Purification of Components of the Translation Elongation Factor Complex of *Plasmodium falciparum* by Tandem Affinity Purification

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*Plasmodium falciparum* is the causative agent of severe human malaria, responsible for over 2 million deaths annually. Of the 5,300 polypeptides predicted to control the parasite life cycle in mosquitoes and humans, 60% are of unknown function. A major challenge of malaria postgenomic biology is to understand how the 5,300 predicted proteins coexist and interact to perform the essential tasks that define the complex life cycle of the parasite. One approach to assign function to these proteins is by identifying their physiological partners. Here we describe the use of tandem affinity purification (TAP) and mass spectrometry for identification of native protein interactions and purification of protein complexes in *P. falciparum*. Transgenic parasites were generated which express the translation elongation factor PfEF-1β harboring a C-terminal PTP tag which consists of the protein C epitope, a tobacco etch virus protease cleavage site, and two protein A domains. Purification of PfEF-1β-PTP from crude extracts followed by mass spectrometric analysis revealed, in addition to the tagged protein itself, the presence of the native PfEF-1α, the G-protein PIEF-1α, and two new proteins that we named PIEF-1γ and PIEF-1δ based on their homology to other eukaryotic γ and δ translation elongation factor subunits. These data, which constitute the first application of TAP for purification of a protein complex under native conditions in *P. falciparum*, revealed that the translation elongation complex in this organism contains at least two subunits of PfEF-1β. The success of this approach will set the stage for a systematic analysis of protein interactions in this important human pathogen.
ond purification step. The PTP method was successfully employed in *Trypanosoma brucei* for the purification and characterization of a multisubunit transcription factor, the U1 small nuclear RNP and RNA polymerase I (17, 19, 27).

Here we report the use of PTF-based tandem affinity purification for the analysis of protein networks in *P. falciparum*. We have successfully used this strategy to purify the translation elongation factor complex and determine the identity of its molecular components. Furthermore, we have developed a Gateway-based entry clone for rapid cloning of *P. falciparum* open reading frames upstream of the PTP tag prior to transfection of the parasite. We present data demonstrating the successful use of this strategy to purify the native phosphoethanolamine methyltransferase Ppm of *P. falciparum*.

**Materials and Methods**

**Construction of transfection plasmids.** To make the PEF-1β-PTP-fused fragment for cloning in the pDCI vector (6), forward primer 5'-CGTCGAGATG CTAGTAGGGCCAACTTTATATTTACGTA-3' (with the added XhoI site underlined and the start codon in bold) and reverse primer 5'-ATAGTTTGAGGG CCGCCCAATTTTGTTAAAGGAAAAGATTTC-3' (with the added NotI site underlined and the stop codon deleted) were used in combination to amplify the PEF-1β open reading frame from *P. falciparum* total cDNA. Forward primer 5'-AAGAATTCGCGGCCGAGAAATGCAGTTGAGCTCTTGGT-3' (with the added NotI site underlined and reverse primer 5'-CCGCCCTGCAATGACGTGGT GCTTCCGCGGATCCGGTCT-3' (with the added XhoI site underlined and the stop codon in bold) were used to amplify the PTP tag coding sequence cloned in a nPT-PURO-PTP vector (28). The PEF-1β and PTP fragments were digested by NotI and then ligated to create a fusion at the NotI site. The ligation product was recovered by PCR using a combination of PEF-1β forward and PTP reverse primers and subsequently digested by XhoI and ligated at the XhoI site of the pDCI vector. Directional cloning and the correct sequence of the fused fragment were confirmed by sequencing. To construct the pcHDr-PTpm-PTP expression vector, PPTM cDNA in the pENTR/D-TOPO vector, PTP tag in the pDONR2-P3 vector, the HSP86-5' promoter in pDONR4-P3 (32), and the pcHDr-3/4 destination vector (32) were combined in an LR reaction. Recombinant plasmids were propagated in *Escherichia coli* and extracted by Maxi-prep (QIAGEN) columns for transfection of *P. falciparum*.

**Transfection of *P. falciparum*.** Synchronized *P. falciparum* strain 3D7 parasites cultured in complete medium (6) at 2% hematocrit and 7% parasitemia (prior to purification step). The PTP method was successfully used to transfect the parasite. We present data demonstrating the successful use of this strategy to purify the native phosphoethanolamine methyltransferase Ppm of *P. falciparum*.

