The malaria parasite, *Plasmodium falciparum*, spends part of its life cycle inside the erythrocytes of its human host. In the mature stages of intraerythrocytic growth, the parasite undertakes extensive remodeling of its adopted cellular home by exporting proteins beyond the confines of its own plasma membrane. To examine the signals involved in export of parasite proteins, we have prepared transfected parasites expressing a chimeric protein comprising the N-terminal region of the *Plasmodium falciparum* exported protein-1 appended to green fluorescent protein. The majority of the population of the chimeric protein appears to be correctly processed and trafficked to the parasitophorous vacuole, indicating that this is the default destination for protein secretion. Some of the protein is redirected to the parasite food vacuole and further degraded. Photobleaching studies reveal that the parasitophorous vacuole contains subcompartments that are only partially interconnected. Dual labeling with the lipid probe, BODIPY-TR-ceramide, reveals the presence of membrane-bound extensions that can bleb from the parasitophorous vacuole to produce double membrane-bound compartments. We also observed regions and extensions of the parasitophorous vacuole, where there is segregation of the luminal chimera from the lipid components. These regions may represent sites for the sorting of proteins destined for the trafficking to sites beyond the parasitophorous vacuole membrane.

Due to the compartmentalization of eukaryotic cells, a sophisticated protein trafficking system is an integral requirement for homeostasis and growth. Proteins destined for compartments other than the cytoplasm are synthesized with intrinsic signals that determine their transport within the cell. Small peptide motifs often form the necessary targeting determinant (1). For example, an N-terminal hydrophobic sequence forms part of the typical secretory signal that directs proteins across the endoplasmic reticulum (ER) - membrane (2–5). Similarly, N-terminal amphipathic and bipartite sequences target proteins to the chloroplast and mitochondria (6–8).

The malaria parasite, *Plasmodium falciparum*, spends part of its life cycle inside mature human erythrocytes. The parasite invades this quiescent host cell and develops within a parasitophorous vacuole (PV). An unusual and highly specialized secretory system enables the malaria parasite to survive within a cell that lacks its own machinery for protein synthesis and trafficking. Indeed, the parasite targets proteins, not only to compartments within its own confines, but to the PV, in which it resides, as well as the PV membrane (PVM), the erythrocyte cytoplasm, and host cell membrane (9–11).

Efforts have been made to understand the trafficking signals that target parasite proteins to different compartments within and outside the parasite. Proteins destined for the ER, the parasite plasma membrane (PPM), the PV, or the PVM appear to have a “classical” hydrophobic N-terminal signal sequence (i.e. a stretch of 10–15 hydrophobic amino acids commencing 3–17 amino acids from the N terminus) (12, 13). For example, the secretory signal of the PVM-located integral membrane protein, exported protein-1 (Exp1, also called antigen 5.1, QF119, or cross-reactive antigen) has a characteristic N-terminal signal (14), which is cleaved by the malaria parasite at a site adjacent to glutamate 23 (15). This signal sequence is recognized by the translocation machinery of higher eukaryotes (16). However, a number of proteins that are directed past the PVM to the erythrocyte cytosol do not have classical N-terminal signals. Instead, proteins such as the knob-associated histidine-rich protein (KAHRP) have a hydrophobic stretch of amino acids starting 20–80 amino acids from the N terminus (13, 17). This “internal” hydrophobic signal does not appear to function in heterologous trafficking systems, since KAHRP is not translocated across the ER membrane in a cell-free system using mammalian microsomes (9, 16, 18).

Recently, the development of the technology for transfection of malaria parasites has permitted an analysis of the signals that direct proteins to different compartments. For example, Wickham et al. (19) prepared a series of constructs in which gene fragments encoding the N-terminal regions of KAHRP exported protein-1; GFP, green fluorescent protein; KAHRP, knob-associated histidine-rich protein; PVM, parasitophorous vacuolar membrane; PIEMP1, *P. falciparum* erythrocyte membrane protein-1; PIERC, *P. falciparum* endoplasmic reticulum-located calcium-binding protein; SLO, streptolysin O; TVN, tubulovesicular network; NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble NSF attachment protein receptor; PV, parasitophorous vacuole; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; FV, food vacuole; EM, electron microscopy.

