Chapter 6:

Protein acyltransferase activity of *Plasmodium falciparum* DHHC proteins

Protein acyl transferase (PAT) activity of *P. falciparum* DHHC proteins

The results described in the previous chapters characterised the localisation and essentiality of *P. falciparum* and *P. berghei* homologues of the family of proteins –namely the DHHC protein family- known to catalyse protein palmitoylation in other eukaryotic organisms. However, whether any of these *Plasmodium* DHHC-domain-containing proteins actually have palmitoyl transferase activity has never been formally shown. In this chapter, a novel assay was developed to directly test the PAT activity of *P. falciparum* DHHC proteins characterised in Chapter 5, and was used to determine whether individual PfDHHC proteins are substrate specific.

6.1. PAT activity assay – the method

The PAT activity of several of the DHHC proteins in other eukaryotic organisms, such as yeast and humans, have been previously shown using tritiated palmitoyl-CoA in *in vitro* radiolabeling assays, or using tritiated palmitic acid in *in vivo* metabolic labelling assays. For example, in the *in vitro* radiolabeling assays, the DHHC protein of interest is mixed with the putative target protein in the presence of tritiated palmitoyl-CoA, and the PAT activity of the DHHC protein determined by measuring the incorporation of tritiated palmitate. These assays successfully demonstrated the PAT activity of DHHC proteins, such as Erf2 and Akr1 in yeast, and DHHC9 in humans [1-3].

The use of radiolabeling however is time-consuming, with blots containing tritiated proteins sometimes requiring months of exposure. Thus, for the purposes of this project, a PAT activity assay incorporating metabolic labelling and click chemistry was developed to increase the throughput and to allow for specific experimental questions to be followed in the time available. The principle of the PAT activity assay was to express a potential palmitoylated target protein in the presence and absence of a PfDHHC protein, and then to purify only palmitoylated versions of the target protein using metabolic labelling and click chemistry methods (described in Section 1.6.2). The activity of the PfDHHC protein of interest could then be assessed by comparing the level of target protein palmitoylation in the presence and absence of the PfDHHC protein. If, palmitoylation of the target protein only occurred in the presence of the PfDHHC protein, this would demonstrate the PAT activity of said PfDHHC protein. Comparison of palmitoylation levels in the presence of different PfDHHCs could then be used to test whether the PAT activity of a particular PfDHHC protein is substrate specific.

The PAT activity assay was developed using the Human Embryonic Kidney 293 (HEK293) cell expression system, which has been used previously for the successful production of recombinant *P. falciparum* proteins [4]. Potential *P. falciparum* target proteins were codon-optimised for expression in mammalian cells and C-terminally-tagged with a c-Myc tag, to allow their detection. These constructs were then expressed in HEK293 cells by transient transfection, and co-expressed with either a putative PfDHHC –also codon-optimised for expression in mammalian cells but C-terminally-tagged with a FLAG tag for detection- or with an empty CD4 control vector which does not express any PfDHHC. After 18 hours post-transfection, the cells were then metabolically labelled for 6 hours with the palmitic acid analogue, 17-ODYA, at a concentration of 25 µM, or mock-treated with DMSO as a control. After 6 hours of labelling with 17-ODYA, proteins were extracted from the HEK293 cells (using a Triton X-100-based buffer) and then reacted with biotin-azide under standard click chemistry reactions (described in Section 1.6.2 and in the Materials and Methods), resulting in the biotinylation of all 17-ODYA-labelled proteins. Biotinylated proteins were then streptavidin affinity purified, and the 'palmitoylation status' of the proteins was determined by their enrichment in the 17-ODYA-treated sample compared to the DMSO-treated control sample. The presence of the target protein in the 17-ODYA-treated sample was specifically determined using the C-terminal c-Myc tag. As the PfDHHC is tagged with a different tag, autopalmitoylation of the PfDHHC could also potentially be determined by looking for the presence of the PfDHHC in the 17-ODYA-treated sample using its C-terminal FLAG tag. Expression of both *P. falciparum* target proteins and PfDHHC proteins in HEK293 cells could also be confirmed by immunoblotting of whole protein extracts and by immunofluorescence assay using the c-Myc and FLAG tags. A schematic detailing the steps of this assay is shown in Figure 6.1.

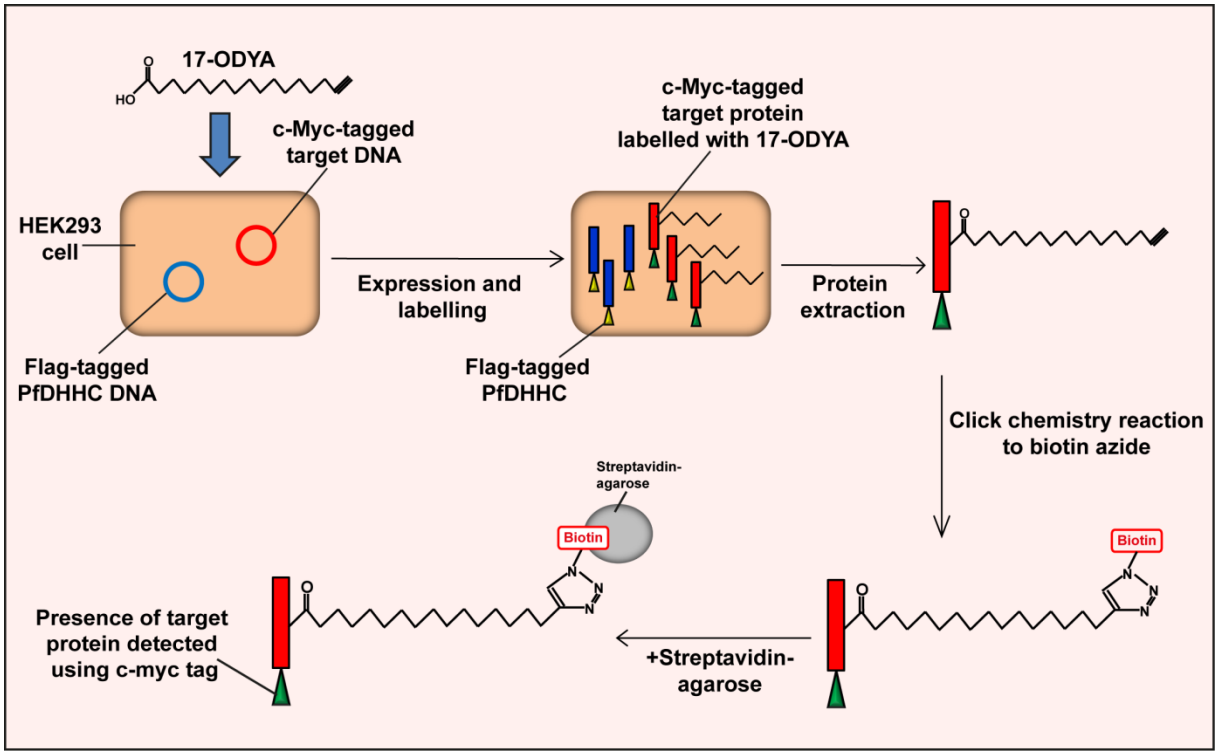

Figure 6.1: PAT activity assay. This assay incorporates the expression of codon-optimised *P. falciparum* proteins in a mammalian cell expression system along with metabolic labelling and click chemistry methods of palmitoyl-protein purification. HEK293 cells were co-transfected with FLAG-tagged PfDHHC DNA along with the c-Myc-tagged DNA of a potential target palmitoyl-protein, both of which were codon-optimised for expression in human cells. The HEK293 cells were then treated with the metabolic label, 17-ODYA. Proteins were extracted and reacted to biotin-azide under standard click chemistry conditions, resulting in the biotinylation of 17-ODYA-labelled proteins. Biotinylated proteins were affinity-purified using streptavidin-agarose and the presence of the target protein detected using antibodies against the c-Myc tag. As a standard control, transfected cells were also mock-labelled with DMSO instead of 17-ODYA. As a control for the background palmitoylation of target proteins by endogenous HEK293 PAT proteins, cells were also co-transfected with the c-Myc-tagged target protein and an empty CD4 control vector, in the absence of the PfDHHC. The level of target protein palmitoylation when in the presence of the PfDHHC compared to when in the absence of the PfDHHC was expected to be an indication of whether the particular PfDHHC was responsible for the palmitoylation of the target protein.

