Tetracysteine-Based Fluorescent Tags to Study Protein Localization and Trafficking in Plasmodium falciparum-Infected Erythrocytes

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Abstract

Plasmodium falciparum (Pf) malaria parasites remodel host erythrocytes by placing membranous structures in the host cell cytoplasm and inserting proteins into the surrounding erythrocyte membranes. Dynamic imaging techniques with high spatial and temporal resolutions are required to study the trafficking pathways of proteins and the time courses of their delivery to the host erythrocyte membrane.

Methodology and Findings: Using a tetracysteine (TC) motif tag and TC-binding biarsenical fluorophores (BAFs) including fluorescein arsenical hairpin (FlAsH) and resorufin arsenical hairpin (ReAsH), we detected knob-associated histidine-rich protein (KAHRP) constructs in Pf-parasitized erythrocytes and compared their fluorescence signals to those of GFP (green fluorescent protein)-tagged KAHRP. Rigorous treatment with BAL (2, 3 dimercaptopropanol; British anti-Lewisite) was required to reduce high background due to nonspecific BAF interactions with endogenous cysteine-rich proteins. After this background reduction, similar patterns of fluorescence were obtained from the TC- and GFP-tagged proteins. The fluorescence from FlAsH and ReAsH-labeled protein bleached at faster rates than the fluorescence from GFP-labeled protein.

Conclusion: While TC/BAF labeling to Pf-infected erythrocytes is presently limited by high background signals, it may offer a useful complement or alternative to GFP labeling methods. Our observations are in agreement with the currently-accepted model of KAHRP movement through the cytoplasm, including transient association of KAHRP with Maurer’s clefts before its incorporation into knobs in the host erythrocyte membrane.

Introduction

Upon invading erythrocytes, Plasmodium falciparum (Pf) parasites extensively remodel their host cells [1]. Each parasite surrounds itself with a parasitophorous vacuole membrane (PVM), which separates erythrocyte cytoplasm from the parasite-residing space. The absence of endogenous protein trafficking mechanisms in erythrocytes requires Pf parasites to install processes by which synthesized proteins can be transported to the surface of the host erythrocyte.

Some Pf-produced proteins are localized in electron-dense protrusions (“knobs”) at the parasitized erythrocyte (PE) surface [2,3]. These proteins provide points of cytoadherence to endothelium of microvessels, enabling the parasitized erythrocytes to sequester and avoid elimination by the spleen [4,5,6,7,8,9]. Pf erythrocyte membrane protein 1 (PfEMP1) and knob-associated histidine-rich protein (KAHRP) are two important components of knobs [6,7]. KAHRP serves as an essential structural component of knobs, binds to the membrane skeleton of the host erythrocyte, and helps to anchor the acid terminal segment (ATS) of PfEMP1 [10,11], which is responsible for cytoadherence through antigenically variant regions that recognize receptors such as CD36, TSP (thrombospondin), and ICAM-1 (Inter-Cellular Adhesion Molecule 1) [12,13,14]. A current model of intraerythrocytic protein delivery to knobs invokes parasite export of proteins across the PVM to the erythrocyte cytoplasm, transient association with Maurer’s clefts and, finally, docking at the erythrocyte membrane [15,16].

Since the original report of GFP for biological imaging [17], applications of this protein have greatly expanded to include different color variants with different emission spectra [18]. Previous work using GFP-tagged KAHRP, PfEMP1 and other proteins has identified amino acid sequences of protein export motifs, translocons at PVM and putative steps in the pathways by which constituent proteins of knobs reach the host erythrocyte membrane [19,20]. Dynamic fluorescence techniques such as fluorescence recovery after photobleaching (FRAP) and Förster resonance energy transfer (FRET) can also be used to investigate
intracellular trafficking and protein-protein interactions. FRAP has provided valuable insights on protein trafficking involving various compartments of the PE, such as the parasitophorous vacuoles [PVs], PV extensions, and erythrocyte cytoplasm [14]. However, the trafficking process may be perturbed by imperfect maturation of GFP fusion proteins, complications with oligomerization, or reduction of protein diffusion rates due to the non-negligible size of GFP (25 kDa to 27 kDa; 238 amino acids) [21,22].