---

*A* This work was supported by the National Health and Medical Research Council, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed. Tel.: 61-3-94791375; Fax: 61-3-94792467; E-mail: L.Tilley@LaTrobe.edu.au.

The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; BODIPY-TR-ceramide, N-(4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diazia-3-vlphenoxy)acetly)sphingosine; DIC, differential interference contrast; PPM, parasite plasma membrane; Exp1, eXport 1.
were appended to the reporter protein, green fluorescent protein (GFP). These constructs were introduced into P. falciparum using a stable transfection system (8, 19, 20), and the locations of the proteins were assessed by fluorescence microscopy. The studies indicated that the atypical N-terminal signal sequence of KAHRP contains information that is both necessary and sufficient for entry into the ER and trafficking to the PV. These studies suggested that secretion into the PV is the default pathway for export of proteins in the malaria parasite. The studies of Wickham et al. (19) also indicated that a separate sequence element in the histidine-rich region of KAHRP is needed for the translocation of this protein across the PVM. Trafficking of proteins to the erythrocyte cytosol appears to be a two-step process, with the PV acting as an intermediate compartment (19, 21). Following release into the erythrocyte cytosol, soluble KAHRP appears to associate briefly with the cytoplasmic surface of Maurer’s clefts and eventually is assembled into “knob” structures underneath the erythrocyte membrane (19).

A separate transfection study employed constructs encoding the classical N-terminal signal sequence of Exp1 appended to luciferase (22). Using a transient transfection system and a sensitive biochemical assay for detection of the luciferase tag, these authors reported that about 40% of the tagged Exp1 was transported and that additional signal sequence information is required to trap the exported proteins in the PV.

The discrepancy between the data obtained from these two experimental approaches prompted us to reexamine the Exp1 signal sequence in a stable transfection system using GFP as the reporter. In this work, we have prepared a construct encoding the N-terminal signal sequence of Exp1 fused to GFP and used the plasmid to transfect P. falciparum-infected erythrocytes. Our results confirm that transit through the ER to the PV is the default pathway for protein transport and that additional signal sequence information is required to trap the exported proteins in the PV.

The studies of Wickham et al. (19) also indicated that a separate sequence element in the histidine-rich region of KAHRP is needed for the translocation of this protein across the PVM. Trafficking of proteins to the erythrocyte cytosol appears to be a two-step process, with the PV acting as an intermediate compartment (19, 21). Following release into the erythrocyte cytosol, soluble KAHRP appears to associate briefly with the cytoplasmic surface of Maurer’s clefts and eventually is assembled into “knob” structures underneath the erythrocyte membrane (19).

MATERIALS AND METHODS

PCR and Cloning.—The sequence of P. falciparum exported antigen-1, Exp1, was obtained from the National Center for Biotechnology Information database (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST/; accession number P04926) (23, 24). The sequence encoding amino acid residues 1–155 of Exp1 (P. falciparum 3D7 strain (EXP1-(1–155)) was amplified by PCR from genomic DNA using primers 5’-AGGATATGGATGCATCTTGTACACCCTTTG and 5’-CCTAGGATACTTTAATAACACCCTT (restriction sites are underlined). The resulting fragment, bearing the BglII and AvrII restriction sites, was first cloned into the pCR2.1 TOPO vector (Invitrogen) and then placed upstream of the mut 2 EGFP coding region (rabbit antiserum against 3D7 strain obtained from Prof. Robin Anders, La Trobe University) and Exp1 (rabbit antiserum kindly provided by Prof. Klaus Lingelbach, Philipps-University, Marburg, Germany). For immunoblot analyses, asynchronous P. falciparum cultures were harvested, subjected to SDS-PAGE (12% acrylamide), transferred to polyvinylidene difluoride membrane, probed with antisera and visualized by horseradish peroxidase-conjugated secondary antibodies (Sigma), and developed with enhanced chemiluminescence (ECL) reagent. BODIPY-TR-ceramide was obtained from Molecular Probes and used to label parasitized erythrocytes as described by Behbehani and Habib (20). Briefly, parasitized erythrocytes were resuspended in complete medium (5% parasitemia, 10% hematocrit) and incubated in the presence of 1 µM BODIPY-TR-ceramide in complete medium at 37°C for 60 min, washed three times in complete medium and examined by fluorescence microscopy. The extent of conversion of the exogenously added ceramide to sphingomyelin was assessed by extracting the lipids and subjecting them to thin-layer chromatography as described by Haldar et al. (31).