This assay was adapted from work described in [5], where tagged versions of human DHHC proteins of interest were co-expressed in mammalian cells, along with putative target proteins tagged with a different epitope tag, and metabolically labelled with 17-ODYA. In the previous assay described in [5], the target proteins were immunoprecipitated using their epitope tags and the click chemistry reaction was performed on the immunoprecipitates using rhodamine azide, which allows the visualisation of labelled proteins by in-gel fluorescence. Due to the lack of facilities for in-gel fluorescence, the PAT activity assay developed here was performed using biotin-azide in the click

chemistry reaction instead. The labelled proteins were then purified by streptavidin affinity purification and the presence of the proteins visualised using their C-terminal epitope tags. Additionally, click chemistry reactions were performed on total protein extracts rather than immunoprecipitates.

The effect of the presence of the putative PfDHHC protein on the palmitoylation of the target protein was determined by comparing the level of target protein palmitoylation in the presence, as well as in the absence of a given PfDHHC (that is, when the target protein was co-expressed with only the empty CD4 control vector). Due to the potential overlapping functionality of the DHHC-PATs, and how little is currently known about their substrate specificity in any eukaryotic species, some background palmitoylation of the target protein could occur due to endogenous PATs present in HEK293 cells. However, it was expected that if the PfDHHC of interest was responsible for palmitoylating the target protein, the amount of palmitoylated target protein would be greater when in presence of the PfDHHC, compared to when in the absence of the PfDHHC.

6.2. Expression and localisation of *P. falciparum* proteins in HEK293 cells

6.2.1. Expression of P. falciparum proteins in HEK293 cells

All five PfDHHC proteins which were studied in Chapter 5 –PfDHHC3, 5, 7, 8 and 9- were codon-optimised for expression in HEK293 cells and introduced into HEK293 expression vectors [6], along with sequence coding for a C-terminal FLAG tag in order to allow the detection of the PfDHHC proteins, as described in the Materials and Methods. The HEK293 expression vectors used here also contained immunoglobulin-like domains 3 and 4 of rat CD4 (thought to aid folding of heterologous proteins and usually also used as an epitope tag), which would normally be present following the open reading frame of the PfDHHC proteins [7]. However, as CD4 was not required for the purposes of this assay, a STOP codon was introduced downstream of the C-terminal FLAG tag and upstream of CD4. Thus the recombinant FLAG-tagged PfDHHC proteins were not expected to contain CD4.

A number of potential *P. falciparum* target proteins were considered based on their localisation in relation to the localisation of the PfDHHC proteins (established in Chapter 5) and on the identification of putative palmitoylated cysteines within the target proteins (described in Chapter 3). Due to time constraints, only two potential target proteins –PfSec22 (PFC0890w) and Armadillo Repeats-Only (ARO) (PFD0720w) - were studied here. PfSec22 is annotated as a SNARE protein containing a single TM domain in *Plasmo*DB (http://plasmodb.org). The site-ID palmitome

purification experiments (described in Chapter 3) identified a peptide from the N-terminal region of PfSec22, which contained two cysteine residues, Cys2 and Cys8, either or both of which could potentially be palmitoylated. PfSec22 has been found to be involved in ER to Golgi transport in *P. falciparum* and appears to be localised predominantly at the ER [8]. PfSec22 could therefore potentially be palmitoylated by PfDHHC5, which also localises to the ER, or PfDHHC3, which localises to the Golgi. PfARO is a dually-acylated protein which localises to the rhoptries, where it is attached to the membrane of the rhoptries by its myristate and palmitate anchors [9]. Although PfARO was not identified in the site-ID palmitome purification experiments (Chapter 3), this protein has been experimentally shown to be palmitoylated in *P. falciparum*, and either or both of two cysteines located within the first 20 N-terminal amino acids, Cys5 and Cys6, are thought to be the potential palmitoylation sites [9]. As PfARO appears to be targeted to the rhoptries, it could thus potentially be palmitoylated by PfDHHC7, which is also located at the rhoptries, or by PfDHHC3, which PfARO could potentially encounter in the Golgi during transport to the rhoptries. These two potential target proteins were also codon-optimised for expression in HEK293 cells and introduced, along with a C-terminal c-Myc tag, followed by a STOP codon, into the same HEK293 CD4-containing expression vector. An 'empty' expression vector containing only CD4 was used as the negative vector control for the target proteins.

6.2.2. Subcellular localisation of P. falciparum proteins expressed in HEK293 cells

In order to confirm that the *P. falciparum* proteins were expressed properly in HEK293 cells, as well as to determine where the parasite proteins localised to in these heterologous mammalian cells, immunofluorescence microscopy was performed on HEK293 cells transiently transfected with each of the proteins of interest, using antibodies against the FLAG tag and c-Myc tag respectively.

(1) Subcellular localisation of FLAG-tagged PfDHHC proteins expressed in HEK293 cells

All five PfDHHC proteins of interest (PfDHHC3, 5, 7, 8 and 9) were transfected into HEK293 cells using polyethylenimine (PEI). After 24 hours with the transfection mixture, cells were fixed in 4% formaldehyde and immunofluorescence staining was performed using antibodies against the FLAG tag. Fluorescence staining was detected for four of the PfDHHCs (PfDHHC3, 5, 7, and 9) (Figure 6.2A), indicating that these four parasite proteins were expressed in mammalian cells. Immunofluorescence signal was not detected for PfDHHC8. However, PfDHHC8 was also not detectable by both immunoblot and immunofluorescence assay when tagged in *P. falciparum* (Chapter 5). This, along with the lack of detection in HEK293 cells,

suggests that this protein may be expressed at levels that are too low for detection, even in mammalian cells, or else issues with protein solubility preclude its detection using the current extraction procedures.

The subcellular localisations of the four PfDHHC proteins were determined by comparing their immunofluorescence signals to that of two mammalian proteins with established localisations: calnexin (ER marker) [10] and cadherin (plasma membrane marker) [11] (Figure 6.2A). It must be noted however, that although the cadherins are integral membrane proteins found at the plasma membrane, they are mainly required for cell-cell adhesion by forming adherens junctions between cells. In these experiments, mainly single cells with no cell-cell junctions were studied, and thus membrane localisation of the cadherins may not be as clear. Nevertheless, staining of the HEK293 cells with antibodies against cadherin still displayed a mainly peripheral, and therefore presumably plasma membrane signal. A low level of cytoplasmic staining was also detected, which may be due to the presence of soluble cadherins, and such staining has been described by the antibody manufacturer (Abcam).

In HEK293 cells, PfDHHC3 staining co-localised with that of calnexin, suggesting an ER localisation (Figure 6.2A). In *P. falciparum*, this DHHC protein is targeted to the Golgi (Section 5.1.3). However, it appeared that when expressed in mammalian cells, this protein localised instead to the ER, perhaps because as a parasite protein, it may lack the mammalian exit signal that would direct it to the Golgi in mammalian cells.

PfDHHC5 staining did not co-localise with either calnexin or cadherin, indicating that this protein was not localised to the ER or the plasma membrane in HEK293 cells (Figure 6.2A). PfDHHC5 was localised to the ER in *P. falciparum* (Section 5.1.3). However, PfDHHC5 contains ankyrin repeats in its N-terminus, and other N-terminal ankyrin repeat-containing DHHC proteins, such as DHHC17 in humans [12] and Akr1 in yeast [2], have been localised to the Golgi. Thus, it would perhaps not be unexpected if PfDHHC5 were to be targeted to the Golgi when expressed in a mammalian cell. Although immunofluorescence co-staining was performed with antibodies against two different Golgi-localised proteins (syntaxin-6 and 58K Golgi protein) both these antibodies did not produce good Golgi immunofluorescence staining and unfortunately, the localisation of PfDHHC5 to the Golgi cannot be proven at this time.

PfDHHC7 co-localised with cadherin staining in HEK293 cells, indicating a plasma membrane localisation (Figure 6.2A). In *P. falciparum*, PfDHHC7 was localised to the rhoptries (Section 5.1.3), specialised organelles at the apical end of the parasite, close to the cell periphery. As no such organelle exists in mammalian cells, localisation to the plasma membrane instead is not unexpected.

Lastly, PfDHHC9 staining co-localised with that of calnexin (Figure 6.2), indicating that PfDHHC9 was localised to the ER in mammalian cells. In *P. falciparum* (Section 5.1.3) PfDHHC9 was localised to the IMC, another specialised parasite organelle consisting of flattened membrane-bound vesicles close to the plasma membrane. Although not much is known about the formation of the IMC, some proteins which associate with the IMC in *P. falciparum* have been shown to be trafficked to the IMC via the ER [13]. As the IMC does not exist in mammalian cells, it could be that in HEK293 cells, PfDHHC9 is retained in the ER instead.