In recent years, other alternative labeling techniques have also been developed, including the tagging of target proteins with short peptides that carry a six amino acid tetracysteine (TC) motif, -Cys-Cys-Xaa-Xaa-Cys-Cys- (where Xaa is an amino acid other than Cys) [23]. This six amino acid tag binds membrane-permeable biarsenical fluorophores (BAFs) such as FlAsH and ReAsH [24]. Before binding to the TC tag, FlAsH and ReAsH are nonfluorescent in the form of FlAsH-EDT$_2$ or ReAsH-EDT$_2$, where EDT (1,2-ethanediol) moieties quench the fluorescence by vibrational deactivation. Upon BAF binding to the TC motif, the EDT is displaced and BAF becomes fluorescent [24]. Because BAFs are highly membrane permeable, unbound molecules within the cell can be washed away. These unique properties of the BAF have enabled two-color pulse-pulse labeling and time series studies of protein-protein interactions, protein synthesis and trafficking in live cells [25,26,27,28]. In a typical two-color application, two BAFs with distinct emission spectra are applied to TC-tagged proteins to image and track proteins produced at different times. In contrast, in GFP-based imaging techniques such imaging and tracking is available only with a photoactivatable form of GFP [29]. Another potential advantage of TC-tagging is the ability of ReAsH to support the photoconversion of diaminobenzidine (DAB) into osmiophilic residues, which can be observed by electron microscopy [25]. Finally, TC tags are short so they are less likely than GFP tags to interfere with protein expression or to affect protein structure; like GFP tags, TC tags do not require antibody binding for imaging.

Here we report on a study of the potential applicability and limitations of FlAsH and ReAsH labels bound to TC-tagged KAHRPs in PEs. The fluorescence images from this study are in agreement with a leading model of KAHRP trafficking to the host erythrocyte membrane.

**Materials and Methods**

**DNA constructs**

Plasmid pH2-KAHRP(+His)-GFP (Fig. 1A, [14]), kindly provided by Dr. Alan Cowman, was used as a template to PCR amplify the N-terminal sequence of KAHRP, its histidine-rich region, and GFP. Plasmid pDC-pvcrt-o-MH (a modified version of plasmid pDC-CAT that includes 16 codons for adjoining Myc and His$_6$ tags at the C’ terminus of pvcrt-o or other gene of interest [30]; not shown) was used to directly clone the amplified coding sequences of KAHRP(+His)-TC or KAHRP(+His)-GFP-TC between SpeI and XmaI restriction sites. We designed a single forward oligonucleotide primer containing a SpeI restriction site followed by the N-terminal region of KAHRP: Fp1-KAHRP 5’ ggcataagATGAAAAAGTTTATAGAAGACACACCCCAGTTGAGAAAGAAAGGCTTTCC 3’ (SpeI restriction site is underlined). Reverse oligonucleotide primers were designed to include coding sequences of the TC-containing motif (amino acid sequence RTGAGG; TC residues underlined) and the C-terminal sequence of either KAHRP(+His) alone or of KAHRP(+His)-GFP; these reverse primers were designed without stop codons, to allow a potentially continuous open reading frame.

**Figure 1. Transfection plasmids and detection of GFP fusion proteins.** (A) Plasmid pH2-KAHRP(+His)-GFP [14] was used to amplify the first 60 amino acids of KAHRP containing the putative hydrophobic signal sequence followed by the histidine rich region with or without GFP fusion. 3’ hsp86, histidine-rich protein-2 3’ UTR; hDHFR, human dihydrofolate reductase gene; 5’ CAM, PI calmodulin promoter region; 5’ hsp86, heat-shock protein-86 promoter region; 3’ pCt, P. chabaudi dihydrofolate reductase terminator 3’ UTR; 5’ AM, Myc (M) and His$_6$ (H) tags are encoded immediately after the XmaI restriction site (Sp) immediately before the first codon of KAHRP cloned into pDC-pvcrt-o-MH (not shown); Rp1-GFP-TC, reverse primer used to amplify GFP, add an EcoRV restriction site (Ev) in frame between GFP and the TC tag (for subcloning of other genes), and add a XmaI restriction site (Xm) after the TC sequence, for cloning into pDC-pvcrt-o-MH; Rp2-His-TC, reverse primer used to amplify the histidine rich region of KAHRP, add an EcoRV restriction site (Ev) while maintaining the reading frame of KAHRP(+His) and the TC tag (for future subcloning of other genes), and add a XmaI restriction site (Xm) after the TC sequence, for cloning into pDC-pvcrt-o-MH; (B) Transfection plasmid pDC-KHT contains the sequence for the fusion protein KAHRP(+His)-TC from pH2-KAHRP(+His)-TC. Myc (M) and His$_6$ (H) tags are encoded immediately after the XmaI restriction site. (C) Transfection plasmid pDC-KHGT contains the sequence of KAHRP(+His)-TC in frame with codons for a PRGKTYF terminus (F) that begin at the XmaI site. 5’ pCt, P. chabaudi dihydrofolate reductase promoter region; 3’ hsp86, heat-shock protein-86 3’ UTR. (D) Immunoblot image shows antibody detection of GFP as a M, 28,000 band, detection of KAHRP(+His)-GFP-TC as a M, 42,000 band, and detection of M, 39,000 and M, 29,000 bands from KAHRP(+His)-GFP protein. No band was detected from control non-transformed 3D7 PE.