**FIG. 1.** (A) Map of the plasmid pDC1-PEF-1β-PTP. (B) Outline of the PEF-1β-PTP cassette. The PTP tag consists of protein C, the TEV protease site, and two protein A epitopes. (C) Analysis of expression of native and PTP-tagged PEF-1β in wild-type (3D7) and transgenic (TR) parasites using the PAP reagent and anti-PEF-1β antibodies as described in Materials and Methods. Anti-Ptpm antibodies were used as a control to detect the native Ptpm enzyme. M, protein marker. The dots indicate degradation products.
the protein A moiety. Immunoblot signals were measured using the Image J version 1.37v software.

RESULTS

Expression of PTP-tagged PfEF-1β in P. falciparum. To establish tandem affinity purification as a tool to purify protein complexes from P. falciparum under native conditions and in quantities sufficient for mass spectrometric identification, we chose the translation elongation factor PEF-1β as a bait. PEF-1β is a member of a conserved family of translation elongation nucleotide exchange factors that play an essential role in the GTP-dependent elongation step of protein synthesis. This step involves binding of aminoacyl-tRNA to the ribosomal A site, formation of a peptide bond, and translocation of the newly formed peptidyl-tRNA to the P site (15). EF-1β is responsible for the regeneration of a GTP-bound EF-1α necessary for each elongation cycle. A previous study in P. falciparum using radiolabeling and immunoprecipitation demonstrated an interaction between PEF-1β and PEF-1α (16). Furthermore, this study revealed that PEF-1α exists in a protein complex that may play an essential function in protein translation (16). For the purification of PEF-1β, its coding region was fused to the PTP tag sequence (Fig. 1). The resulting fusion was cloned under the regulatory control of the calmodulin gene promoter into the pDC1 vector (6), which harbors a mutated human dihydrofolate reductase (DHFR) gene that confers resistance to the antimalarial drug WR99210 (Fig. 1A). Fol-
lowing transfection of the *P. falciparum* 3D7 clone, stable transfectants were selected and analyzed for the expression of the fusion protein by Western blotting. As a control, wild-type 3D7 parasites were also examined. As shown in Fig. 1C, a band of 55 kDa of the expected size of the fusion protein could be detected in 3D7-PfEF-1/H9252-PTP parasites but not in 3D7 using the PAP reagent that recognizes the protein A moiety of the fusion protein. Immunoblotting using anti-PfEF-1/H9252 antibodies revealed two bands of 55 and 36 kDa corresponding to the sizes of the PTP-tagged and native PfEF-1/H9252, respectively, whereas only one band of 36 kDa could be detected in the 3D7 clone. As a positive control, antibodies against the *P. falciparum* phosphoethanolamine methyltransferase, Pfpmt, detected a single band of 30 kDa in both 3D7 and 3D7-PfEF-1β-PTP parasites.

**Purification of the PfEF-1β protein complex.** To purify protein complexes that include PfEF-1β, crude extracts were prepared from 3D7-PfEF-1β-PTP transgenic parasites and passed through an IgG column followed by cleavage of the bound material by TEV protease (Fig. 2A). Input extract and released material were analyzed by immunoblotting using the PAP reagent to detect the protein A moiety and anti-ProtC antibodies to detect the protein C moiety (Fig. 2B). The latter antibody detected a band of 55 kDa in the crude extract and a smaller size protein (39 kDa) in the TEV protease-released material consistent with the cleavage of the C-terminal double protein anolamine methyltransferase, Pfpmt, detected a single band of 30 kDa in both 3D7 and 3D7-PfEF-1β-PTP parasites.
A motif (Fig. 2B). Accordingly, no bands could be detected in the TEV protease-released material using the PAP reagent (Fig. 2B). TEV protease-released proteins were then passed through a column containing an immobilized HPC4 monoclonal antibody that recognizes the ProtC epitope and binds to it with very high affinity in the presence of calcium (K_d ~ 1 nM). The latter property enabled the final elution of the PIF-1β-PfEF-1αProtC fusion protein and any other proteins that were in complex with it by adding a buffer containing EGTA. The eluted material was separated by SDS-PAGE and further analyzed by immunoblotting using the anti-ProtC monoclonal antibody and the PAP reagent (Fig. 2B). The immunoblot assay demonstrated that both chromatography steps were highly efficient, since the flowthrough fractions from both columns were nearly depleted of tagged PIF-1β. Densitometric quantification of immunoblot signals indicated ~13% recovery of the purified proteins in the final eluates relative to their abundance in total extracts. Consistent with previous radiolabeling and immunoprecipitation studies (16), antibodies raised against PIF-1α detected this protein in the final eluate (Fig. 2B), thus validating the coexistence of PIF-1β and PIF-1α in a complex during the P. falciparum intraerythrocytic life cycle. Interestingly, immunoblotting of the purified fractions, including the final eluate using antibodies against PIF-1β, revealed in addition to the tagged PIF-1β-PfEF-1α, the presence of the native PIF-1β protein (Fig. 2B). This suggests that the translation elongation factor complex contains more than one subunit of PIF-1β. To demonstrate the specificity of the purification, all fractions were tested by immunoblotting using antibodies against Pfpm1, which is involved in parasite phospholipid biosynthesis (20–22, 33). No Pfpm1 signal could be detected in the IgG or TEV eluates, whereas this protein was clearly present in the crude extracts (Fig. 2B). As a control for the tandem affinity purification procedure, extracts from wild-type 3D7 parasites were subjected to the same purification strategy as transgenic parasites. As expected, no detectable protein bands could be observed using anti-ProtC antibody or the PAP reagent, and using antibodies against PIF-1β, PIF-1α, and Pfpm1, only the endogenous proteins could be detected in the total extract and the flowthrough of the IgG column (Fig. 2C).