(2) Subcellular localisation of c-Myc-tagged PfARO and PfSec22 in HEK293 cells

The two potential target proteins, PfARO and PfSec22, were transfected into HEK293 cells respectively using polyethylenimine (PEI). After 24 hours, cells were fixed in 4% formaldehyde and immunofluorescence staining was performed using antibodies against the c-Myc tag. Immunofluorescence staining was successfully detected for both proteins (Figure 6.2B), indicating that both these proteins were expressed in mammalian cells. The subcellular localisations of these two proteins were determined by comparing their immunofluorescence signals to that of two mammalian proteins with established localisations: calnexin (ER marker) [10] and cadherin (plasma membrane marker) [11].

PfARO staining appeared to be mainly cytoplasmic, with some partial co-localisation with cadherin at the cell periphery (Figure 6.2B), indicating that although mainly localised to the cytoplasm, some PfARO proteins were also localising to the plasma membrane. In *P. falciparum*, ARO is localised to the rhoptries, where it associates with the cytosolic face of the rhoptry membrane, most likely via the myristate and palmitate attachments on its N-terminal domain. Removal of either of these acylation motifs causes ARO localisation to become cytoplasmic [9]. As this protein has no TM-domains, and is being expressed in mammalian cells without its endogenous DHHC protein, the mainly cytoplasmic localisation suggests that the majority of PfARO is not palmitoylated, while the fraction which is localised to the plasma membrane could be due to background palmitoylation by endogenous HEK293 PATs.

PfSec22 co-localised with calnexin staining, indicating that this protein was localised to the ER in HEK293 cells (Figure 6.2B). PfSec22 is annotated on *Plasmo*DB as a SNARE protein, and has been found to be predominantly localised in the ER in *P. falciparum* [8]. Both yeast and human versions of Sec22 are involved in ER to Golgi transport, and are also mainly found in the ER membrane [14]. A protein-protein BLAST (blastp) search using the PfSec22 amino acid sequence revealed some homology to both yeast and human Sec22 (identities of approximately 30%). Thus, the localisation of PfSec22 to the ER membrane of HEK293 cells is expected.

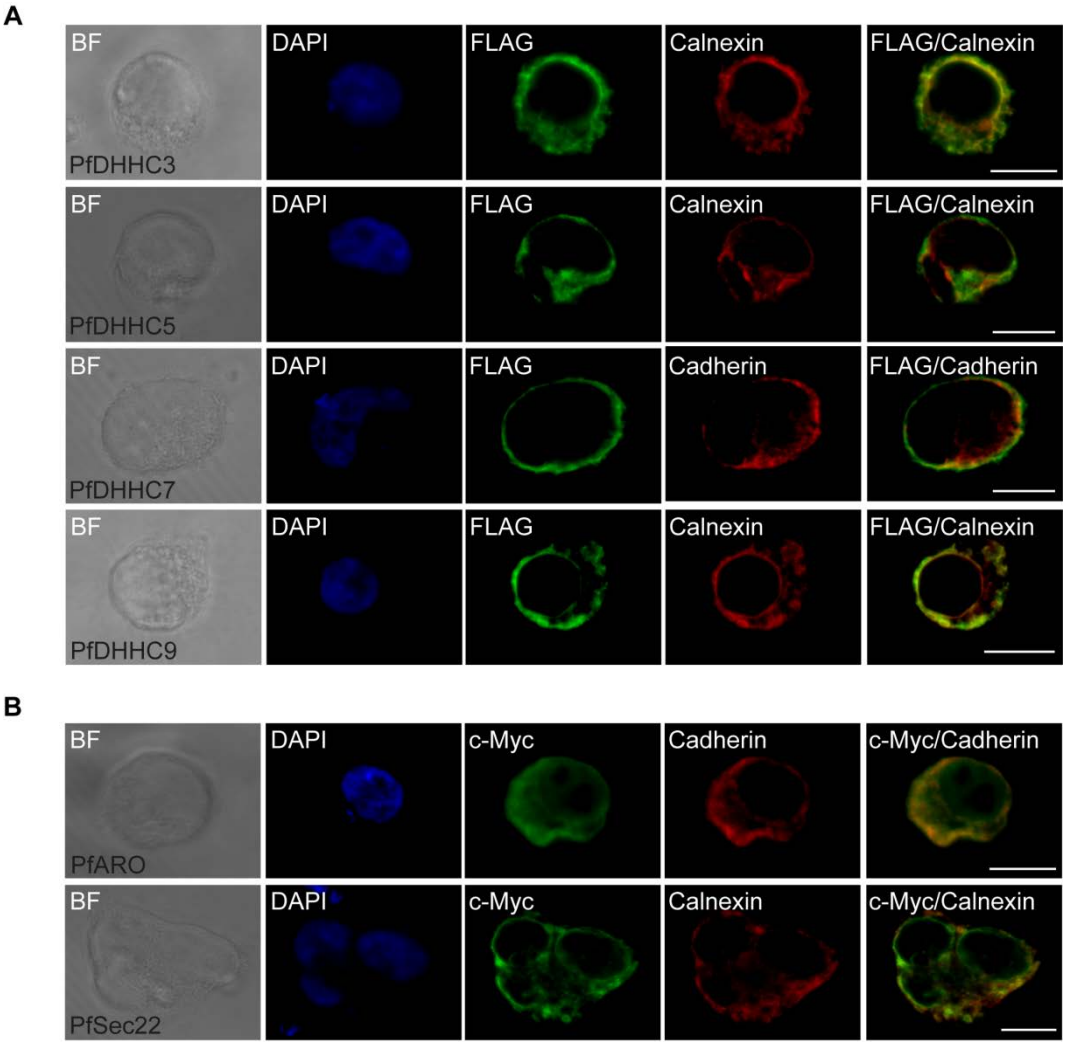

Figure 6.2: Localisation of codon-optimised *P. falciparum* proteins in HEK293 cells. (A) Codon-optimised *P. falciparum* DHHC proteins (PfDHHC3, 5, 7, and 9) were expressed in HEK293 cells and expression of the proteins determined by immunofluorescence assay using antibodies against the FLAG tag. Localisation of the PfDHHC proteins (green) was determined by comparing the immunofluorescence signal to that of the following known localisation markers (red): calnexin (endoplasmic reticulum marker) and cadherin (plasma membrane marker). Nuclear staining by DAPI is shown in blue. **(B)** Codon-optimised *P. falciparum* proteins PfARO and Pfsec22 were expressed in HEK293 cells. Expression was determined by immunofluorescence assay using antibodies against the c-Myc tag. Both PfARO and PfSec22 (green) were localised by immunofluorescence against the following mammalian localisation markers (red): calnexin (endolasmic reticulum marker) and cadherin (plasma membrane marker). Nuclear staining by DAPI is shown in blue. Scale bar: 10 µm.

In summary, all the *P. falciparum* proteins of interest, with the exception of PfDHHC8, were successfully expressed using the HEK293 mammalian cell expression system, albeit with quite different intracellular localisations compared to their endogenous localisations in *P. falciparum*, highlighting a major confounder of heterologous expression systems. PfDHHC3 and 9 localised to the ER, PfDHHC7 localised to the plasma membrane and PfDHHC5 may be potentially localised to the Golgi. Meanwhile, the two potential target proteins, PfARO and PfSec22, were also successfully expressed using the HEK293 cell expression system. PfARO displayed a mostly cytoplasmic localisation, with some localisation at the plasma membrane, while PfSec22 was localised to the ER.

6.2.3. Immunoprecipitation of c-Myc-tagged PfSec22 and PfARO in the presence of PfDHHC proteins

In order to determine whether the *P. falciparum* potential target proteins could be visualised by immunoblot using antibodies against the c-Myc tag, PfSec22 and PfARO were expressed in HEK293 cells, each either in the presence of one of the PfDHHC proteins (PfDHHC3, 5, 7, 8 and 9), or in the presence of the empty CD4 control vector. PfSec22 and PfARO were then immunoprecipitated from the protein extracts using antibodies against the c-Myc tag. The immunoprecipitates were separated by SDS-PAGE and the presence of PfSec22 and PfARO was determined by immunoblot, using antibodies against the c-Myc tag from a different species.

PfSec22 is predicted to be 26 kDa, and previously published immunoblots of *P. falciparum* protein extracts with polyclonal antibodies raised against recombinant PfSec22 produced bands running at approximately this size [8]. However, this analysis of recombinant PfSec22 immunoprecipitated from HEK293 cells, using α-c-Myc tag antibodies, resulted in two clear bands: a stronger lower molecular weight band at approximately 10 kDa and a weaker higher molecular weight band at approximately 17 kDa (Figure 6.3). This discrepancy in apparent molecular weight could be due to the fact that this parasite protein is being expressed recombinantly in mammalian cells, where the protein folding and processing may differ from that of parasite cells, thus resulting in the aberrant migration of the protein. Additionally, post-translational modifications of the protein which occur in the parasite might not occur when the protein is expressed in mammalian cells and *vice versa*, thus causing a difference in the expected molecular weight. Alternatively, the difference in apparent molecular weight could be due to aberrant migration because of the presence of coiled-coil structure in the PfSec22 SNARE protein.