Confocal Fluorescence Microscopy—Parasitized erythrocytes expressing Exp1-(1–155)-GFP were mounted wet on a glass slide, covered by a coverslip, and imaged within 10 min at ambient temperature (maintained at 20°C) using a Leica TCS confocal microscope (ConfoLab Facility, Clayton, Monash University, Clayton, Victoria, Australia) equipped with a 100 planachromat oil immersion objective (1.4 numerical aperture). The 488- and 568-nm lines of a 60 milliwatt krypton-argon laser were used to excite GFP and BODIPY-TR, respectively. The fluorescence from each probe was detected through a 530/30-nm band pass filter and a 590-nm long pass filter, respectively. Differential interference contrast (DIC) images were generated using the transmitted light.

Photobleaching experiments were typically conducted as follows. Two prebleach images were obtained, and the laser was focused on a single spot (representing a diffraction-limited point) defined on the prebleach image. The point was illuminated for a specified time (1 s) using a bleach pulse at maximum laser power at the excitation wavelength characteristic of the fluorophore to be bleached. The measure of fluorescence recovery after photobleaching (FRAP) measurements, post-bleach images were obtained immediately after the high intensity illumination (delay ~1 s) and at time intervals after bleaching. For fluorescence loss in photobleaching (FLIP) measurements, four high intensity bleach pulses were applied at 30-s intervals prior to the acquisition of each image. The gain on the photomultiplier was kept high, and the laser power was adjusted using an acousto-optical tunable filter to a level sufficient to minimize photobleaching of the sample during image acquisition. Images were typically obtained at 0.049-nm pixel resolution and were the average of four acquisitions (~2-s total image acquisition time).

Image Analysis.—Confocal images were processed and analyzed using commercially available software (available on the World Wide Web at rsb.info.nih.gov/ij). For photobleaching measurements, images were processed using a median filter followed by smoothing (both employing a 1-pixel radius) to improve the signal/noise ratio and were corrected for background. The images were also corrected for the small degree of bleaching occurring during image acquisition using a correction factor obtained by subtracting the intensity of the two prebleach images exponentially to the remaining images. The bleaching between successive images was typically 2–5%. Two criteria were rigorously employed when quantitating the intensity of regions of interest between successive images: (i) the regions of interest should not contain saturated pixel values (i.e. all pixel values should be less than 255), and (ii) there should be a significant movement of signal between the regions of interest. The confocal images presented in this paper have been adjusted for presentation purposes to highlight and accentuate features of interest and do not represent the actual images analyzed.

The spatial distribution of bleaching was visualized by constructing
Protein Trafficking in the Malaria Parasite

Fig. 1. Organization of the EXP1 gene and gene product. A, schematic diagram of the EXP1 gene. Exons 1–3 are indicated by rectangles, and introns and gene flanking sequences are shown by solid lines. B, amino acid sequence of 3D7 Exp1. The N-terminal signal sequence (which terminates at the proteolytic cleavage site) is underlined, and the hydrophobic core of the signal is double underlined. The putative transmembrane domain is indicated by a solid line.