**Molecular determination of the components of the P. falciparum translation elongation factor complex.** To demonstrate the utility of the method for mass spectrometric identification of proteins that are copurified with PIF-1β, the proteins of the final eluate were separated by SDS-PAGE and visualized by UV transillumination using Sypro ruby, a highly sensitive fluorescent stain for proteins that is compatible with mass spectrometric analysis. Eight major protein bands could be detected in the final eluate (Fig. 3A). As a control, the same purification conducted with extracts from wild-type 3D7 parasites did not reveal any detectable parasite protein bands (Fig. 3B). For identification of the proteins detected in the Sypro ruby staining of the PIF-1β purification, bands were excised, digested with trypsin, and subjected to liquid chromatography-tandem mass spectrometry. For each band, several peptides could be detected (Table 1). These were used to identify the corresponding proteins by screening against the available P. falciparum predicted proteins. Consistent with the immunoblotting data obtained using anti-PIF-1β, anti-ProtC, and anti-PIF-1α antibodies against the eluate, the tagged and native PIF-1β (NCBI 23613651) as well as PIF-1α (NCBI 3410705) were all identified in the purified complex. Interestingly, two additional proteins, PFC0870w and PF13_0214, that also share 55% identity and 72% similarity with PIF-1β, primarily in the predicted nucleotide exchange factor subunit of the fungal and plant elongation factor complex (Fig. 3C). PIF-1β also shares 55% identity and 72% similarity with PIF-1α in the predicted nucleotide exchange domain. PIF-1γ is a 434-amino-acid polypeptide highly homologous to the \( \delta \) subunit of the fungal and plant elongation factor complex (not shown). Additional bands detected in the stained gel were found to correspond to degradation forms of native and tagged PIF-1β as confirmed by mass spectrometry and immunoblotting using anti-PIF-1β and anti-ProtC anti-