The presence of two clear bands raises the question as to whether the higher molecular weight band corresponds to the palmitoylated version of PfSec22. Although protein palmitoylation does not

consistently result in a shift in molecular weight, this has been shown to occur in some cases. For example, immunoprecipitation of the palmitoylated *P. falciparum* invasion motor complex protein, GAP45, from *P. falciparum* schizonts, produces two bands, and only the higher molecular weight band of the doublet corresponds to palmitoylated GAP45 [15]. Interestingly, the intensity of the higher molecular weight band of PfSec22 immunoprecipitated from HEK293 cells was strongest when PfSec22 was co-expressed with PfDHHC5, and the higher molecular weight band was readily detected when PfSec22 was co-expressed with PfDHHC3 and 7. In contrast, the higher molecular weight band was only weakly detected when PfSec22 was co-expressed with PfDHHC8 and 9, and the empty CD4 control vector. If indeed the higher molecular weight band is palmitoylated PfSec22, this difference in intensity of the band may be an indication of which PfDHHC is responsible for palmitoylating PfSec22.

PfARO is predicted to be 30 kDa, and previous work involving the immunoblot of *P. falciparum* schizonts, using antibodies against PfARO, has produced bands migrating at the expected size [9]. Here, PfARO immunoprecipitated from HEK293 cells using α-c-Myc antibodies revealed two clear bands: a lower molecular weight band at approximately 28 kDa and a higher molecular weight band at approximately 45 kDa. Additionally a faint third band is observed above the 46 kDa marker (Figure 6.3). The discrepancy in the molecular weight of the 28 kDa band from the predicted molecular weight of PfARO is much smaller than the discrepancy of PfSec22 above, but could also be due to differences in protein folding or processing when expressed recombinantly in a mammalian cell line. As with PfSec22, the higher molecular weight band (at approximately 45 kDa) could be due to palmitoylation of PfARO. The intensity of the 45 kDa band was strongest when PfARO was co-expressed with PfDHHC3 and 5, and the intensity of the band was weaker when co-expressed with PfDHHC7 and 9. The 45 kDa band was weakest when PfARO was co-expressed with PfDHHC8 and with the empty vector.

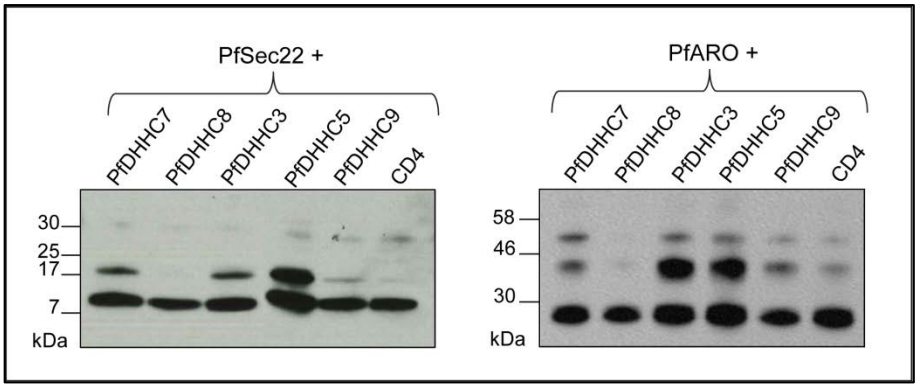

Figure 6.3: Immunoprecipitation of PfSec22 and PfARO proteins using antibodies against the c-Myc tag. HEK293 cells were co-transfected with plasmids coding for the expression of c-Myc-tagged PfSec22 or PfARO, along with the indicated FLAG-tagged PfDHHC proteins (PfDHHC3, 5, 7, 8 and 9) or the empty control vector (CD4). Pfsec22 and PfARO were immunoprecipitated from cell lysates using an α-c-Myc antibody. The proteins were separated by SDS-PAGE and visualised by immunoblot, using α-c-Myc antibody from a different species.

In summary, both PfSec22 and PfARO could be successfully expressed in HEK293 cells and immunoprecipitation of both these proteins resulted in two distinct bands. For both these proteins, the higher molecular weight band differed in intensity depending on which PfDHHC it was co-expressed with, which could suggest specificity in their palmitoylation. In order to directly test whether these higher molecular weight bands were actually the palmitoylated versions of the target proteins, 17-ODYA metabolic labelling and click chemistry reactions were carried out on co-transfected cells in order to perform the PAT activity assay described above.

6.3. PAT activity assay of PfDHHC proteins with PfSec22 and PfARO

The PAT activity assay (as described above) was performed for both PfSec22 and PfARO, co-transfected with each of the four PfDHHC proteins that could be expressed in HEK293 cells (PfDHHC3, 5, 7 and 9), or a CD4 empty vector control. As the expression of PfDHHC8 was unable to be detected in HEK293 cells by immunofluorescence microscopy, suggesting low expression of the protein, any difference observed in the palmitoylation of the target protein could thus also be due to the lack of expression of PfDHHC8, rather than the fact that PfDHHC8 did not palmitoylate the particular target. Thus, PfDHHC8 was not used for any further analysis.

6.3.1. PAT activity assay of PfDHHC proteins with PfSec22

PfSec22 was co-expressed in HEK293 cells in the presence of each of the four PfDHHCs (PfDHHC3, 5, 7 and 9), or with only the CD4 empty vector control. Eighteen hours post-transfection, cells were metabolically labelled with 17-ODYA, or treated with DMSO as a control, for a further six hours, after which cells were lysed for protein extraction. An aliquot of each whole cell lysate was kept for confirmation of protein expression. The PAT activity assay was performed on the remaining cell lysates as described above and in the Materials and Methods. Both the initial cell lysates and the PAT activity assay click chemistry elutions were separated by SDS-PAGE and the presence of PfSec22 was specifically determined by immunoblot using antibodies against the c-Myc tag.

Immunoblot of the initial cell lysates indicated the presence of the two bands observed previously (Section 6.2.3): a lower molecular weight band at approximately 10 kDa and a higher molecular weight band at approximately 17kDa (Figure 6.4). However, immunoblot of the elutions after the click chemistry reaction was performed resulted in a single band running at approximately 17 kDa, and this band was present only in samples treated with 17-ODYA and not in the samples treated with DMSO (Figure 6.4). The detection of this band only in 17-ODYA-treated samples and not in DMSO-treated samples confirmed that the 17 kDa band corresponds to palmitoylated PfSec22. The molecular weight of the palmitoylated PfSec22 band corresponded exactly with the higher molecular weight band observed in the initial cell lysate and in the immunoprecipitation analysis of PfSec22 described above (Section 6.2.3 and Figure 6.3). Therefore, this indicated that the higher molecular weight band visible in immunoblots and immunoprecipitates was the palmitoylated version of PfSec22, and the absence of any lower molecular weight band in the 17-ODYA-labelled samples confirms that the lower molecular weight 10 kDa band was non-palmitoylated PfSec22.