RESULTS

Amino Acids 1–35 of Exp1, When Fused to GFP, Allow Secretion of the Chimera in Transfected P. falciparum—The arrangement of the gene and the protein sequence encoded by EXP1 (3D7 strain) are shown in Fig. 1. Exon 1 of the EXP1 gene encodes the first 40 amino acids of the protein (Fig. 1, A and B). The first 22 amino acids of this protein have been shown to comprise a classical signal sequence that functions both in vivo and in vitro to direct proteins into the ER (14–16).

Recently, it has been suggested that the N-terminal region of Exp1 also contains sequence elements that direct the protein beyond the PV (22). To further examine this suggestion, we generated a chimera comprising an N-terminal fragment of Exp1 linked to the reporter GFP. A portion of the EXP1 gene that encodes the first 35 amino acids of the protein was joined upstream of themut 2 enhanced GFP coding sequence in the transfection vector pH2 (pH2-EXP1-(1–35)-GFP) (Fig. 1C). This region of Exp1 includes the signal sequence comprising 13 hydrophobic amino acids flanked by charged residues and the cleavage site between residues 22 and 23. The resultant GFP fusion protein was expressed from a stably maintained episome (8, 19) within the transformed 3D7 P. falciparum blood stage parasites.

Parasites expressing the GFP chimera protein (Exp1-(1–35)-GFP) were obtained 55 days after transfection and were maintained in culture in the presence of a 10 μM concentration of the antifolate drug, WR99210. They showed similar growth rates to untransfected parasites and maintained the plasmid even under lower (100 μM) drug concentrations. This suggests that the expression of the GFP chimera did not confer a growth disadvantage on the parasites.

To confirm expression of the Exp1-(1–35)-GFP fusion protein in the P. falciparum transfecteds, we performed Western blots on parasite-infected erythrocytes (Fig. 2). When probed with antibodies recognizing GFP, no reactivity was observed in uninfected erythrocytes or in the untransfected parental line, 3D7. By contrast, the transfected parasites expressed proteins that were resolved as a doublet of 28 and 26 kDa. The predicted size of the full-length Exp1-(1–35)-GFP chimera is 30 kDa. It is likely that the 28-kDa band represents a processed form of Exp1-(1–35)-GFP in which the 22-amino acid signal sequence has been removed. The 26-kDa band presumably represents a further degradation product. Lower molecular weight degradation products of GFP chimeras have been reported previously (8, 19).

The expression of the Exp1-(1–35)-GFP reporter in different stages of the intraerythrocytic cycle of transfected P. falciparum was examined using confocal fluorescence microscopy (Fig. 3). The Exp1-(1–35)-GFP chimera was expressed under the control of the hsp86 promoter and is thus produced at all stages of intraerythrocytic growth (32). By contrast, endogenous Exp1 is expressed most strongly in mature stage parasites (33) (data not shown). The protein appears to be largely located in the PV that surrounds the parasite (Fig. 3). Some parasites showed a “necklace of beads” pattern around the periphery of the parasite (Fig. 3, B and D) as has been reported previously for a chimera of an N-terminal fragment of KAHRP with GFP (19). In some doubly infected erythrocytes (Fig. 3D), only one of the parasites appeared to be expressing the transfection construct. The second parasite has presumably expelled the plasmid but is protected from the deleterious effects of WR99210 by its sister parasite. In more mature stages of the intraerythrocytic development of the parasite, protrusions from the PV were commonly observed (Fig. 3, B, C, and E). The protrusions largely appeared to remain connected to the PV. As suggested previously (19), the short protrusions of the PV may reflect evaginations of the PV into blind appendices that form part of the tubulovesicular network (TVN). In some cells, fluorescence was also observed in a compartment within the cytoplasm of the parasite, which appears to be the food vacuole (PV) (Fig. 3, C and E). In the schizont stage, a segmented pattern around a highly fluorescent central remnant body was obtained (Fig. 3F). This indicates that the fusion protein surrounds the individual merozoites, which again is consistent with trafficking of the protein to the PV. These results suggest that the first 35 amino acids of the Exp1 protein are sufficient for entry into the secretory system and secretion from the parasite into the PV but do not contain information that would direct the protein into the erythrocyte cytosol.
Exp1-(1–35) Is Present in the PV and FV of Transfected P. falciparum but Is Not Released into the Erythrocyte Cytosol—The volume of the erythrocyte cytosol is much larger than that of the PV (22). Therefore, it remained formally possible that a significant proportion of the Exp1-(1–35) chimera was exported as a soluble protein to the erythrocyte cytosol but was below the level of detection using fluorescence microscopy. In order to confirm the location of the reporter protein, transfected parasites were subjected to selective permeabilization protocols employing SLO and saponin. Treatment of the transfected parasitized erythrocytes with 4 or 8 hemolytic units of SLO released more than 97% of the hemoglobin from the parasitized erythrocytes (data not shown), but did not release PfERC (Fig. 4, C–E). In some cases, distortions and evaginations of the PV were observed (Fig. 3C). Some regions or extensions of the PV may be sites of sorting of Lipid and Protein Components—To further examine the organization of Exp1-(1–35)-GFP within the parasite, we have labeled the transfected parasites with BODIPY-TR-ceramide as described by Behari and Haldar (30). The BODIPY-TR-ceramide probe is taken up from the medium into the erythrocyte membrane and transfers to membrane structures within the infected erythrocyte cytosol (Fig. 6). Since the probe may have detergent-like properties, we have used very low levels (1 μM) of the BODIPY-TR-lipid probe (i.e. ~1 probe molecule:400 endogenous lipid molecules) in an effort to avoid disruption of the membranes of the parasitized erythrocytes. Under these conditions, only a very small amount of the ceramide was con-