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with the Myc and His×6 tags encoded by the pDC-pvcrt-o-MH plasmid. To facilitate the future cloning of other genes of interest followed by the Tc motif in our transfection plasmids, we also included a six-nucleotide EcoRV restriction site in these reverse oligonucleotide primers, which maintains the reading frame of the Tc motif: Rp1-GFP-Tc 5'-GGCTTCGCAACCCGACCCGACCCGGCCACCGACCCGACGC-3' and Rp2-His-Tc 5'-GCTTCCGGGGCGCCACCCGGCCACCCGACGCACGCGGGCCACCGACCCGACCCGACCCGGCCACCGACCCGACGC-3' and Rp2-His-Tc 5'-GCTTCCGGGGCGCCACCCGGCCACCGACCCGACCCGGCCACCGACCCGACGC-3' and Rp2-His-Tc 5'-GCTTCCGGGGCGCCACCGACCCGACGCACGCGGGCCACCGACCCGACCCGACCCGGCCACCGACCCGACGC-3'.

KHT and pDC-KHGT plasmids are deposited in GenBank was not removed for these studies. The sequences of the pDC-plasmids, pDC-KHGT and pDC-KHT, were checked for the presence of PCR fragments amplified from genomic DNA of Pf3D7 parasites. To facilitate the future cloning of other genes of interest, we also digested the KHT plasmids with restriction enzymes that generate cohesive ends, such as EcoRV and HindIII, for cloning into other expression vectors.

Because this extra cytosine did not affect the GFP and TC motif: Rp1-GFP-TC 5'-GGCTTCGCAACCCGACCCGACCCGGCCACCGACCCGACGC-3' and Rp2-His-Tc 5'-GCTTCCGGGGCGCCACCCGGCCACCGACCCGACGCACGCGGGCCACCGACCCGACCCGACCCGGCCACCGACCCGACGC-3' (the Xmal restriction site for cloning into pDC-pvcrt-o-MH is in lower case and underlined; the EcoRV restriction site is italicized and underlined; the CCPGCC codons underlined) Fragments KAHRP(+His)-GFP-Tc and KAHRP(+His)-TC were amplified from pHII2-KAHRRP(+His)-GFP with primers Fp1-KAHRRP plus Rp1-GFP-Tc and with Fp1-KAHRRP plus Rp2-KAHRRP-Tc, respectively, using 30 repetitions of the following PCR-thermo cycle: 30 s denaturation at 94°C; 60 s annealing at 60°C (for KAHRP(+His)-GFP-Tc) or at 65°C (for KAHRP(+His)-TC); and 120 s extension at 68°C. The purified fragments were cloned into pGEM-T Easy vector (Stratagene, Santa Clara, CA), confirmed by sequencing (Clinical Laboratory Improvements Amendments Molecular Diagnostics & Sanger Sequencing Group, Frederick, MD), digested with SalI and Xmal, and ligated into pDC-pvcrt-o-MH which had also been digested with SalI and Xmal. The resulting plasmids, pDC-KHGT and pDC-KHT, were checked for integrity and orientation of the inserts by restriction analysis and DNA sequencing. Results showed that plasmid pDC-KHGT contains the sequence of KAHRP(+His)-TC in frame with the codons of the Myc and His×6 tags, as expected (Fig. 1B). Plasmid pDC-KHGT, however, contains an inserted extra cytosine immediately upstream the Xmal restriction site; this extra cytosine does not affect the codons of the KAHRP(+His)-GFP-Tc sequence but it places the downstream Myc and His×6 tag codons out of frame, so that the encoded protein terminates immediately upstream the CCPGCC codons underlined; the RV restriction site is italicized and underlined; the CCPGCC codons underlined) Fragments KAHRP(+His)-GFP-Tc and KAHRP(+His)-TC were amplified from pHII2-KAHRRP(+His)-GFP with primers Fp1-KAHRRP plus Rp1-GFP-Tc and with Fp1-KAHRRP plus Rp2-KAHRRP-Tc, respectively, using 30 repetitions of the following PCR-thermo cycle: 30 s denaturation at 94°C; 60 s annealing at 60°C (for KAHRP(+His)-GFP-Tc) or at 65°C (for KAHRP(+His)-TC); and 120 s extension at 68°C. The purified fragments were cloned into pGEM-T Easy vector (Stratagene, Santa Clara, CA), confirmed by sequencing (Clinical Laboratory Improvements Amendments Molecular Diagnostics & Sanger Sequencing Group, Frederick, MD), digested with SalI and Xmal, and ligated into pDC-pvcrt-o-MH which had also been digested with SalI and Xmal. The resulting plasmids, pDC-KHGT and pDC-KHT, were checked for integrity and orientation of the inserts by restriction analysis and DNA sequencing. Results showed that plasmid pDC-KHGT contains the sequence of KAHRP(+His)-TC in frame with the codons of the Myc and His×6 tags, as expected (Fig. 1B). Plasmid pDC-KHGT, however, contains an inserted extra cytosine immediately upstream the Xmal restriction site; this extra cytosine does not affect the codons of the KAHRP(+His)-GFP-Tc sequence but it places the downstream Myc and His×6 tag codons out of frame, so that the encoded protein terminates instead with the amino acid sequence PRGTKTYF (Fig. 1C).

