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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

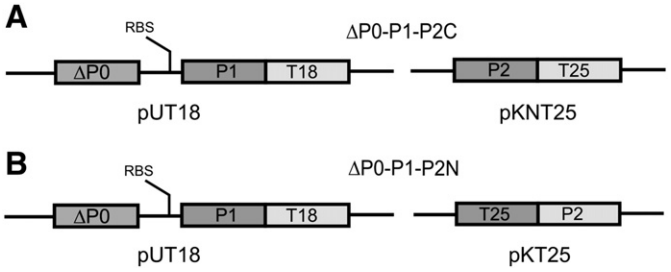

Fig. 1. Schematic representation of expression cassettes used for uL10/P0–P1–P2 trilateral interaction study in bacterial two hybrid experiment. A — P2 protein expressed from pKNT25 vector as a C-terminally tagged protein. B — P2 protein expressed from pKT25 vector as a C-terminally tagged protein.

kanamycin (25 μg/ml) to select double transformants. Then, the cells were transferred onto medium supplemented with X-Gal (Fermentas) and incubated for 48 h at two different temperatures 30 °C and 37 °C. As a positive control, we used a pair of pKT25-zip and pUT18-zip vectors, in which leucine zippers from the GCN4 protein were cloned in frame with adenylate cyclase fragments. A β-galactosidase activity assay was performed on permeabilized cells using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate.

2.4. Biophysical analyses

Size-exclusion chromatography (SEC) was performed on an Akta Purifier FPLC system from GE Healthcare Life Sciences equipped with the Superose 12 HR 10/30 FPLC gel filtration column. For native conditions, the column was equilibrated with a buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, and 10 mM $MgCl_2$; for denaturating conditions, the column was equilibrated with the aforementioned buffer supplemented with 8 M urea and the protein sample was equilibrated for 12 h in the denaturating buffer. The flow-rate was 0.2 ml/min. The protein elution profile was monitored at 280 nm and analyzed using UNICORN program v. 4.0 supplied with the FPLC system. Used molecular markers for SEC: Thyroglobulin (bovine) 670,000 Da, elution volume — 8,39 ml; γ-globulin (bovine) 158,000 Da, elution volume — 11,28 ml; ovalbumin (chicken) 44,000 Da, elution volume — 12,95 ml; myoglobin (horse) 17,000 Da, elution volume — 14,61 ml; vitamin B12 1,350 Da, elution volume — 18,81 ml.

Circular dichroism spectroscopy (CD) spectra were collected on a Jasco J-715 spectropolarimeter equipped with a PFD-350S Peltier-type thermostatic cell holder. The CD melting temperature curves were determined by monitoring changes in the ellipticity intensity at 222 nm as a function of temperature. Thermal denaturation experiments were performed in the range of 20–90 °C at a heating rate of 0.58 °C/min. Thermal scans were collected using 1 mm cells at a protein concentration of 5 mg/ml. Chemical unfolding of protein samples was performed at various concentrations of urea or guanidine hydrochloride buffered in 10 mM Tris–HCl pH 7.5; the samples were equilibrated for at least 12 h at 21 °C. The transitions were monitored by recording the CD signal at 222 nm.

2.5. Mass spectrometry

The complex was analyzed using a high mass Q-TOF-type instrument adapted for a QSTAR XL platform (MDS Sciex) [32]. The low frequency extended mass range of the second quadrupole permits isolation of ions up to 35,000 m/z. For the analysis, the protein solutions were buffer-exchanged into 200 mM ammonium acetate (pH 7.5) using Micro Bio-Spin chromatography columns (Bio-Rad) and introduced into a mass spectrometer *via* nanoflow capillaries to acquire spectra of the native ribosomal stalk. The composition of the *P. falciparum* stalk complex was identified by tandem MS. We isolated defined m/z values in the quadrupole, corresponding to a single charge state of the stalk complex and applied stepwise increasing collision energy (30–80 V) to disrupt the complex and induce dissociation of individual proteins.