FIG. 2. Western blot analysis of parasites transfected with the Exp1-(1–35)-GFP construct. Total cell lysates from ~10⁶ cells (10% parasitemia) were lysed by freezing and thawing and subjected to electrophoresis (10–15% acrylamide), transferred to polyvinylidene difluoride membrane, and probed with anti-GFP antibody (Roche Molecular Biochemicals; 1:1000 dilution). UI, UT, and TR represent uninfected erythrocytes and untransfected and transfected parasitized erythrocytes, respectively.

FIG. 3. Expression of Exp1-(1–35)-GFP chimeric proteins at different stages of the intraerythrocytic life cycle of P. falciparum. The first image in each set represents the DIC image, and the second is the fluorescence signal from the GFP chimeric protein, with an overlay of these images in the third panel. A, ring stage parasite showing PV labeling. B, erythrocyte infected with two late ring stage parasites, only one of which is expressing the Exp1-(1–35)-GFP transgene. The fluorescence signal resembles a necklace of “beads,” some of which are marked with arrows. C–E, trophozoite stage parasites showing PV expression. Some cells retain the necklace of beads pattern (D). In some cases, distortions and evaginations of the PV were observed (C and E, yellow arrowheads). In some cells, GFP was observed in the PV (C and E, blue arrowheads). F, schizonts show a segmented pattern with a highly fluorescent central remnant body. Fluorescence from GFP was captured in live cells using a Leica TCS NT confocal microscope. Bar, 5 μm.
verted to sphingomyelin (data not shown) as has been reported previously (30).

The BODIPY-TR-ceramide probe labeled the erythrocyte membrane and the periphery of the parasite (Fig. 6). In doubly labeled cells, it is clear that both the GFP chimera and the BODIPY-TR probe are associated with the PV; however, the luminal Exp1-GFP protein appears to be restricted to subcompartments within the PV (Fig. 6, blue arrowheads).

The BODIPY-TR-ceramide was also present in protrusions and loops extending from the PV and in small structures attached to the erythrocyte membrane (Fig. 6, A, B, and D). The loop structures are presumably the double membrane-bound extensions of the PV known as the tubulovesicular network (30, 39) (Fig. 6E). The small structures underlying the erythrocyte membrane are likely to be Maurer’s clefts (22, 40, 41) (Fig. 6E). The GFP chimera is present in some regions of the PV/TVN but is absent from others. It is possible that the regions and extensions of the PV that lack GFP (Fig. 6, white open head arrows) represent sites of sorting of components destined for trafficking beyond the PV. A diagrammatic representation of the different membrane structures in these doubly labeled cells is shown in Fig. 6E.