To facilitate the future cloning of other genes of interest, we also digested the KHT plasmids with restriction enzymes that generate cohesive ends, such as EcoRV and HindIII, for cloning into other expression vectors.

Parasite culture and parasite transformation

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Protein analysis by immunoblotting

Mature parasitized erythrocytes were isolated for immunoblotting on L5 magnetic separation columns [Milenyi Biotec, Auburn, CA] as described elsewhere [33]. From the column, approximately 5 x 10^7–10^8 mature PE were obtained in 2 mL RPMI complete media, centrifuged at 2,200 rpm for 5 min, and washed with a phosphate buffered saline (PBS, Na2HPO4 795 mg/L; KH2PO4 144 mg/L; NaCl 9000 mg/L; pH 7.4) solution. Parasitized cells were freed of hemoglobin by treatment with 150 mg/mL saponin in a total reaction volume of 500 mL PBS for 10 min on ice. After centrifugation at 13,000 rpm for 5 min, the pellet was recovered, resuspended in PBS, pelleted again, and resuspended in 1% triton X-100 in ice-cold PBS in the presence of serum and cytochrome c (Roche Diagnostics, Mannheim, Germany). Samples were combined with NuPAGE 10% Bis-Tris polyacrylamide gel (Invitrogen), and transferred to a polyvinylidene fluoride (PVDF) membrane.

Fluorescence photobleaching was measured on live cells under continuous exposure to focused light at the excitation wavelength. Images were captured at a frame rate of 3 frames/s for 20 s at 9.98 mW of incident light through a 100× objective for GFP and FlAsH, and at 10.88 mW for ReAsH. No anti bleaching reagents were added to the media.

Wide-field fluorescence light microscopy

Wide-field fluorescence images were recorded using a 100× (N.A. 1.4) objective in a Leica DMI 6000B inverted optical microscope (Leica Microsystems, Bannockburn, IL). Micrographs were acquired using a Hamamatsu Orca ER digital CCD camera (Hamamatsu Photonics System, Bridgewater, NJ) and Image Pro image acquisition software (Media Cybernetics, Bethesda, MD). Green fluorescence from GFP and FlAsH bound to TC-tagged proteins was recorded using an XF100-2 fluorescence filter set (excitation 475AF40; emission 535AF45; Omega Optical, Brattleboro, VT); red fluorescence from ReAsH bound to TC-tagged proteins was recorded using an XF102-2 filter set (excitation 560AF55; emission 645AF75; Omega Optical, Brattleboro, VT). The excitation power for each optical filter set was measured with a wavelength-corrected, calibrated power meter (S120C, Thorlabs Inc., NJ) by placing a silicon photodiode detector head at the focal plane of the objective lens. The scale of the intensity adjustment knob for field diaphragm control was calibrated against the power measurement.