3. Results

3.1. P. falciparum P1 and P2 proteins do not form a stable heterodimer

To understand the molecular architecture of the parasite stalk complex, first we attempted to characterize the P1/P2 interplay. We cloned, overexpressed, and purified individual P1, P2 and the deletion form of uL10/P0 (ΔuL10/$\Delta P0_{127-316}$) proteins from *P. falciparum* using the *E. coli* heterologous system based on the pT7-7 vector. All the proteins were efficiently expressed; however, the uL10/P0 and P1 proteins were located in the insoluble protein fraction as inclusion bodies. uL10/P0 and P1 were purified using Ni-affinity chromatography in denaturing conditions, and subsequently the proteins were refolded in water-based buffer. Analysis using SEC in native conditions showed that the uL10/P0 and P1 proteins were eluted as a single peak in the void volume of the column, indicating that purified proteins exist in the oligomeric state with molecular masses in the range of hundreds of kDa, exceeding the resolution of the SEC column. On the contrary, the P2 protein was expressed in the soluble protein fraction and the SEC analysis showed several peaks in the range of 120–40 kDa, demonstrating that it exists in oligomeric forms with lower molecular masses than those of the P1 and uL10/P0 proteins (data not shown). It should be pointed out that the behavior of parasite P-proteins resembles that found in other species, where individual P-proteins produced in a heterologous system tend to form oligomers [33–36]. However, as it was shown previously in yeast, human, and other organisms [8,35,37–39], P-protein folding is a cooperative event, and the P1–P2 dimer is the least, while the uL10/P0-$(P1–P2)_2$ pentamer the most favorable stable entity of P-proteins. Therefore, we attempted to form a P1–P2 protein complex. Surprisingly, the *in vitro* method developed previously based on the denaturation/renaturation procedure of P1 and P2 proteins [40] was found unsuccessful, and the hetero-dimer was not reconstituted. SEC analysis showed that the P1 protein did not stay in the solution, and was found as a high molecular mass oligomer. On the other hand, the P2 protein was soluble, however was present in homo-oligomeric forms (Supplementary figure, Fig. S1). Several buffer conditions (in the presence and absence of reducing agents) and refolding strategies (continuous or step-wise dialysis) were tested to rule out methodological problems. In any case we saw no improvement in the solubility or formation of the complex. Thus, we used the *in vivo* bicistronic expression system for co-expression of both P1 and P2 proteins in *E. coli*. The 6× His-tag was attached to one of the co-expressed proteins, enabling affinity purification of the complex. To rule out the possibility that the 6× His-tag position may affect protein–protein interactions, we fused 6× His-tag either to the N-terminus of the P1 protein or to the C-terminus of P2 (Fig. 2). Co-expression of two proteins (regardless of the 6× His-tag position) resulted in partial solubilization of the P1 protein, indicating that P2 exerted a positive effect on P1 folding (Fig. 2, A and B). Especially, expression with the 6× His-tag placed on the C-terminus of P2 resulted in higher efficiency of P1/P2 expression and the purification was more effective. However, the P1–P2 complex was unstable in solution, and over time the P1 protein was found as a high molecular mass oligomers undergoing precipitation, whereas P2 remained soluble. The SEC analysis of the purified P1/P2 proteins revealed high heterogeneity of the P1–P2 complex (Fig. 2C), showing several oligomeric forms. Optimization trials were launched to enhance both expression and proper folding of analyzed proteins, however we did not see improvement in any specific conditions tested. These included expression in various bacterial strains, induction with various IPTG concentrations as well as application of osmotic stress with sorbitol or heat shock to induce chaperone synthesis.