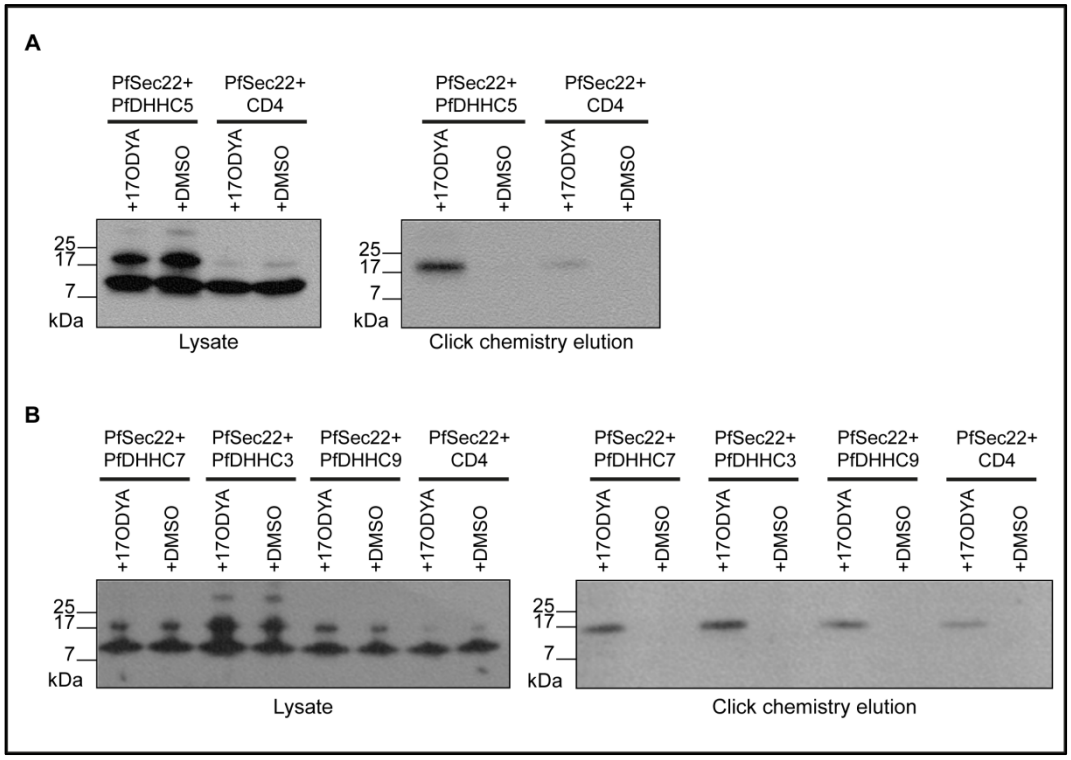

Figure 6.4: PAT activity assay demonstrating the PAT activity of PfDHHC proteins on the target protein, PfSec22. HEK293 cells were co-transfected with plasmids expressing c-Myc-tagged PfSec22, along with the indicated FLAG-tagged PfDHHC protein or the empty vector control (CD4). Cells were either treated with the metabolic label, 17-ODYA, or mock-treated with DMSO. Proteins were extracted and an aliquot of each lysate kept aside to confirm protein expression. The remaining lysates were put through click chemistry reactions to biotin-azide, which resulted in the biotinylation of 17-ODYA-labelled proteins. These biotinylated proteins were subsequently affinity purified by streptavidin-agarose and eluted by boiling in SDS. Samples from the initial whole cell lysates and the click chemistry elutions were separated by SDS-PAGE and the presence of c-Myc-tagged PfSec22 in each of the samples was observed by immunoblot using antibodies against the c-Myc tag. **(A)** HEK293 cells co-transfected with c-Myc-PfSec22 + FLAG-PfDHHC5 or c-Myc-PfSec22 + CD4 empty vector. **(B)** HEK293 cells co-transfected with c-Myc-PfSec22 + FLAG-PfDHHC7, c-Myc-PfSec22 + FLAG-PfDHHC3, c-Myc-PfSec22 + FLAG-PfDHHC9 or c-Myc-PfSec22 + CD4 empty vector.

The intensity of the PfSec22 palmitoylated band in the click chemistry elutions was clearly greater when PfSec22 was co-expressed with PfDHHC5 than when PfSec22 was co-expressed with the empty vector (Figure 6.4A). This indicates that while there may be some background level of PfSec22 palmitoylation by endogenous HEK293 PATs, the co-expression of PfSec22 with PfDHHC5 significantly increased the palmitoylation of PfSec22. This is the first direct proof that a *P falciparum* DHHC protein has PAT activity.

PfDHHC3, 7 and 9 all similarly increased the intensity of the palmitoylated PfSec22 band, compared to when PfSec22 was co-transfected with the empty control vector (Figure 6.4B). This suggests that

all four PfDHHCs tested can palmitoylate PfSec22 and demonstrates the PAT activity of these 4 PfDHHC proteins.

While the ability to palmitoylate PfSec22 was clearly not restricted to a single PfDHHC, the increase in the intensity of the PfSec22 palmitoylated band in the click chemistry elutions above the intensity of the band due to background palmitoylation appeared to be greatest when PfSec22 was co-expressed with PfDHHC5 (Figure 6.4A). However, it must be noted that these click chemistry elutions are not strictly quantitative, as the intensity of the band is also affected by the efficiency of the metabolic labelling. Additionally, each lane is protein material taken from different transfections, and each transfection was individually streptavidin affinity purified, which may lead to differences in the amount of protein present. These differences cannot be completely discounted when a difference in band intensity is observed. Furthermore, the metabolic label 17-ODYA is actually an inhibitor of cytochrome P450 metabolism [16]. Cells treated with 17-ODYA are thus generally less healthy, and treatment with 17-ODYA appears have a slight effect on cell growth and protein expression, especially if treatment is excessive. These effects on cell growth and protein expression can affect the amount of protein produced for the PAT assay and can thus also contribute to differences in band intensity.

Nevertheless, even in the click chemistry elutions, the intensity of the PfSec22 palmitoylated band was consistently greater when PfSec22 was in the presence of the PfDHHC proteins, indicating that these DHHC proteins were indeed palmitoylating PfSec22. Additionally the difference in the intensities of the higher molecular weight palmitoylated band of PfSec22, in the presence of the different DHHC proteins, was also previously observed when PfSec22 was immunoprecipitated and analysed by immunoblot (Figure 6.3), as described in Section 6.2.3. In this case, as the cells were not treated with either 17-ODYA or DMSO, the difference in band intensity can be assumed to be mainly due to the presence of the particular PfDHHC protein.

Taken together, the results from the immunoprecipitation of PfSec22 and the metabolic labelling and click chemistry reaction suggest that of the PfDHHCs tested, the palmitoylation of PfSec22 appears to be greater in the presence of PfDHHC5, followed by PfDHHC3 and 7. Although PfDHHC9 also appears to be able to palmitoylate PfSec22, the amount of palmitoylation seems very low, and PfDHHC9 may not normally palmitoylate PfSec22 in the parasite. The palmitoylation of PfSec22 by 3 different PfDHHC proteins is not surprising given that DHHC-PATs are known to have overlapping functionalities in other organisms and may act redundantly by palmitoylating similar substrates [17].

The palmitoylation of PfSec22 by PfDHHC5 (which was shown to localise to the ER in *P. falciparum*, as described in Chapter 5) and PfDHHC3 (which was shown to localise to the Golgi in *P. falciparum*, as described in Chapter 5) is consistent with the fact that PfSec22 is a SNARE protein involved in transport between the ER and Golgi, and is mainly localised in the ER. Palmitoylation of PfSec22 by PfDHHC7 is more unexpected as PfDHHC7 is localised to the rhoptries in *P. falciparum*. However, due to their different localisations, PfSec22 would probably not come in contact with PfDHHC7. Thus, this may be an example of how the particular localisation of the DHHC protein might in part regulate the target proteins that it palmitoylates.

6.3.2. PAT activity assay of PfDHHC proteins with PfARO

PfARO was co-expressed in HEK293 cells in the presence of each of the four PfDHHCs (PfDHHC3, 5, 7, and 9), or with only the CD4 empty vector control. Eighteen hours post-transfection, cells were metabolically labelled with 17-ODYA or treated with DMSO as a control, for a further 6 hours, after which cells were lysed for protein extraction. An aliquot of each whole cell lysate was kept for confirmation of protein expression. The PAT activity assay was performed on the remaining cell lysates, as described above and in the Materials and Methods. Both the initial cell lysates and the PAT assay click chemistry elutions were separated by SDS-PAGE, and the presence of PfARO was specifically determined by immunoblot using antibodies against the c-Myc tag.

Immunoblot of the initial cell lysates indicated the presence of the same bands observed previously for PfARO (Section 6.2.3): a lower molecular weight band at approximately 28 kDa and a higher molecular weight band at approximately 45 kDa, along with a faint third band just above the 46 kDa marker (Figure 6.5). However, immunoblot of the elutions after click chemistry was performed resulted in a single band running at approximately 45 kDa, and this band was present only in samples treated with 17-ODYA and not in the samples treated with DMSO (Figure 6.5). The detection of this band only in 17-ODYA-treated samples and not in DMSO-treated samples indicated that this band corresponded to palmitoylated PfARO. As the apparent molecular weight of this band was approximately 45 kDa, this band corresponded to the 45 kDa molecular weight band observed in the initial cell lysate and in the immunoprecipitation analysis of PfARO (Figure 6.3). Therefore, this indicated that this 45 kDa band was the palmitoylated version of PfARO, and the lower molecular weight band at approximately 28 kDa must then correspond to non-palmitoylated PfARO. The faint third band above the 46 kDa marker was not observed in the click chemistry elutions, indicating that it is not likely to be corresponding to a doubly- or multiply-palmitoylated version of PfARO. This third

band could instead perhaps correspond to the formation of a dimer, or perhaps to a version of PfARO which was modified by a different PTM, such as phosphorylation, which could also cause a shift in the apparent molecular weight.