Photobleaching comparisons of GFP vs. BAFs

Fluorescence photobleaching was measured on live cells under continuous exposure to focused light at the excitation wavelength. Images were captured at a frame rate of 3 frames/s for 20 s at 9.98 mW of incident light through a 100× objective for GFP and FlAsH, and at 10.88 mW for ReAsH. No anti bleaching reagents were added to the media.
Reduction of nonspecific binding background of BAFs

Although Griffin et al. [24] estimated a low binding of the endogenous cysteine-rich regions of cellular proteins to BAFs, other reports showed different levels of nonspecific background staining, which could result in poor signal-to-noise ratios (S/N) depending on the type of cell line and target protein [34,35]. Methods to overcome the nonspecific background have included the use of BAL (2,3 dimercaptopropanol; British anti-Lewisite) or EDT (ethanediolthiol) to reduce the affinity of BAFs for endogenous peptides containing CXXXC or CXXC [23,36,37] or other Cys-rich amino acid sequences.

Our initial images of 3D7 non-transformed parasites exposed to FlAsH or ReAsH showed unacceptable background fluorescence. To reduce nonspecific background, the cells were pre-treated with 650 μmol/L BAL in incomplete RPMI 1640 (without serum, albumin and phenol-red, RPMI-inc) for 15 min at 37°C, followed by washes with RPMI-inc at room temperature. Use of RPMI-inc during labeling and washing avoided unnecessary background effects from the interaction of albumin with BAFs [37].

The BAL-treated samples were then exposed to 2.5 μmol/L BAFs for 30 min at 37°C. ReAsH labeling was followed by 3 consecutive washes with 250 μmol/L BAL in warm RPMI-inc to remove ReAsH-BAL complexes and unbound or loosely bound ReAsH. An additional treatment with 250 μmol/L BAL in RPMI-inc containing 20 μmol/L Disperse Blue (Sigma) for 10 min to 40 min at 37°C was added to displace ReAsH that was tightly bound to nonspecific sites by diethyl-independent hydrophobic interactions [37]. After a final wash with warm RPMI-inc only, the samples were examined by fluorescence microscopy.

FlAsH labeling was followed by 3 washes with 250 μmol/L BAL in warm RPMI-inc; 15 min treatment at 37°C with 250 μmol/L BAL in RPMI containing 20 μmol/L Disperse Blue; 15 min treatment at 37°C with 500 μmol/L BAL in RPMI-inc containing 20 μmol/L Disperse Blue; and a final wash with warm RPMI-inc only.

S/N ratios of ReAsH and FlAsH were calculated using the following equation: \( \frac{F_{\text{specific}} - F_{\text{nonspecific}}}{F_{\text{nonspecific}}} \); where \( F_{\text{specific}} \) represents the average pixel fluorescence intensity from the BAF in transgenic PE, expressing the TC tag, and \( F_{\text{nonspecific}} \) is the average pixel fluorescence intensity of cells with non-transformed 3D7 parasites treated with the same BAF.

To assess endogenous cysteine-rich proteins as a possible source of nonspecific background in the non-transformed 3D7 parental line, we pretreated 3D7 PE with 200 μmol/L coumarin maleimide (CPM) in RPMI-inc for 1 h at 37°C followed by labeling with 2.5 μmol/L ReAsH. CPM excitation and emission were 380 nm and 407 nm, respectively. CPM is highly reactive to thiol groups, exhibits no fluorescence in an unbound state, and has been used to block endogenous cysteines [34]. Analysis of fluorescence intensities was performed using Image Pro image software.

Effect of BAL on Pf in vitro growth

To determine whether BAL would have an adverse effect on the parasite growth after the 15 min BAL pre-treatment for background reduction, we performed in vitro growth inhibition assays with various concentrations of BAL and at two incubation times, 15 min and 1 h. A modified protocol from the SYBR Green cell multiplication detection method established by Smilkstein et al. [38] was used. Synchronized ring stage cultures at 1% parasitemia were incubated for 15 min at 37°C at BAL concentrations of 0 mmol/L to 5 mmol/L and then washed with RPMI-inc. The PE were then resuspended in complete RPMI and cultured for 72 h. Parasite growth was measured by DNA multiplication detected by SYBR Green.