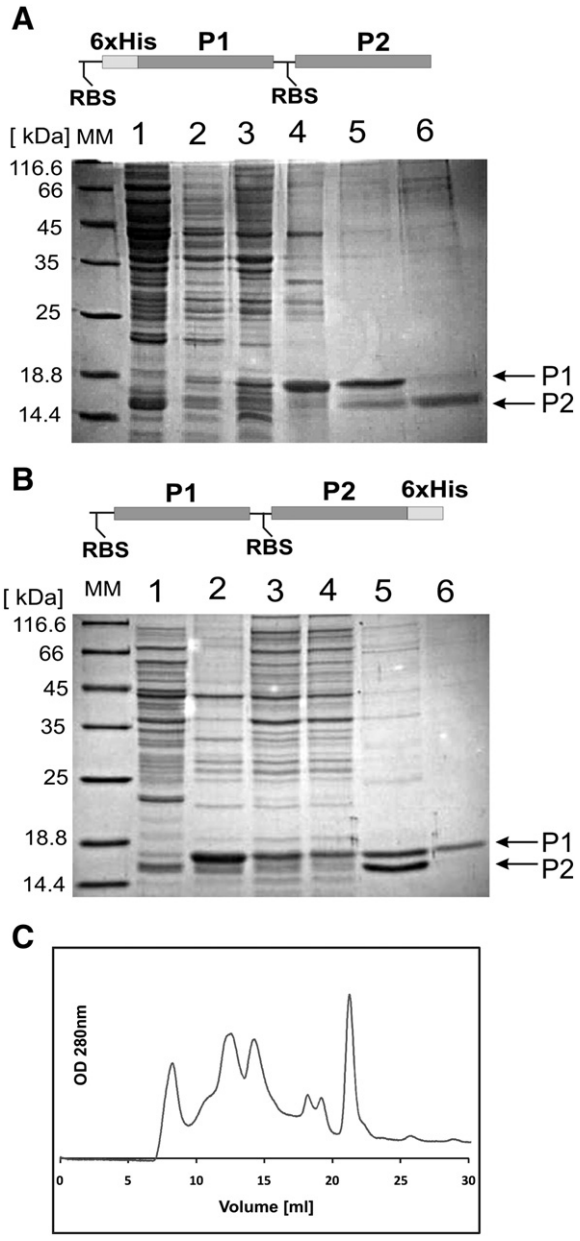

Fig. 2. SDS-PAGE analysis of co-expression of *Plasmodium falciparum* P1 and P2 proteins. A — Bicistronic expression of 6×His-P1 and P2 proteins. B — Bicistronic expression of P1 and P2-6×His proteins. Upper panels, schematic representation of the bicistronic cassette used for expression, RBS — position of the ribosome-binding site. Line MM, molecular mass standards; Line 1 and 2, whole cell extract from *Escherichia coli* with bicistronic cassette, without and with IPTG expression induction, respectively; Line 3, and 4, soluble and insoluble protein fractions, respectively; Line 5, imidazole-eluted fraction from the Ni-column; Line 6, protein fraction eluted with 8 M urea from the Ni-column. C — size exclusion-chromatography of the purified P1–P2-6×His fraction.

3.2. uL10/P0 protein is crucial for P1–P2 heterodimer formation

The low stability of P1/P2 complex may suggest that assembling the *P. falciparum* ribosomal stalk might be driven by a different mechanism than that in other eukaryotes studied. To answer the question about the *P. falciparum* mode of P-proteins assembling, we used a bacterial two-hybrid system (BACTH) to analyze the interplay among the uL10/P0, P1, and P2 proteins. A set of genetic constructs was prepared, where genes for the P1 and P2 proteins were fused with genes for the reporter protein (adenylate cyclase fragments) being the integrated part of the bacterial two-hybrid system [31]. In the case of the uL10/P0 protein, we used a deletion form of uL10/P0, the so-called P-domain, which contains binding sites for the P1–P2 heterodimer (amino-acid residues 197–316, defined *in silico* on the basis of the sequence similarity to the yeast uL10/P0 protein). No interactions were observed while analyzing the bilateral interplay among the uL10/P0, P1, and P2 proteins; unexpectedly, unlike in other organisms, we detected no stable interactions between P1 and P2, which confirm our *in vitro* observations (Fig. 3A and B). Based on previous work, which has shown that the uL10/P0 protein is regarded as a scaffolding protein, we used the BACTH system, modified to allow tracing the tri-lateral interactions among uL10/P0, P1 and P2 to reveal the mode of stalk assembling. The system was adapted to analyze the P1/P2 interactions in the presence of uL10/P0. A bicistronic expression cassette was used for simultaneous expression of the ΔuL10/ΔP0$_{197\text{-}316}$ fragment (hereafter referred to as ΔuL10/P0) and the P1 protein in fusion with an adenylate-cyclase fragment, while expression of P2, fused with another part of the enzyme, was driven from another vector (see Fig. 1 Material and Method paragraph). Simultaneous expression of those three stalk elements restored β-galactosidase activity, indicating that the interaction between P1 and P2 was strongly stimulated by ΔuL10/ΔP0 (Fig. 3).

Notably, ΔuL10/ΔP0 did not stimulate the P1–P1 or P2–P2 interactions (Fig. 3, ΔP0–P1 and ΔP0–P2). Therefore, in contrast to other species, where heterodimers were observed as stable intermediates, all the three elements are required simultaneously for a stable complex to form. For comparison, we analyzed the yeast P-protein interactome in an analogous experiment using BACTH system. We detected a positive signal of interactions between yeast P1A–P2B and P1B–P2A proteins, as it was reported previously [40,41]. Interestingly, while analyzing tri-lateral interactions among yeast P-proteins we noticed a stimulatory effect of the uL10/P0 protein on the formation of the P1B–P2A heterodimer exclusively, resembling the behavior of parasite proteins (Fig. 3C).

3.3. In vivo formation of the P. falciparum ribosomal stalk structure in the heterologous bacterial system

Considering the fact that the uL10/P0 protein constitutes an important element for stalk assembling, we first made an attempt to reconstruct the stalk using the denaturation/renaturation approach. This *in vitro* method was inefficient and recombinant proteins did not form a stable complex; similarly to the P1/P2 renaturation experiment, a major part of the ΔuL10/P0$_{127\text{–}316}$ fragment and P1 protein precipitated, whereas P2 refolded into several oligomeric forms. Since, the *in vitro* approach was unproductive, we developed a novel system for simultaneous expression of the *P. falciparum* uL10/P0, P1, and P2 proteins in *E. coli*. It should be noted that expression of three full length P-proteins would result in reconstitution of a functional *P. falciparum* ribosomal stalk, loading of which onto the bacterial ribosome could be toxic to the bacterial cells [42,43]. To bypass that obstacle, we constructed a tricistronic expression cassette comprising genes for the ΔuL10/ΔP0 fragment (devoid of the conserved ribosome binding domain, with an intact C-terminal domain), and full-length P1 and P2 (Fig. 4A). Additionally, the gene for ΔuL10/ΔP0 was fused in frame with the DNA sequence encoding GST-tag. The three stalk-proteins were efficiently expressed in a soluble form (Fig. 4B) and were co-purified by single-step affinity chromatography in native conditions. Subsequently, the GST-tag was removed by thrombin cleavage. The pure complex behaved as a homogenous species in SEC and in native-PAGE analyses (Fig. 5), resembling a natively isolated yeast stalk complex [8,10].