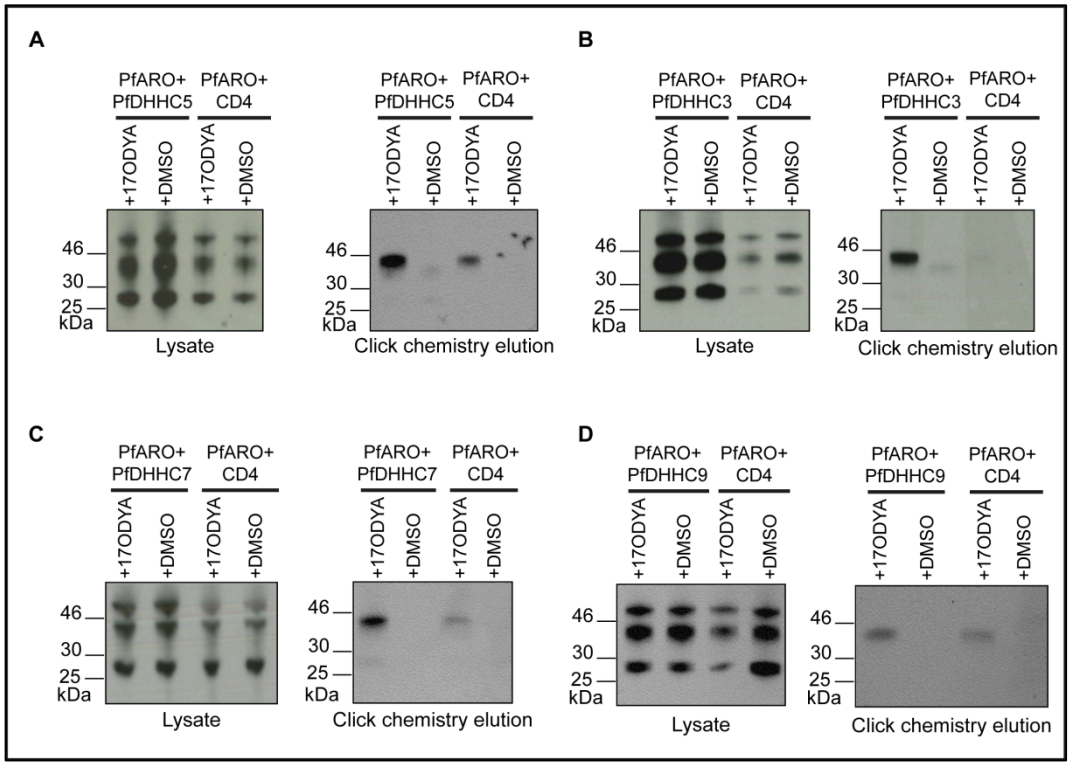

Figure 6.5: PAT activity assay demonstrating the PAT activity of PfDHHC proteins on the target protein, PfARO. HEK293 cells were co-transfected with plasmids expressing c-Myc-tagged PfARO, along with the indicated FLAG-tagged PfDHHC protein or the empty vector control (CD4). Cells were either treated with the metabolic label, 17-ODYA, or mock-treated with DMSO. Proteins were extracted and an aliquot of each lysate kept aside to confirm protein expression. The remaining lysates were put through click chemistry reactions to biotin-azide, which resulted in the biotinylation of 17-ODYA-labelled proteins. Biotinylated proteins were subsequently affinity purified by streptavidin-agarose and eluted by boiling in SDS. Samples from the initial lysates and the click chemistry elutions were separated by SDS-PAGE and the presence of c-Myc-tagged PfARO in each of the samples was observed by Western blot using antibodies against the c-Myc-tag. **(A)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC5 or c-Myc-PfARO + CD4 empty vector. **(B)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC3 or c-Myc-PfARO + CD4 empty vector. **(C)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC7 or c-Myc-PfARO + CD4 empty vector. **(D)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC9 or c-Myc-PfARO + CD4 empty vector.

The intensity of the PfARO palmitoylated band in the click chemistry elutions was clearly greater when PfARO was co-expressed with PfDHHC5 compared to when PfARO was co-expressed with the empty vector (Figure 6.5A). The same thing was also observed for PfDHHC3, 7 and 9, where the intensity of the PfARO palmitoylated band was greater when PfARO was co-expressed with each of these DHHC proteins, compared to when co-expressed with the empty vector (Figure 6.5B, C and D). Unfortunately, in these experiments with PfARO, immunoblot of the initial cell lysates indicated that protein expression in each of the different transfection and labelling conditions appeared to vary, perhaps due to differences in cell numbers or in the health of the cells. This difference in protein expression could contribute to the difference seen in the intensity of the PfARO palmitoylated band in the click chemistry elutions and this means that the increased intensity of the PfARO palmitoylated band in the presence of the PfDHHCs over that of the bands due to background palmitoylation may not be completely due to palmitoylation by the PfDHHCs, but due in part also to the differences in protein expression.

However, as in the case of PfSec22, the difference in the intensities of the higher molecular weight (45 kDa) palmitoylated band of PfARO, in the presence and absence of the different PfDHHC proteins, was also observed when PfARO was immunoprecipitated and analysed by immunoblot (Figure 6.3), as described in Section 6.2.3. In the immunoblot of immunoprecipitated PfARO, protein expression in each transfection condition appeared to be more similar, and thus should not contribute as much to the differences in the intensity of the PfARO palmitoylated band. In this case, the intensity of the PfARO palmitoylated band appeared to be greater in the presence of PfDHHC3, 5, 7 and 9, compared to the intensity of the palmitoylated band in the absence of any PfDHHC (Figure 6.3). This suggests that like PfSec22, PfARO was also able to be palmitoylated by at least 4 different PfDHHC proteins, further demonstrating the potential overlapping functionality of the DHHC proteins.

In summary, the use of the PAT assay with PfARO as a target has again demonstrated the PAT activity of the four *P. falciparum* DHHC proteins studied here –PfDHHC3, 5, 7 and 9. Additionally, the intensity of the PfARO palmitoylated band appeared to be the greatest in the presence of PfDHHC3 and 5 (Figure 6.3), implying that of the four PfDHHCs tested in this PAT activity assay, PfDHHC3 and 5 appeared to have the most PAT activity towards PfARO. As PfARO localises to the rhoptries in *P. falciparum*, it was expected that palmitoylation of PfARO might mainly be performed by PfDHHC7, which also localises to the rhoptries in the parasite. However, the results of the PAT assay show that PfDHHC5 (which localises to the ER) and PfDHHC3 (which localises to the Golgi) appeared to have strong PAT activity towards PfARO, implying that PfARO might be palmitoylated when in the

secretory pathway instead, before attachment to the rhoptry membrane. PfDHHC7 meanwhile may be required for the palmitoylation of other rhoptry proteins. For example, RAP1, another rhoptry-localising protein, was identified as palmitoylated by the site-ID palmitome purifications (described in Chapter 3), and could potentially be a target for PfDHHC7. Alternatively, another possibility could be that some of these parasite DHHC proteins could perhaps be non-functional when expressed in a mammalian cell expression system. It is interesting that PfDHHC5 was clearly able to palmitoylate both PfARO as well as PfSec22, perhaps indicating better expression, or increased activity, of this particular PfDHHC protein in mammalian cells compared to others.

Lastly, PfDHHC9, which localises to the IMC in *P. falciparum*, did not appear to have strong PAT activity towards either PfARO or PfSec22 (at least in these PAT activity assays), despite exhibiting an ER localisation in HEK293 cells. This indicates that despite the change in localisation, which allows PfDHHC9 to come in contact with potential targets in the ER, palmitoylation of these targets does not occur as much as when in the presence of the other PfDHHCs, suggesting that PfDHHC9 may possibly only palmitoylate a specific set of target proteins, which are likely to be IMC-localised proteins. This could perhaps be an example of the potential substrate specificity of this DHHC protein, although this is by no means proven at this time.

6.4. Point mutations of potential palmitoylation sites in PfSec22 and PfARO

The results described above have shown that PfSec22 and PfARO are indeed palmitoylated proteins and are palmitoylated mainly by PfDHHC3 and 5. For both these palmitoyl-proteins, evidence exists indicating that cysteine residues in the N-terminal regions of these proteins may potentially be the sites of palmitoylation, as described in Section 6.2.1. In order to determine which cysteine residues in both these target proteins were the palmitoylated cysteines, point mutations, which mutated the potentially palmitoylated cysteine residue into an alanine residue, were performed on the PfSec22 and PfARO HEK293 expression constructs, as described in the Materials and Methods. These mutated target proteins were then expressed in HEK293 cells, along with the appropriate PfDHHC protein, in order to determine whether any difference in the level of target protein palmitoylation was observed. If the particular cysteine residue was indeed the site of palmitoylation, it was expected that mutation of that cysteine residue into an alanine residue would result in a decrease in the level of target protein palmitoylation.