**Organization and Dynamics of Exp1-GFP within the PV**—We have used FRAP to examine the organization and dynamics of Exp1-(1–35)-GFP within the PV and in protrusions and blebs adjacent to the PV (Fig. 7). A small region of a labeled infected erythrocyte was bleached using a high power laser pulse of 1-s duration. After the pulse, the cell was imaged immediately and at different time intervals thereafter. As seen in Fig. 7A, a 1-s bleach pulse directed onto one of the “beads” of fluorescence in the PV resulted in a loss of fluorescence that was localized to the region that was pulsed. During the 120-s postbleach period, there was 50% recovery of the fluorescence signal into the bleached area (Fig. 7F). This indicates that the bleached bead is not an isolated structure but is part of some larger structure in which diffusion of Exp1-(1–35)-GFP can occur. The degree of connectivity between different beads within the PV was investigated in greater detail using the FLIP experiment shown in Fig. 8A. A small region corresponding to one of the PV beads was exposed to a series of 1-s bleach pulses, and the loss of fluorescence intensity in other regions of the PV was examined. These data show that the population of mobile GFP chimeras that was able to repopulate the region of the bleached bead was quickly depleted. In addition, the B image demonstrates that the repeated exposure of isolated beads to the high intensity laser pulse did not produce a substantial loss of fluorescence in other regions of the PV. This indicates that whereas there is some connectivity between individual beads and a larger compartment, much of the protein population is unable to diffuse from one subcompartment to the next within the time scale examined.

The PV often had a smoother appearance in erythrocytes infected with more mature parasites (Fig. 7B). As with the previous example, application of a 1-s bleach pulse resulted in some loss of fluorescence in the region exposed to the high intensity laser pulse. However, in this case, the loss of fluorescence occurred throughout a larger region of the PV. After 30 s, significant recovery of fluorescence into this bleached area was observed. The analysis shown in Fig. 7G indicates that the increase in fluorescence in the bleached region is associated with a small decrease in fluorescence intensity in other regions of the PV. This suggests that the recovery of fluorescence in the bleached region may reflect the diffusion of Exp1-GFP from the unbleached regions of the PV. This was confirmed by performing a FLIP analysis on parasites exhibiting a smooth PV morphology (Fig. 8, B and C). In contrast to the observations on parasites with a “beaded” PV, repeated bleaching of one region of the PV resulted in a relatively uniform loss of fluorescence.

---

**Fig. 4. Release of EXP1-(1–35)-GFP by sub-fractionation of the transfectants.** Highly synchronized transfectants, grown to trophozoite stage after synchronization (~0.5 x 10⁶ cells), were solubilized in either 450 units (4HU) or 900 units (8HU) of SLO or 0.09% saponin and separated into supernatant (S) and pellet (P) fractions by centrifugation. These fractions were separated by SDS-PAGE (12% acrylamide), blotted onto polyvinylidene difluoride membrane, and probed with antibodies recognizing GFP (1:1000 dilution), S-antigen (1:500 dilution), and PfERC (1:500 dilution).