3.4. P. falciparum stalk proteins assemble as a pentameric complex uL10/P0-(P1–P2)$_2$

We addressed the stoichiometry of the recombinant *P. falciparum* stalk complex composed of uL10/P0–P1/P2 proteins by nano-electrospray mass spectrometry in non-denaturing conditions. This approach allows maintaining non-covalent interactions between proteins and

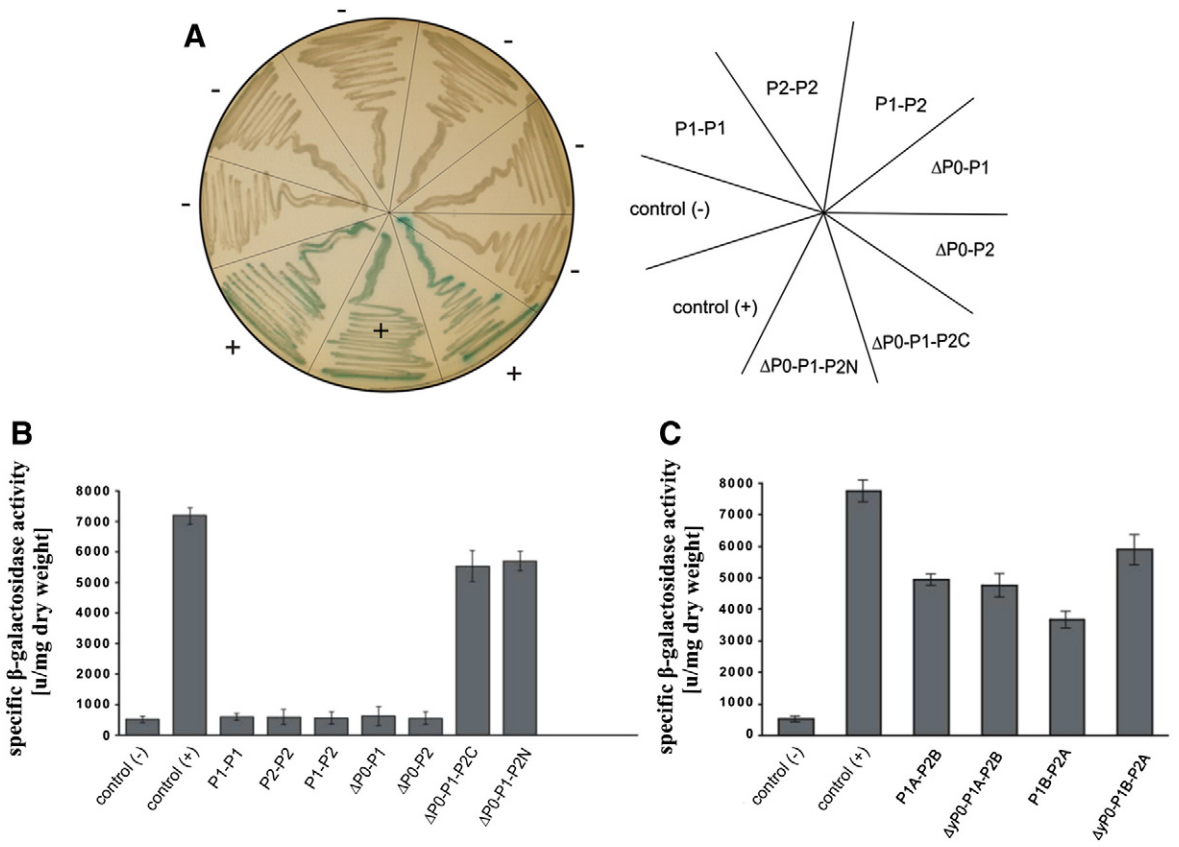

Fig. 3. Bacterial two-hybrid analysis of P-protein interactions. A — Growth of *E. coli* cells transformed with respective vectors on the plate with LB medium supplemented with X-gal. The schemes on the right show the experimental set-ups. B — Quantitative analysis of the interaction between the *P. falciparum* P-proteins expressed as units of β-galactosidase. C — Quantitative analysis of the yeast P-protein interactions, using P1A, P1B, P2A, P2B, and yeast (y) uL10/P0 proteins. Positive control, leucine zippers from the GCN4 protein; negative control, empty vectors.