Results

Expression of KAHRP(+His)-GFP-TC

We constructed two transfection plasmids from the original plasmid that expresses KAHRP(+His)-GFP [14]; plasmid pDC-KHT, which encodes KAHRP(+His) followed by TC, Myc and His6 tags but not GFP (Fig. 1B); and pDC-KHTG, which encodes KAHRP(+His) followed by GFP, the TC tag, and a PRGTKTYF terminus that resulted from a single nucleotide frame shift at the Xmal site (Fig. 1C). The KAHRP(+His) domain in these constructs provides a histidine-rich region necessary for protein delivery to erythrocyte surface [14]. To confirm expression of intact fusion protein from plasmid pDC-KHTG, we used anti-GFP antibodies to probe immunoblots of the transformed parasite line 3D7-KAHRP(+His)-GFP-TC (Fig. 1D). Positive controls included purified recombinant GFP protein and extracts of the transformed line 3D7-KAHRP(+His)-GFP (containing plasmid pH2-CAHRP(+His)-GFP DNA); negative control was an extract of non-transformed 3D7 parasites. In the positive control, purified recombinant GFP (predicted molecular mass of 27 kDa) was detected as a single band with relative molecular weight (\( M_r \)) of 28,000 (Fig. 1D), while two bands with \( M_r = 29,000 \) and \( M_r = 39,000 \) were detected from 3D7-KAHRP(+His)-GFP parasites. These two bands from transformed 3D7-KAHRP(+His)-GFP parasites are in agreement with the original report by Wickham et al. [14], in which KAHRP(+His)-GFP has a predicted molecular mass of 37.6 kDa due to possible cleavage at the predicted Cys34-Ser35 site. The slightly greater \( M_r \) on our immunoblots likely reflects altered electrophoretic mobility due to the histidine-rich nature of KAHRP.GFP fusion proteins have been reported to show an additional band of \( M_r = 29,000 \) corresponding to a GFP-cleavage product [14,39]. The expressed protein from our pDC-KHTG-transformed 3D7 parasites shows a 42,000 \( M_r \) band, consistent with the addition of the TC tag and PRGTKTYF terminus (together approximately 2.7 kDa). Absence of a detectable cleavage band on the 3D7-KAHRP(+His)-GFP-TC immunoblot may be due to an effect of the TC tag and/or the PRGTKTYF terminus on susceptibility of the protein to proteolysis.

Optimization of FlAsH and ReAsH fluorescence labeling

In pilot experiments we found that background fluorescence intensity from non-transformed parasites in PE treated directly with FlAsH or ReAsH was unacceptably high (Figure 2A and 2B). Therefore, various treatments of the PE with 650 μmol/L BAL before labeling and washes with 250 to 500 μmol/L BAL+20 μmol/L Disperse Blue after BAL labeling were explored.

With non-transformed 3D7 parasites treated with ReAsH and FlAsH, we were able to achieve markedly reduced but still detectable nonspecific background with a 650 μmol/L BAL pre-treatment (prior to BAL labeling) followed by three consecutive washes with 250 μmol/L BAL and a further wash with either 250 μmol/L BAL (ReAsH) containing 20 μmol/L Disperse Blue or 500 μmol/L BAL containing 20 μmol/L Disperse Blue (FlAsH) (Fig. 2, C, D). Further experiments showed that the pre-treatment BAL concentration could be lowered to 100 μmol/L without greatly increasing the background, but 50 to 100 μmol/L BAL in the post-labeling washes was not enough to adequately control the background fluorescence. Little or no nonspecific background was evident in non-parasitized erythrocytes labeled with BAFs after BAL treatment.

Transformed 3D7-KAHRP(+His)-GFP parasites treated with ReAsH showed good retention of positive signal after treatment to reduce background (Fig. 2E); slightly higher signals were detected.
from parasites treated with FlAsH (Fig. 2.F). However, some cell-to-cell variations in the emission intensity within the same culture were noticed, probably due to age differences or infections with multiple parasites. In addition, we observed that BAL treatment not only reduces the unspecific background, but also the overall positive signal.

Fluorescence images of dual GFP- and ReAsH-labeled 3D7-KAHRP(＋His)-GFP-TC parasites showed a comparable retention and distribution of fluorescence signal after applying the procedures for background reduction (Fig. 2G).