6.4.1. Point mutation of Cys2 in PfSec22 does not affect PfSec22 palmitoylation by PfDHHC5

As mentioned in Section 6.2.1, two cysteines in the N-terminal region of PfSec22 (Cys2 and Cys8) were identified as potentially palmitoylated by the site-ID palmitome purification experiments described in Chapter 3. In order to determine whether Cys2 was the site of PfSec22 palmitoylation, a point mutant was made, where Cys2 was mutated into an alanine residue, and co-expressed in HEK293 cells along with PfDHHC5, the DHHC protein which produced the greatest increase in PfSec22 palmitoylation over background palmitoylation (Section 6.3.1). The PfSec22 Cys2 point mutant (named PfSec22-C2dA) was then immunoprecipitated from protein extracts using antibodies against the c-Myc tag, and visualised by immunoblot using antibodies against the c-Myc tag from a different species.

Immunoblot analysis of PfSec22-C2dA immunoprecipitates resulted in the same two bands observed previously (Section 6.2.3): the lower molecular weight band running at approximately 10 kDa and the higher molecular weight band running at approximately 17 kDa, which was determined above to correspond to the palmitoylated version of the protein (Figure 6.6A). In the presence of PfDHHC5, there was no difference in the intensity of the palmitoylated band of PfSec22-C2dA compared to the intensity of the palmitoylated band of wild-type PfSec22 (Figure 6.6A). Additionally the intensity of the PfSec22-C2dA palmitoylated band (when co-expressed with PfDHHC5) was still greater than that of the band due to background palmitoylation (when wild-type PfSec22 was co-expressed with the empty vector) (Figure 6.6A). This indicates that the mutation of Cys2 into an alanine residue did not have an effect on the level of PfSec22 palmitoylation by PfDHHC5. It can thus be inferred that Cys2 of PfSec22 does not appear to be the site of palmitoylation. This suggests that Cys8 is likely to be the palmitoylated cysteine instead. Although it was intended to perform a point mutation of Cys8 as well, due to time contraints, the data was not yet available during the writing of this dissertation.

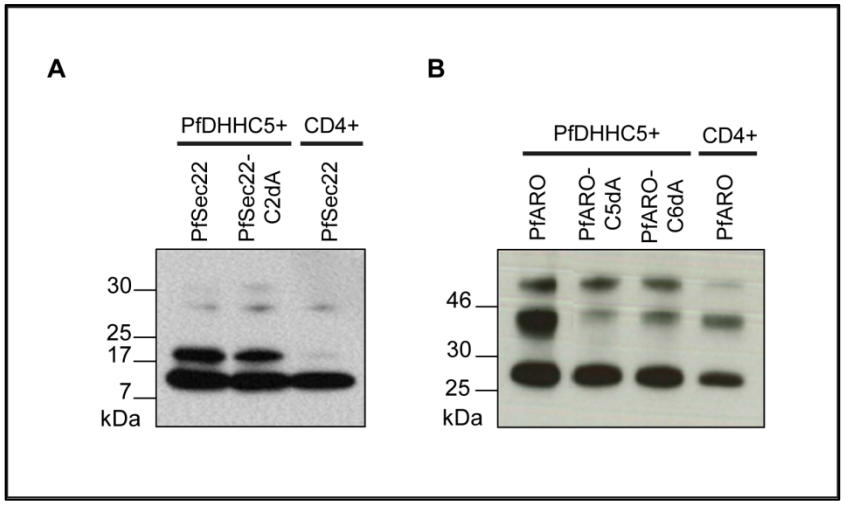

Figure 6.6: Immunoprecipitation of mutant PfSec22 and PfARO proteins using antibodies against the c-Myc tag. HEK293 cells were co-transfected with plasmids coding for the expression of mutant c-Myc-tagged PfSec22 or PfARO (which had the predicted palmitoylated cysteine residues point mutated to alanine residues), along with FLAG-tagged PfDHHC5 or the empty vector (CD4). The mutant PfSec22 and PfARO proteins were immunoprecipitated from cell lysates using an α-c-Myc antibody. The proteins were separated by SDS-PAGE and visualised by immunoblot, using α-c-Myc antibody from a different species. **(A)** HEK293 cells co-transfected with c-Myc-PfSec22 + FLAG-PfDHHC5, c-Myc-PfSec22-C2dA + FLAG-PfDHHC5 or c-Myc-PfSec22 + CD4 empty vector. **(B)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC5, c-Myc-PfARO-C5dA + FLAG-PfDHHC5, c-Myc-PfARO-C6dA + FLAG-PfDHHC5 or c-Myc-PfARO + CD4 empty vector.

6.4.2. Point mutation of Cys5 and Cys6 in PfARO reduces palmitoylation by PfDHHC5

PfARO has been previously shown to be palmitoylated, and two cysteine residues in the N-terminal region of PfARO (Cys5 and Cys6) are thought to be the potential sites of palmitoylation [9]. In order to determine whether Cys5 or Cys6 was the palmitoylated cysteine, point mutants were made for both Cys5 and Cys6, where each cysteine residue was mutated into an alanine residue individually. Each of these point mutants were co-expressed in HEK293 cell, again along with PfDHHC5. The PfARO Cys5 point mutant (name PfARO-C5dA) and the PfARO Cys6 point mutant (named PfARO-C6dA) were each immunoprecipitated from protein extracts using antibodies against the c-Myc tag, and visualised by immunoblot using antibodies against the c-Myc tag from another species, as described above.

Immunoblot of the immunoprecipitates from both PfARO point mutants resulted in the same bands as previously observed (Section 6.2.3): the lower molecular weight band running at approximately 28 kDa and the higher molecular weight band running at approximately 45 kDa, which was determined above to correspond to the palmitoylated version of the protein (Section 6.3.2) (Figure 6.6B).

Additionally, the third band running above the 46 kDa marker was also present in the immunoprecipitates from both point mutants (Figure 6.6B).

In the presence of PfDHHC5, there was a decrease in the intensity of the palmitoylated band of PfARO-C5dA compared to the intensity of the palmitoylated band of wild-type PfARO (Figure 6.6B). In fact, the intensity of the PfARO-C5dA palmitoylated band did not appear to be greater than that of the band due to background palmitoylation (when wild-type PfARO was co-expressed with only the empty vector control, not with PfDHHCs) (Figure 6.6B). This indicated that the mutation of Cys5 to an alanine residue appeared to affect the level of PfARO palmitoylation by PfDHHC5, thus suggesting that Cys5 may be a palmitoylated cysteine.

The same result was observed for the PfARO-C6dA point mutant, where in the presence of PfDHHC5, there was a decrease in the intensity of the palmitoylated band compared to that of wild-type PfARO (Figure 6.6B). In this case as well, the intensity of the PfARO-C6dA palmitoylated band did not appear to be greater than that of the band due to background palmitoylation (Figure 6.6B). This indicated that the mutation of Cys6 into an alanine residue also appeared to affect the level of palmitoylation by PfDHHC5, suggesting therefore that Cys6 of PfARO may also be a palmitoylated cysteine.

The results described here suggest that both cysteine residues (Cys5 and Cys6) in the N-terminal region of PfARO appear to be the sites of palmitoylation, and mutation of either of these cysteine residues into alanine residues results in a decrease in the level of PfARO palmitoylation. Presumably, the continued presence of the palmitoylated band when either of the cysteine residues was individually mutated could be due to the palmitoylation of the non-mutated cysteine residue. It would be interesting to determine whether, if both cysteine residues were to be simultaneously mutated, the palmitoylated band would no longer be present, indicating that palmitoylation had been abolished. This would further confirm that both Cys5 and Cys6 of PfARO were in fact the sites of palmitoylation. Unfortunately, due to time constraints, this data was not yet available at the time of writing this dissertation.

6.7. Site-directed mutagenesis of the cysteine in the DHHC domain of PfDHHC5

Not much is known about the exact mechanism of palmitate transfer by the DHHC proteins, and the role played by the highly conserved DHHC signature motif is as yet unknown [18]. Most of the DHHC proteins studied so far have been found to be palmitoylated when incubated individually with

palmitoyl-CoA *in vitro*, indicating that these DHHC proteins are autopalmitoylated [1-3, 19]. This led to the hypothesis that palmitate transfer occurs via a palmitoyl-enzyme intermediate. As autopalmitoylation is abolished when the cysteine residue of the DHHC domain is mutated, that cysteine residue is thought to be a possible candidate for the site of modification during the formation of the palmitoyl-enzyme intermediate, although autopalmitoylation of another cysteine residue is also possible [18].