determining the exact molecular mass of the intact stalk complex. Further, it allows measurement of the masses of individual protein components, sequentially dissociated from the complex under increased collision energy conditions. The measured mass spectrum of the *P. falciparum* stalk complex showed a series of peaks corresponding to the mass of 63,280 Da (Fig. 6, lower panel and Table 1), which is in good agreement with the theoretical mass of the pentamer (63,118 Da). Some increase in mass is expected due to buffer ions and water molecules retained by the complex [44]. To confirm the composition of the stalk complex, the quadrupole 19+ charge state of the complex was isolated and subsequently applied to increasing collision energy to induce stalk dissociation (Supplementary figure, Fig. S2). This yielded two series of products: the individual dissociated P-proteins at 1400–2100 m/z and corresponding "stripped" stalk complexes at 4000–5000 m/z (Fig. 5 upper panel, Table 1). The measured masses of the individual P-proteins agree very well with their theoretical masses (Table 1). Notably, in the MS spectrum, the P1 peaks were of considerably higher intensity than the P2 series, which implies that P1 dissociated more readily from the stalk complex than P2, resulting in appearance of a ΔuL10/P0-P1-$(P2)_2$ "stripped" species (Fig. 6 upper panel); in similar yeast stalk analysis, ΔuL10/P0–$(P1)_2$–P2 complexes were observed instead [8]. It is noteworthy that no P1–P2 heterodimers were detected, supporting our previous data, which excluded formation of a stable heterodimer.

3.5. Denaturation of the ΔuL10/P0–$(P1–P2)_2$ complex is fully cooperative and follows a two-state transition mechanism

The stability of the pentameric complex was analyzed in solution by far-UV CD. First, we traced the temperature-induced complex unfolding. The thermal denaturation curve shows a fully cooperative unfolding behavior, which is consistent with the two-step unfolding model (Fig. 5, inset A). The transition midpoint for the thermal denaturation was estimated as 54.4 °C. In addition, the stability of the complex was analyzed in a chemically induced unfolding experiment, as a function of urea or Gdn-HCl concentration. The chemical denaturation curves describe a fully cooperative unfolding process similar to that observed previously for a native yeast stalk complex, consistent with the two-step unfolding model (Fig. 5, inset A). The chemical denaturation transition mid-points were determined as 2.36 M and 3.17 M for Gdn-HCl and urea, respectively. Again, we observed no stable folding intermediates during both thermal and chemical denaturation. Based on the presented data, we conclude that the *P. falciparum* ribosomal stalk is assembled through a simultaneous cooperative interaction among all the three stalk-proteins without an intermediate heterodimeric form observed in other species.

4. Discussion

The canonical form of P-proteins is the pentameric complex uL10/P0–$(P1–P2)_2$, which is a part of the GTPase Associated Center on the large ribosomal subunit. In spite of general structural and functional resemblance of P-proteins among all eukaryotes, there are minute/subtle differences that make the P-proteins a species-specific element of the 60S ribosomal subunit. Although it has been reported that the P-proteins exist mainly in the pentameric form [8,45], recent reports indicate that the P2 proteins may exist as individual oligomers in *P. falciparum,* indicating their unique physiological role [27,46]. This phenomenon is further supported by the fact that protozoan P-proteins constitute separate and evolutionary most divergent branch on the phylogenetic tree, underscoring their molecular distinction. In order to understand this unique group of proteins, we have taken an integral approach to study the cross-talk among all *P. falciparum* P-stalk proteins. The molecular behavior of *P. falciparum* proteins resembles the properties of P-proteins