**Table 1. Liquid chromatography-tandem mass spectrometry-identified peptides of proteins purified by TAP of PIF-1β**

| Protein Description | Peptide Sequence |
|---------------------|-----------------|
| PIF-1β              | QIVVGVNKK       |
|                     | IGGGITVPVVR     |
|                     | STTGGHITYK      |
|                     | SGDSALVLSEPK    |
|                     | GYVADSITKNEPAK  |
|                     | YEFVTPDAPGHK    |
|                     | QIVGVPNMDTVK    |
|                     | FTAVQIIVHGPUPIK |
|                     | AGMVNLNAPSVAVSECK |
|                     | EVLEEARPGDINGFNVK |
|                     | VDFIFPSGFEDGLNIEK |
|                     | SVEHIKEVLEEARPGDINGFNVK |
|                     | VGYOADKVDIFPSGFEDGLNIEK |
| PIF-1β-PfEF-1α      | IYQHKI      |
|                     | SITDIIAK     |
|                     | DTYPHLFR    |
|                     | OKIVDENIK   |
|                     | DSASNLLLEKK |
|                     | KLPAVAFGLYK  |
|                     | JENIDLDNEEDK |
|                     | IVDENIKWGEEVK |
|                     | AGGDDDDNNDILFDDDDNTK |
|                     | LHMSCIYDDFVNTNELIKEK |
|                     | AAGGDDDDNNDILFDDDDNTKDSASNLLEKEK |
| PIF-1β               | IYQHKI      |
|                     | SITDIIAK     |
|                     | DTYPHLFR    |
|                     | OKIVDENIK   |
|                     | DSASNLLLEKK |
|                     | SITDIIAKPIK |
|                     | DSASNLLLEKK |
|                     | JENIDLDNEEDK |
|                     | JENIDLDNEEDK |
|                     | AAGGDDDDNNDILFDDDDNTKDSASNLLEKEK |
| PIF-1γ               | LENNFSK   |
|                     | HIEDTLK    |
|                     | YLCISHR    |
|                     | LLAPKNDVR  |
|                     | GDTIDPFEKMK |
|                     | LDINNTOJDK |
|                     | LYDTSNOK   |
|                     | YVFACDQNK  |
|                     | LDINNTOJDKK|
|                     | VOTVASECFNVK|
|                     | YVFACDQNKK |
|                     | ETVDRPLVDK |
|                     | ETVDRPLVDK |
|                     | LNMPFENGKDVK |
|                     | TYDINGFSLYMK |
|                     | GDTIDPFEKMKHDPSEFYHIK |
|                     | DDNNNNNNADGNHADLSSLDEAELK |
|                     | DDNNNNNNADGNHADLSSLDEAELK |
| PIF-18              | TPFAPGFLK   |
|                     | SSSLIDIKPGENTDLDEVLK |

*The phosphorylated serine residues are underlined.*
bodies (Fig. 1B and 2B). Together these data provide the first evidence that tandem affinity purification can be used to purify the complete translation elongation factor complex of \textit{P. falciparum}. Interestingly, further analysis of the data revealed that of the 18 peptides of PfEF-1\textbeta revealed by mass spectrometry, two peptides were found to be phosphorylated on serine 263 (Table 1).

**Gateway-based PTP tagging of \textit{P. falciparum} proteins.** The results described above show that the PTP-based strategy can be used for purification and identification of protein complexes in \textit{P. falciparum}. For rapid construction of targeting vectors harboring various \textit{P. falciparum} open reading frames fused to PTP and to facilitate the large-scale purification of proteins under native conditions or the determination of protein networks in this parasite, we have developed a PTP entry vector compatible with the Gateway-based expression vectors reported by van Dooren and colleagues (32). As a proof of principle we used this strategy to construct a targeting vector harboring a fusion between the phoshoethanolamine methyltransferase open reading frame, \textit{PfPMT}, and the PTP sequence (Fig. 4A) and used it to transfect \textit{P. falciparum} 3D7 clone parasites. Transgenic parasites harboring this vector expressed the Pfpmt-PTP protein fusion using a monoclonal antibody against protein C and polyclonal antibodies against Pfpmt and PfEF-1\textbeta (as control). Identified protein bands are indicated. TEV, tobacco etch virus protease; M, protein marker; TE, total extract; TR, transgenic parasites; FT, flowthrough; TE-EL, eluate from IgG column; Pr.C, protein C; F-EL, final eluate from anti-ProtC column. Asterisks indicate native protein degradation products. About 0.28\% (vol/vol) of the total protein extract (TR-TE), 1.0\% of the TEV protease eluate (TE-EL), and 2.5\% of the final EGTA eluate (F-EL) were loaded in the respective lanes.

**DISCUSSION**

The major challenge of malaria biology following the decoding of several \textit{Plasmodium} genomes has been to understand how a complex life cycle spent between two hosts, within different host cells, in different cellular locations, and under different morphological, physiological, and metabolic states is controlled by less than 6,000 proteins. Large-scale microarray and mass spectrometry analyses during different life cycles of \textit{P. falciparum} have revealed a highly coordinated expression pattern in this parasite (1, 2, 7, 13, 14, 35). It remains, however, unknown how, at any given time during the parasite’s life cycle, different proteins interact to accomplish their essential tasks. A large-scale two-hybrid screen using \textit{P. falciparum} cDNA derived from mixed stages of the parasite intraerythrocytic life cycle has been reported (10). The pair-wise interactions reported have not been validated in \textit{P. falciparum}, and their physiological relevance remains to be established, for most of the interactions were performed in a heterologous system (i.e., yeast) and involved fragments rather than full-length cDNAs (10). Furthermore, due to the mixed population of RNAs from different intraerythrocytic stages, some interactions may occur between different proteins in vitro, whereas in vivo these proteins do not coexist at the same time.

