316) and full length P1/P2 proteins into the pGEX4T-1 expression vector. DNA for the ΔuL10/ΔP0 fragment was PCR amplified using specific primers, cleaved by BamHI/EcoRI restriction nucleases, and cloned in frame with the gene for the GST-tag protein, where a specific thrombin cleavage site is present between both proteins. DNA coding sequences for the P1 and P2 proteins were amplified with the aid of PCR as well, and subsequently cloned into the pGEX4T-1-ΔuL10/$\Delta P0_{197–316}$ genetic construct using unique restriction sites EcoRI/SalI and SalI/NotI for P1 and P2, respectively. Efficient expression of the GST-ΔuL10/$\Delta P0_{197–316}$ was facilitated by plasmid-borne regulatory sequences. However, in the case of genes for the P1 and P2 proteins, the RBS and spacer sequences were introduced as described above for the bicistronic system to improve expression. All the genetic constructs were verified by DNA sequencing.

### 2.2. Protein expression and purification

All recombinant proteins were expressed or co-expressed in *E. coli* strain BL21(DE3) cells (Stratagene). Recombinant uL10/P0 or P1 proteins were purified by affinity chromatography on the Ni-column (Sigma-Aldrich) in denaturing conditions, following the manufacturer's procedure. The recombinant P2 protein and the P1–P2 dimer obtained by co-expression from the bicistronic system were purified by affinity chromatography on the Ni-agarose column (Sigma-Aldrich) in native conditions, according to the manufacturer's procedure. All proteins were stored in 50 mM Tris–HCl buffer pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2$. The recombinant proteins ΔuL10/$\Delta P0_{197–317}$-P1–P2 were co-expressed using the tricistronic system and purified by affinity chromatography on the GST-trap column (Sigma-Aldrich), according to the manufacturer's instructions. There was one variation, *i.e.* the column bound recombinant proteins GST-ΔuL10/$\Delta P0_{197–317}$-P1–P2 were treated with human thrombin (Sigma-Aldrich) at a concentration of 20 units/ml of GST-trap resin in 37 °C for 1 h to cleave off the deletion form of ΔuL10/$\Delta P0_{197–317}$.

### 2.3. Bacterial two-hybrid experiment

We used a bacterial two-hybrid system based on a reconstituted signal transduction pathway, using complementary fragments T25 and T18 that constitute the catalytic domain of *Bordetella pertussis* adenylate cyclase. Association of the two-hybrid proteins results in functional complementation between the T25 and T18 fragments and leads to cAMP synthesis, and this in turn activates the reporter gene [31]. DNA carrying genes for all the individual *P. falciparum* P proteins, P1, P2, and the ΔuL10/ΔP0 fragment (amino acids residues 197–316) as well as yeast P1 (P1A and P1B), P2 (P2A and P2B), and the ΔuL10/ΔP0 fragment (amino acids residues 199–312) were PCR amplified using sets of specific primers. All amplified DNA fragments were sub-cloned into pUT18 or pUT18C and pKNT25 or pKT25. In the case of the DNA fragments for the P1 and P2 proteins, DNA sequences were cloned into pUT18/pUT18C vectors using SalI/BamHI unique restriction sites and into pKNT25/pKT25 vector using BamHI/EcoRI sites. The ΔuL10/ΔP0 protein fragment was cloned into the pUT18 vector using SalI/EcoRI specific restriction sites. Further, a bicistronic expression cassette was constructed, comprising genes for the ΔuL10/ΔP0 protein and a gene for the P1 (P1A or P1B for yeast) protein fused in frame at the N-terminus of the T18 adenylate cyclase fragment. Both genes in the bicistronic cassette were cloned into the pUT18 vector using unique restriction sites, SalI/BamHI and BamHI/EcoRI for ΔuL10/ΔP0 and P1, respectively. Efficient expression of both proteins is facilitated by the plasmid-borne regulatory sequence for the ΔuL10/ΔP0 fragment and by the RBS and spacer sequence for the P1 fusion protein, as described above for the bicistronic expression of P-proteins. Schematic representation of the expression cassettes used for the trilateral interactions analyses are present in Fig. 1. The analysis was done in a way in which vectors expressing the tested proteins fused to adenylate cyclase fragments were transformed into *E. coli BTH101* reporter cells. Transformants were grown on LB medium supplemented with ampicillin (50 μg/ml) and