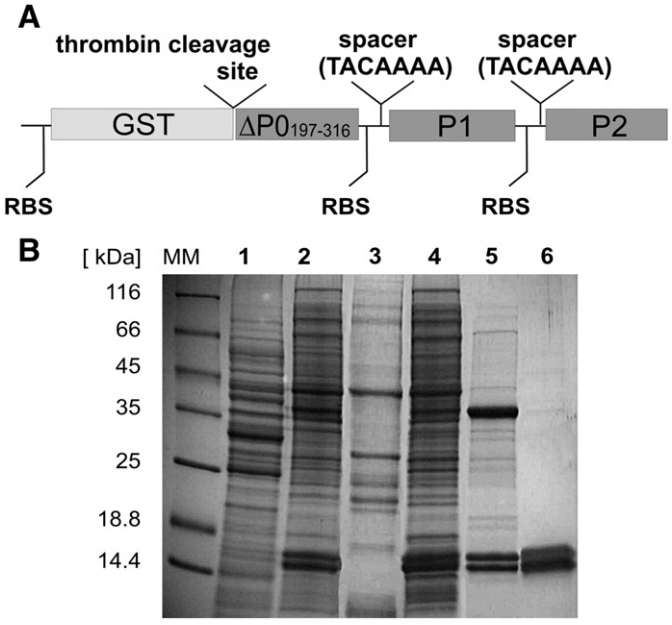

Fig. 4. Co-expression of the P-proteins using a tricistronic expression cassette. A — Schematic representation of the genetic cassette used for co-expression of all P-proteins; GST — glutathione S-transferase tag; RBS — introduced ribosome binding site; spacer and thrombin cleavage site are also indicated. B — SDS-PAGE analysis of protein fractions obtained during consecutive steps of complex purification. MM — molecular mass markers; Line 1 and 2, whole cell extract from *E. coli* with the expression cassette, without and with IPTG expression induction; Line 3, and 4, insoluble and soluble protein fractions, respectively; Line 5, GST-ΔuL10/$\Delta P0_{197-316}$-P1–P2 fraction eluted from the glutathione-sepharose-column; Line 6, protein fraction after cleavage of the GST-tag with thrombin from the ΔuL10/$\Delta P0_{197-316}$-P1–P2 complex.

from other species, where individual P-proteins are present in various oligomeric forms *in vitro* [37,40,47]. Recently, a P2 homo-oligomer was found as a biologically relevant species in *P. falciparum* [48,49], suggesting that it may represent one of the functional forms of P2 in some stages of parasite development. Although the parasite individual P-proteins in general reflect the behavior of stalk proteins from other classes of organisms, the reconstruction of the P1–P2 or uL10/P0–P1–P2 complexes using a previously developed *in vitro* denaturation/renaturation procedure for the P-proteins was found to be unsuccessful. In contrast, it was shown in several reports that in yeast [40,50], human [37], or silkworm [51] heterodimers and pentamers were spontaneously formed in analogous experiments. Our surprising observation got further confirmation in the results of the bacterial two-hybrid experiment. This system was used instead of the commonly used yeast approach to avoid the influence of endogenous yeast P-proteins potentially influencing the cross-talk between analyzed P-proteins [52]. In the BACTH approach, we detected no bilateral interactions between individual *P. falciparum* P-proteins. These observations are seemingly inconsistent with the expression trials where oligomeric forms of the P2 proteins were detected. A possible explanation for such discrepancy is that restoration of the adenylate cyclase active form is spatially forbidden by P2/P2 high molecular mass oligomers. In analogous conditions, both P1A/P2B and P1B/P2A yeast pairs gave a positive interaction signal. Our results indicate that although *P. falciparum* P-proteins are able to interact with each other, they do not form stable hetero-dimeric P1–P2 forms. Co-expression of all parasite P-proteins led to the development of strong β-galactosidase activity, showing that uL10/P0 is indispensable for the stable P1–P2 interaction. On the other hand, introduction of the uL10/P0 protein into the yeast P1/P2 experimental system resulted in further stabilization of the P1B–P2A, but not P1A–P2B association, underlying differences between *Plasmodium* sp. and yeast P-proteins complex assembling.

Since the simultaneous expression of the uL10/P0, P1 and P2 proteins is necessary for a stable P1–P2 interaction and, consequently, P-stalk assembly, we developed a co-expression system of all the P-proteins in bacterial cells using a tricistronic expression cassette. We used full length P1, P2 and the deletion form of the uL10/P0 protein containing a C-terminal P-domain. The rRNA domain of uL10/P0 is highly homologous across life domains, therefore the full length uL10/P0 protein associated with P1 and P2 was found to be able to load onto bacterial ribosome,