**Fig. 5. Effect of brefeldin A treatment of ring stage parasites on the export of Exp1-(1–35)-GFP.** Erythrocytes infected with tightly synchronized (6–10 h) ring stage transfectants were incubated for 18 h in the presence of 5 μg/ml BFA (lower panels) or an equivalent volume of methanol (control; upper panels) and examined by fluorescence microscopy. The confocal images shown (left to right) are as follows: DIC, GFP fluorescence, and an overlay. Bar, 5 μm.
A localized loss of fluorescence was observed following a 1-s bleach pulse (Fig. 7C), indicating that recovery of signal occurred to ∼50% within the 60-s postbleach period (Fig. 7H), suggesting that this extension is connected to a larger compartment, presumably the PV. Again, however, the relatively slow half-time for recovery indicates that there are some restrictions to diffusion through the solute continuum between the TVN and the PV proper. Moreover, in some cells, the extensions appear to have lost the continuum with the PV. As shown in Fig. 7D, there was no recovery of the arrowed PV extension after the bleach pulse. Blebbing of regions of the TVN has been reported previously (19). We also found that the GFP chimera that is located within the PV is not connected by a solute continuum to the PV. As shown in Fig. 7E, bleaching of the PV compartment did not affect the fluorescence signal from the PV, and there was no recovery of signal into the PV within the 120-s postbleach period. Interestingly, bleaching of the PV compartment revealed another compartment within the parasite that contains the GFP chimera (Fig. 7E, asterisk). This may represent an endocytic packet of the PV en route to the FV or a subcompartment within the parasite secretory system en route to the PV.

We have also used FRAP analysis to examine the organization and dynamics of the BODIPY-TR-ceramide probe. An examination of doubly labeled cells confirmed the observation that some of the BODIPY-TR-labeled membrane compartments had separated from the PV (Fig. 9A, upper panel). These structures were often observed to colocalize with the GFP chimera (Fig. 9A, arrow in lower panel) and to exhibit mobility within the erythrocyte cytosol when monitored over time (Fig. 9A, lower panels). Since the GFP chimera is likely to be present in an aqueous environment within these structures, the colocalization indicates that these structures comprise a double membrane that encloses two luminal compartments, with the GFP chimera located in the outer lumen. The inability to resolve the two bilayers reflects the close apposition of the membranes to form a thin outer lumen whose
FIG. 7. FRAP measurements and analysis of GFP in doubly labeled *P. falciparum*-infected erythrocytes expressing Exp1-(1–35)-GFP. A–E, confocal images obtained as described under “Materials and Methods.” The left panels show the DIC image of the cells. The second panel shows the BODIPY-TR image. The remaining panels show the GFP images obtained at low laser power. These images were obtained immediately prior to bleaching (Prebleach), immediately after bleaching using a high intensity laser pulse for 1 s at the position indicated by the arrow (Postbleach t2) and at times t1 and t2 after the bleach event (Postbleach t1 and Postbleach t2, respectively). A, trophozoite stage-infected erythrocyte with a “necklace of beads” fluorescence pattern; t1 = 60 s, t2 = 120 s. B, trophozoite stage-infected erythrocyte with a smooth PV fluorescence pattern; t1 = 30 s, t2 = 60 s. C and D, trophozoite stage-infected erythrocytes showing PV evaginations; t1 = 30 s, t2 = 60 s. E, a trophozoite stage-infected erythrocyte showing PV fluorescence (arrow); t1 = 30 s, t2 = 60 s. F–H, analysis of the images shown in A–C, respectively. The relative fluorescence within each outlined region (numbered) is shown as a function of time after the bleach event (data are normalized to a value of 100 in the prebleach image). The fluorescence intensities have been corrected for bleaching during image acquisition as described under “Materials and Methods.” Bar, 5 μm.

FIG. 8. FLIP measurements and analysis of GFP in *P. falciparum*-infected erythrocytes expressing Exp1-(1–35)-GFP. Confocal images were obtained as described under “Materials and Methods.” Trophozoite stage-infected erythrocytes showed a “necklace of beads” fluorescence pattern (A) and a smooth PV fluorescence pattern (B and C). The left panel shows the DIC images of the cells. The second panel shows the GFP fluorescence image obtained immediately prior to bleaching (PreFLIP). The region indicated by the arrow was repeatedly subjected to an intense laser pulse (1-s duration, 30-s intervals). Images were collected after the cell had been subjected to 4, 8, and 12 of these pulses (corresponding to the FLIP1, FLIP2, and FLIP3 images, respectively). The B image of the PV region shown in the right panel was constructed using the prebleach (PreFLIP) image and the postbleach images (FLIP2 in A and B and FLIP3 in C). Only those regions of the PV that did not undergo movement were analyzed. The color table for the B image is shown in Fig. 9. Bar, 5 μm.
dimensions are below the level of resolution available using the confocal microscope. Such structures are presumably formed by circularization of extensions of the PV/TVN as shown in Fig. 6E. Similar structures have been observed by EM (39). The absence of a lipid continuum between these structures and the PVM was confirmed by the FRAP experiment shown in Fig. 9A (upper panel). High intensity illumination of the structure at the excitation wavelength of the BODIPY-TR probe resulted in almost complete ablation of the BODIPY-TR fluorescence in this structure but had little effect on the fluorescence signal from the other membranes. In addition, there was insignificant recovery of fluorescence into the structure over a 1-min period. These results are consistent with a loss of connectivity to the PVM and other parasite membranes.