These results agree with the calculated S/N ratio (Table 1). Among TC-expressing parasites treated with BAFs, we found a lower S/N ratio for FlAsH in 3D7-KAHRP(＋His)-TC parasites than in ReAsH treated PEs. FlAsH has higher affinity than ReAsH for specific [23] and nonspecific sites consistent with a lower S/N than for ReAsH (compare Fig. 2 F&D vs. Fig. 2 E&C). GFP signals from 3D7-KAHRP(＋His)-GFP-TC PEs provided the highest S/N ratio as no nonspecific background binding of a GFP fluorophore is involved.

We observed crenation of erythrocytes due to possible environmental changes (chemicals, temperature, osmotic pressure)
Table 1. S/N ratios of GFP, FlAsH and ReAsH fluorescence from 3D7-KAHRP(+His)-GFP-TC and 3D7-KAHRP(+His)-TC-transformed PE.

| Parasite | Signal/Noise |
|----------|--------------|
| Line     | GFP | FlAsH | ReAsH |
| 3D7-KAHRP(+His)-GFP-TC | 3.25±1.77 | – | 2.65±1.71 |
| 3D7-KAHRP(+His)-TC | – | 1.23±0.85 | 3.02±2.29 |

Table 2. Measurements of cell multiplication and 50% and 90% inhibitory concentrations (IC50; IC90).

| Parasite | 15 minutes exposure to BAL | 1 hour exposure to BAL |
|----------|---------------------------|------------------------|
| Line     | IC50 (mmol/L) | IC90 (mmol/L) | IC50 (mmol/L) | IC90 (mmol/L) |
| 3D7      | 1.65 ± 0.33 | 4.43 ± 0.86 | 0.50 ± 0.08 | 1.37 ± 0.33 |
| 3D7-KAHRP(+His)-GFP-TC | 0.78 ± 0.07 | 2.60 ± 0.38 | 0.33 ± 0.01 | 0.65 ± 0.08 |
| 3D7-KAHRP(+His)-TC | 1.25 ± 0.31 | 2.88 ± 0.72 | 0.38 ± 0.02 | 0.90 ± 0.14 |

Values are shown as mean of 3 experiments in duplicate with standard errors. Parasites lines 3D7, 3D7-KAHRP (+His)-GFP-TC and 3D7-KAHRP (+His)-TC were exposed for 15 min, and 1 h to different concentrations of BAL (0 mmol/L–5 mmol/L).

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Fluorescence patterns from TC-tagged KAHRPs in parasitized erythrocytes

To demonstrate the applicability of TC-tagged proteins in the study of trafficking behaviors of Pf-produced KAHRPs, we compared fluorescence patterns from the KAHRP(+His)-GFP protein described by Wickham et al. [14] (Fig. 3A–C), KAHRP(+His)-GFP-TC protein labeled with ReAsH (Fig. 3D–F), and KAHRP(+His)-TC protein labeled with FlAsH or ReAsH (Fig. 3G–L) in PEs containing various stages of transformed 3D7 parasites. In all cases, the observed patterns were comparable to those described by Wickham et al. [14]. Further, images from ReAsH-labeled 3D7-KAHRP(+His)-GFP-TC parasites (Fig. 3D–F) confirmed that the GFP and ReAsH fluorescence patterns co-localized as expected for ReAsH binding to TC adjacent to the GFP tag, and similar protein distributions were observed for FlAsH- or ReAsH-labeled 3D7-KAHRP(+His)-TC protein in PEs containing rings (Fig. 3A, D, G, J) and early and late trophozoites (Fig. 3B–C, E–F, H–I, K–L). Fluorescence from the tagged KAHRP first appeared within ring stage parasites and was associated with the PVM (Fig. 3A, D, G, J). Progression to trophozoites was accompanied by the appearance of fluorescent spots in the host erythrocyte cytoplasm often near the host membrane (Fig. 3B, E, H, K). These fluorescent concentrates of protein have been shown to be associated with Maurer’s clefts [14]. In later trophozoite stages, concentrates of protein and an increased component of fluorescence were associated with the erythrocyte membrane (Fig. 3C, F, I, L).