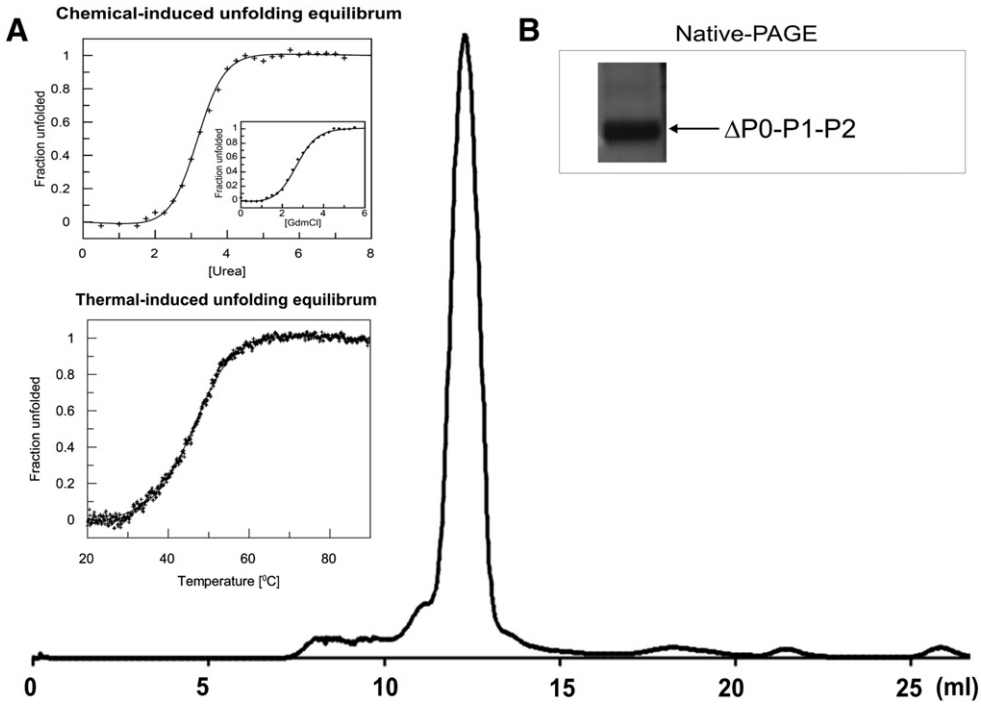

Fig. 5. Size-exclusion chromatography of the purified complex. Size-exclusion chromatography was performed using an analytical column, and the complex was eluted as a single symmetrical peak. Inset A, upper part, chemically induced unfolding of the complex expressed as a function of urea concentration; inset — guanidine chloride (GdmCl) induced unfolding. Lower panel, thermally induced unfolding of the complex. Inset B, native-PAGE analysis, the arrow indicates the position of the complex as a single band.

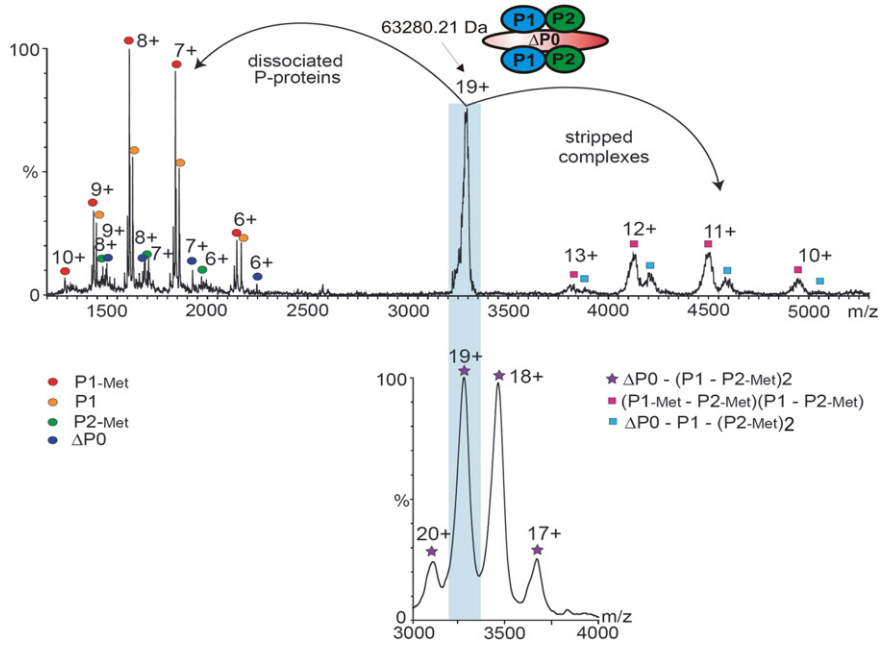

Fig. 6. Mass spectrometry analysis of the *P. falciparum* stalk complex. Mass spectra of the *P. falciparum* P-protein complex acquired under non-denaturing conditions. Lower panel, MS spectrum of the complex with the distribution of charge states; upper panel, tandem MS of the isolated 19+ charge state (marked in blue) of the complex. The 19+ charge state of the stalk pentamer was isolated in the mass spectrometer and high collision energy (80 V) was applied to dissociate the pentamer, resulting in two series of products: individual dissociated proteins at low m/z values and stalk complexes stripped of charged proteins at higher m/z values. The molecular mass and stoichiometry of the complex is indicated.

hampering its functioning [42]. Depletion of the N-terminal domain was obligatory for the high yield of P-protein production still enabling proper stalk complex assembling in bacterial cell. This genetic system allowed expression of all P-proteins in *E. coli* and led to the assembling of a soluble stalk complex *in vivo*. Non-denaturing mass spectrometry showed that the isolated stalk occurred exclusively in a pentameric ΔuL10/P0–(P1–P2)$_2$ form [8,53]. The P-complex was characterized by biophysical methods, showing that denaturing curves exhibit a simple two-state transition mechanism, similar to the natively isolated yeast complex, indicating that the unfolding process is similar in the yeast and *P. falciparum* pentamers. This suggests that folding and assembly are coupled processes where individual P-proteins determine their proper folding and the stable structure acquisition of the whole complex. Despite the functional similarities between P-stalks from various organisms, we saw significant, presumably physiologically relevant, differences in their biochemical properties. Contrary to the yeast P-complex, where P2 is released more readily, the *P. falciparum* P1 protein represents dominant species released from the stalk complex in the tandem MS spectra, implying that it is weakly bound to the uL10/P0. This result is in opposition to the generally accepted models of yeast and human P-stalk structure assembling, where P1 protein was postulated to be responsible for the P1/P2 dimer anchoring [8]. Moreover, the heterologously assembled *P. falciparum* stalk displays lower stability than its native yeast counterpart does, both in solution as well as in the gas phase. Such unusual properties of protozoan P-proteins reflect their significant phylogenetic distinction. While amino acid sequences of the eukaryotic P-protein family belong to a homologous group, protozoan P-proteins represent a separated branch on the phylogenetic tree with severely altered amino acids residues [7,16,17]. The N-terminal domains of both P1 and P2 proteins were defined as key fragments responsible for human [54] and yeast [55] stalk formation. Using *in silico* analysis, we made a comparison of both primary and secondary structures of P-proteins from several organisms. We found four conserved α-helices in the parasite P1 and P2 proteins, similar to those experimentally defined in other eukaryotic P-protein (Fig. 7) [54,56].