The strategy described herein provides a better way to study protein networks in the parasite and at any given time during any stage of the parasite life cycle. We have applied this strategy to the purification of the protein complex that includes the translation elongation factor PfEF-1\textbeta. Evidence for the presence of such a complex in \textit{P. falciparum} was first demonstrated by
radiolabeling of *P. falciparum*-infected erythrocytes followed by immunoprecipitation using anti-PfEF-1β and anti-PfEF-1α antibodies (16). PfEF-1β was found to coimmunoprecipitate with PfEF-1α and vice versa. Interestingly, additional proteins also coimmunoprecipitated with PfEF-1β and PfEF-1α; however, the identity of those proteins remained unknown (16). Employing tandem affinity purification and mass spectrometry, we were able to identify in addition to the tagged PfEF-1β, the native PfEF-1β, and three copurified proteins, PfEF-1α, PfEF-1γ, and PfEF-1δ, all homologs of known translation elongation factor subunits (15). These data suggest that the PTP-based strategy has resulted in the purification of the translation elongation factor EF-1 complex of the parasite. Furthermore, detailed characterization of the purified proteins revealed that serine 263 of PfEF-1γ is phosphorylated. This finding is consistent with a previous study using orthophosphate labeling that showed that some components of the EF-1 complex of *P. falciparum* are phosphorylated (16). The finding that the native PfEF-1β copurifies with the tagged PfEF-1β suggests that at least two PfEF-1β subunits are part of the translation elongation factor complex in *P. falciparum*. To the best of our knowledge, our study is the first of its nature to provide direct evidence that more than one subunit of EF-1β exist in the EF-1 complex.

Unlike human EF-1α, none of the components of the malarial translation elongation complex contains leucine zipper domains. These domains have been proposed to play a role in the protein-protein interactions that define the ordered quaternary structure of the translation elongation complex (15). PfEF-1β shares significant homology with the C-terminal domain of PfEF-1β, which is predicted to be responsible for the nucleotide exchange activity of the protein. Whereas PfEF-1β has been demonstrated to catalyze the GTP/GDP exchange on PfEF-1α, the function of PfEF-1β as an exchange factor remains to be elucidated. Interestingly, while tagged and native PfEF-1β proteins as well as PfEF-1γ and PfEF-1δ concentrated in the final purified complex, only a fraction of PfEF-1α was found to associate with the translation elongation complex. This finding is consistent with previous studies that showed PfEF-1α as a monomeric protein as well as in association with high-molecular-weight protein aggregates (26) and with its well-defined cycle of association and dissociation and transient interactions with the other components of the translation elongation complex. Furthermore, besides its essential role in protein translation, EF-1α has also been shown to interact with tubulin and actin and to be involved in different cellular processes, such as organization of the cytoskeleton, cell differentiation, and apoptosis (for a review, see reference 12).

Unlike PfEF-1β, TAP analysis of Pfpmtd did not reveal detectable copurified proteins. This might reflect an inherent biological property of this enzyme or might be due to low expression of Pfpmtd-PTP, which was under the control of the HSP86 promoter. The latter hypothesis could be tested in future studies using the endogenous *PfMT* promoter or a stronger promoter, such as the *CAM* promoter.

The highly efficient purification of *P. falciparum* proteins demonstrated in this study will set the stage for the purification of other important protein complexes and the identification of new proteins involved in important physiological functions during the parasite’s life cycle. TAP tagging and purification may, for example, help better understand the mechanism of parasite invasion by TAP tagging components of the micronemes and rhoptries or the molecular determinants of protein trafficking to the red blood cell membrane, the food vacuole, or the apicoplast by tagging components of the Mauer’s cleft, the food vacuole membrane, or the apicoplast membrane. This strategy may also be useful in the identification of components of the transcription complexes of the parasite as well as new regulators that specifically bind to specific promoters and regulate their expression during the parasite’s life cycle. Our proposed strategy to combine the Gateway-based cloning and PTP tagging will make it possible to achieve these goals and will facilitate the large-scale functional analysis of the malarial proteome.

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