Photobleaching characteristics of BAF-TC vs. GFP tags in PE

Fluorescence decay rate is an important consideration for both qualitative and quantitative fluorescence imaging. In comparisons of the photobleaching characteristics of GFP in 3D7-KAHRP(+His)-GFP-TC PEs and FlAsH in 3D7-KAHRP(+His)-TC PEs, excitation with 9.98 mW of 475 nm±20 nm light for 20 s reduced the fluorescence intensities to 65% and 73% of their initial values, respectively. In other comparisons, ReAsH-labeled 3D7-KAHRP(+His)-TC parasites consistently bleached at the fastest rates, resulting in 85% decrease of the initial emission signals (Fig. 4). Although the BAF-TC tags exhibit faster photobleaching rates than GFP tags, only a small percentage of BAF fluorescence was bleached away during a typical image acquisition period (less than 500 ms), providing sufficient photostability for a study of protein trafficking in PE.

Discussion

TC-tag labels and BAF offer fluorescence detection complementary to GFP for the study of protein trafficking in PEs. Our results show that ReAsH labeling of KAHRP protein tagged with both TC
and GFP yielded similar fluorescence patterns at red and green wavelengths. Similar patterns were also obtained after FlAsH or ReAsH labeling of KAHRP tagged with TC alone, supporting previous assumptions that the larger GFP tag does not detectably interfere with this protein trafficking [14]. The limiting factor of this technique in malaria PEs is the high background from BAF labeling, consequently interfering with the application of FlAsH-TC or ReAsH-TC to two-color pulse labeling applications.

A major source of nonspecific background fluorescence with BAFs is their binding to CXXC motifs present, for example, in zinc finger proteins, RING finger proteins, protein kinases and cytoskeletal proteins. Coulson et al. [42] found that CCCH-type zinc finger motifs were encoded abundantly in the Pf genome. Additionally, PICRM1 and PICRM2 Pf Cysteine Repeat Modular Domain 1 and 2) [43] include CXXC motifs, which may also compound the high Cys-nonspecific background in Pf infected erythrocytes. Another source for background is non-thiol dependent thiol-dependent BAF binding is the major source of background in Pf parasites. BAL, an antidote to arsenic poisoning [44], is reported to reduce background more efficaciously and with less toxicity to cells than EDT [23,45,46]. To assess BAL toxicity, we determined the IC50 and IC90 for the different Pf lines for periods comparable to the BAL treatment times. The IC50 values of 3D7-KAHRP(+)His)-TC-transformed parasites were above the concentration of BAL treatment prior to BAF labeling (650 μmol/L), and the red and green fluorescence patterns of ReAsH-labeled 3D7-KAHRP(+)His)-TC-transformed parasites were comparable to those reported for KAHRP(+)His)-GFP transformed parasites [14], suggesting that sub-cellular protein localization was not noticeably disturbed by the BAL treatment for background reduction. The slightly lower IC50s of GFP-expressing PE relative to PE not expressing GFP could be due to effects of apoptosis induced by GFP [47].

In future studies it might be possible to improve signal strength over background by engineering tandem TC motifs, as approached in other systems [48]. The use of a stronger promoter for higher exogenous protein expression may also enhance the signal from BAF labeled TC tags. The binding affinity of FlAsH for TC has been reported to be higher than that of ReAsH [23]; in our study, a lower S/N ratio for FlAsH relative to ReAsH suggests that non-specific binding of FlAsH is also greater than that of ReAsH.

We were able to partially but not completely reduce nonspecific thiol-dependent background by using the thiol-containing reagent BAL. As previously reported, pre-treatment with CPM completely blocked background staining [34], but no red fluorescence was evident after subsequent ReAsH labeling (Fig. 2H) confirming that thiol-dependent BAF binding is the major source of background in Pf parasites.

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observed. The roles of these structures in protein trafficking remain to be clarified [49].

In summary, our results from BAF-labeled TC tags are consistent with KAHRP trafficking through the cytoplasm and accumulation at Maurer’s clefts prior to transfer to the host PE membrane, in agreement with the trafficking model proposed by Wickman et al. and Tilley et al. [14,49]. Fluorescence from ring stage transgenic parasites expressing GFP-TC- or TC-tagged KAHRP proteins was within the PV; fluorescence from later stage trophozoites showed signal throughout the cytoplasm, with bright concentrations near or associated with the host erythrocyte membrane. These observations are consistent with transient association of KAHRP at Maurer’s clefts before the protein docks beneath the host erythrocyte membrane.

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