Fundamental dissimilarities were found in the P1 proteins. *P. falciparum* P1 possesses a unique, across *Eucarya* domain, extended N-terminal region (15 amino acids). As it was shown previously [54], the helix α1 in the human P1 is responsible for heterodimer formation and represents the main interface region stabilizing P1–P2 interactions [54]. Thus, the unique N-terminal extension of the parasite P1 protein might severely affect P1/P2 interactions, which may explicate the unexpectedly low stability of *P. falciparum* P1–P2 heterodimer. On the other hand, unique features of N-terminal region, probably responsible for oligomerization properties of P2 protein, were also defined [59]. From this respect the unusual mode of stalk assembling, with no stable heterodimers, might have important biological consequences, facilitating physiologically relevant P2 oligomer's formation. P2 oligomers occur in a developmentally dependent fashion, exhibiting oligomerization at the trophozoite stages in the erythrocytic development cycle; the P2 gets exported to the infected erythrocyte surface at 30 h post-merozoite invasion, concomitant with extensive oligomerization [29,49]. This molecular behavior of the P2 protein does not fall into the generally accepted notion about the behavior of P-proteins, raising questions about the molecular background for such peculiarity. Our data provide insight into the observed phenomenon, showing that *Plasmodium sp.* had developed an original stalk-assembling pathway, allowing the P2 protein to exist separately. The low stability of the *P. falciparum* P-stalk pentamer, together with the lack of propensity to P1/P2 stable association, might represent

Table 1
Individual proteins and protein complexes observed in MS and MS/MS spectra of the *P. falciparum* stalk construct.

Protein/complex	Calculated MW, Da	Measured MW, Da
ΔuL10/ΔP0	13472.2	13472.58 ± 0.19
P1	13013.8	13005.41 ± 1.55
P1$_{-Met}$	12882.6	12874.90 ± 0.26
P2$_{-Met}$	11817.4	11817.28 ± 1.98
(P1$_{-Met}$-P2$_{-Met}$) (P1–P2$_{-Met}$)	49514.87[a]	49575.86 ± 10.18
ΔuL10/ΔP0–P1–(P2$_{-Met}$)$_2$	50112.55[a]	50292.32 ± 7.59
ΔuL10/ΔP0–(P1–P2$_{-Met}$)$_2$	63117.96[a]	63280.21 ± 17.53

[a] Based on measured masses of individual P-proteins.

Fig. 7. Multiple sequence alignment of P1 and P2 N-terminal domains. 3D-NMR — secondary structure (H, position of α-helix) taken from the tertiary structure determined by the NMR approach for the human P1–P2 dimer [54]; Conserved residues in the dimeric interface are marked in red. Secondary structure prediction for parasite P1 and P2 proteins was analyzed with the aid of Jpred and PsiPred programs, respectively [57,58]. The additional N-terminal 15 amino acids of parasite P1 protein is marked in bold.

one of the possible cellular sources of P2 oligomers. We propose that the one-step formation of the stalk pentamer represents the main pathway, which is indispensable for ribosome functioning, whereas the P2 homo-oligomer may represent an off-pathway product with an unknown but physiologically important nonribosomal role.

5. Conclusions

We have shown that the assembly of the *P. falciparum* stalk complex is driven by mutual interplay among all stalk proteins, and the uL10/P0 protein represents a pivotal element in stalk assembly. The P-proteins form a pentameric structure, as is the case for P-stalk complexes from other eukaryotic species; however, the mechanism of the parasite P-stalk formation seems to be distinct. In yeast cells, this is a multistep process with heterodimers P1A–P2B and P1B–P2A assembling as an intermediate step. In turn, in *P. falciparum*, it seems to be a one-step reaction where all three elements are simultaneously required. On the other hand, P1 and P2 being unable to form a P1–P2 heterodimer, favors P2–P2 homo-oligomer formation, which during the parasite evolution was adopted for extraribosomal function [46].

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.10.015.

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