In order to determine whether the cysteine residue in the DHHC domain of the PfDHHC proteins was important for the palmitoylation of target proteins, site-directed mutagenesis was performed in order to mutate that cysteine residue into an alanine residue in the PfDHHC5 HEK293 expression construct, as described in the Materials and Methods. The PfDHHC5 point mutant (named PfDHHC5-CdA) was then co-expressed with PfSec22 or PfARO in HEK293 cells. The target proteins were immunoprecipitated from protein extracts using antibodies against the c-Myc-tag, and visualised by immunoblot using α-c-Myc antibodies from a different species, in order to determine if any difference in target protein palmitoylation was observed.

Immunoblot of PfSec22 resulted in the same two bands as observed previously: one band running at approximately 10 kDa and a second band running at approximately 17 kDa, which was shown above to correspond to palmitoylated PfSec22. In the presence of PfDHHC5-CdA, the intensity of the palmitoylated band of PfSec22 appeared to be the same as the intensity of the palmitoylated band of PfSec22 when in the presence of wild-type PfDHHC5 (Figure 6.7A). The palmitoylated band of PfSec22 due to background palmitoylation (when PfSec22 was co-expressed with the empty CD4 vector) was not clearly seen here, although background palmitoylation of PfSec22 was observed to occur in other experiments. However, in this experiment, protein expression as a whole appeared to be lower than normal, and this was probably why this lower intensity background palmitoylated band was not seen in this case. Nevertheless, the palmitoylated band due to palmitoylation by PfDHHC5-CdA was clearly present, and was greater than when PfSec22 was co-expressed with the empty CD4 vector (Figure 6.7A). This indicates that mutation of the cysteine residue of the DHHC domain did not appear to affect the level of PfSec22 palmitoylation, which argues against a role for this residue in the formation of the palmitoyl-enzyme intermediate, or at least a role that is absolutely required for enzyme activity.

Immunoblot of PfARO again resulted in the same bands as observed previously: one band running at approximately 28 kDa and one band running at approximately 45 kDa, which was shown previously to correspond to the palmitoylated version of PfARO. In this case, the third band running above the 46 kDa marker that was observed in previous experiments was not observed here. However, as

mentioned above, protein expression in this experiment was relatively lower than normal, and as a consequence, this band, which was usually of a lower intensity, could not be seen here. In the presence of the PfDHHC5-CdA, the intensity of the palmitoylated band of PfARO appeared to be the same as the intensity of the palmitoylated band when in the presence of wild-type PfDHHC5 (Figure 6.7B). The intensity of the palmitoylated band was also greater than that of the band due to background palmitoylation (when PfARO was co-expressed with the CD4 empty vector) (Figure 6.7B). Again, this indicates that the mutation of the cysteine residue in the DHHC domain did not appear to affect the level of PfARO palmitoylation.

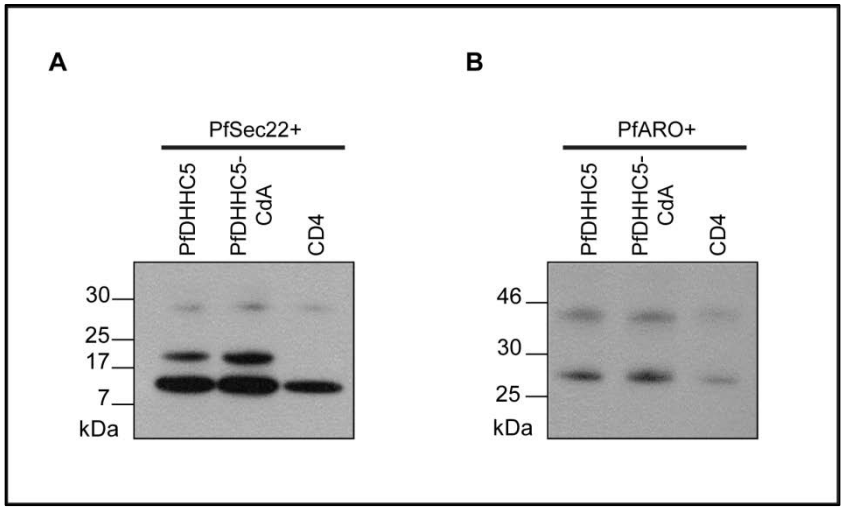

Figure 6.7: Immunoprecipitation of PfSec22 and PfARO proteins when co-expressed with mutant PfDHHC5 using antibodies against the c-Myc tag. HEK293 cells were co-transfected with plasmids coding for the expression of c-Myc-tagged PfSec22 or PfARO along with FLAG-tagged mutant PfDHHC5 (which had the cysteine residue of the DHHC domain mutated into an alanine residue) or the empty vector (CD4). PfSec22 and PfARO proteins were immunoprecipitated from cell lysates using an α-c-Myc antibody. The proteins were separated by SDS-PAGE and visualised by immunoblot, using α-c-Myc antibody from a different species. **(A)** HEK293 cells co-transfected with c-Myc-PfSec22 + FLAG-PfDHHC5, c-Myc-PfSec22 + FLAG-PfDHHC5-CdA or c-Myc-PfSec22 + CD4 empty vector. **(B)** HEK293 cells co-transfected with c-Myc-PfARO + FLAG-PfDHHC5, c-Myc-PfARO + FLAG-PfDHHC5-CdA, or c-Myc-PfARO + CD4 empty vector.

The results described above suggest that, at least in the case of PfDHHC5, the mutation of the cysteine residue of the DHHC domain into an alanine residue did not have an effect on PfSec22 and PfARO palmitoylation. This therefore implies that the cysteine residue of the DHHC domain of PfDHHC5 does not appear to be involved in the transfer of palmitate to target proteins.

For the yeast DHHC proteins, Erf2 and Akr1, mutation of the first histidine residue of the DHHC domain has been found to reduce palmitoylation of their substrate [1, 2]. Interestingly, for both Erf2 and Akr1, mutation of the cysteine residue of the DHHC domain, besides abolishing autopalmitoylation, also resulted in the loss of PAT activity [1, 2]. This was not observed here for PfDHHC5. However, as other residues of the DHHC domain (such as the first histidine residue) appear to also be involved in palmitoylation, it could be that in the case of PfDHHC5, other residues within the DHHC domain are important for palmitate transfer to the substrate, whilst the cysteine residue is involved in the autopalmitoylation of the DHHC protein itself. Unfortunately, due to the size of the PfDHHC5 protein (80.8 kDa) and to its solubility in detergents, PfDHHC5 expressed in HEK293 cells was unable to be immunoprecipitated or visualised by immunoblot. Thus, it is unknown whether the mutation of the cysteine residue in the DHHC domain affects autopalmitoylation of PfDHHC5 at this time.

Alternatively, other models of protein palmitoylation suggest that instead of acting as the palmitoyl-enzyme intermediate, the DHHC domain may be involved in the allosteric regulation of the DHHC protein, controlling substrate specificity and binding, whilst palmitate transfer occurs on other cysteine residues [18]. As the results described here indicate that mutation of the cysteine residue did not affect target protein palmitoylation, this could imply that the cysteine residue of the DHHC domain is not involved in substrate specificity or binding, if that is indeed the role of the DHHC domain.

It would be interesting to determine whether the same results are observed for the other *P. falciparum* DHHC proteins studied here, as well as to investigate which residue within the DHHC domain is actually important for the palmitoylation of target proteins, in order to shed some light on the exact mechanism of palmitate transfer performed by the DHHC proteins.

Conclusions

The PAT activity assay developed here has successfully shown that four PfDHHC proteins of interest (PfDHHC3, 5, 7 and 9) exhibit PAT activity and can palmitoylate *P. falciparum* proteins expressed in a

heterologous system. This assay also confirms that two potential palmitoyl-proteins, PfSec22 and PfARO, are indeed palmitoylated. Futhermore, this assay has revealed that like the DHHC proteins of other eukaryotic organisms, the *Plasmodium* DHHC proteins have overlapping functionalities, where several different PfDHHC proteins appear to be able to palmitoylate the same substrate protein, although factors such as the localisation of the DHHC protein in relation to the localisation of the target protein may affect target protein palmitoylation. Lastly, at least in the case of PfDHHC5, the cysteine residue of the DHHC domain does not appear to be directly involved in palmitate transfer, implying that the other residues of this highly conserved domain may be the residues involved in target protein palmitoylation.

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