In other cases, some of these structures appeared to remain connected to the PV. As shown in Fig. 9B, a high intensity pulse of 1-s duration directed at the tip of an extension of the PV resulted in partial bleaching of the BODIPY-TR signal in this compartment. In contrast to the previous example, the degree of fluorescence loss at the bleach site was limited, and the bleached area extended throughout a large area of the PV proximal to the illuminated region (see B image). This is consistent with significant diffusion of BODIPY-TR-ceramide occurring during the bleach event and image acquisition time and is in the correct range for the diffusion of a membrane lipid (42). This is further confirmed by the QR image shown in Fig. 9, which shows that there is subsequent recovery into the bleached region. These data indicate that the protrusion is connected to the PVM by a lipid continuum. The laser pulse also caused some bleaching of an adjacent region of the erythrocyte membrane. Diffusion of the probe within the host cell membrane allowed recovery of fluorescence in this compartment on a similar time scale. It is important to note that the bleach pulse had no effect on the fluorescence signal from the GFP chimera in this doubly labeled cell (Fig. 9B, bottom panels).

**DISCUSSION**

Proteins destined for extraparasitic locations in malaria parasite-infected erythrocytes are thought to transit through the ER and the PV prior to transfer to the erythrocyte cytosol (19, 43, 44); however, the nature of the polypeptide secretory signals that direct proteins to their correct destinations is currently the subject of some debate. An analysis of the sequences of exported parasite proteins reveals some rather unusual motifs. Proteins that are destined for sites within the confines of the PV have classical hydrophobic N-terminal signal sequences (i.e. a stretch of 10–15 hydrophobic amino acids close to the N terminus). By contrast, many proteins that are directed past the PV to the erythrocyte cytosol do not have classical N-terminal signals. Proteins such as KAHRP, the mature parasite-infected erythrocyte surface antigen and the ring-infected erythrocyte surface antigen have a hydrophobic stretch of amino acids starting 20–80 amino acids from the N terminus (see Table I in Ref. 43). An exception to this rule is HRP2, which has a classical hydrophobic signal and yet is largely located in the erythrocyte cytosol (45, 46). It is possible that information determining the final destination of the protein is contained within the N-terminal signal. Alternatively, both classical and recessed hydrophobic signal sequences may function interchangeably to direct the translocation of proteins into the ER (with the PV as the default destination), with additional trafficking information encoded elsewhere in the polypeptide sequence. Work from Wickham et al. (19) indicated that the noncanonical signal sequence of KAHRP is sufficient for transport to the PV but that additional signal information is needed for export beyond this point. By contrast, the work of Burghaus and Lingelbach (22) suggested that the classical
signal sequence of Exp1 directed a chimera to the erythrocyte cytosol. They proposed that the erythrocyte cytosol is the default destination and that additional signal information is needed to retain proteins in the PV.

In this work, we have reexamined the trafficking signals within the exported protein, Exp1. Exp1 was originally described as an antigen of \textit{P. falciparum} that is transported from the parasite to the PV and, to some extent, to membrane-bound compartments in the erythrocyte cytosol (14, 23, 24, 33). Exp1 is an integral membrane protein with the C-terminal domain facing the erythrocyte cytosol (14). We prepared a chimeric gene construct encoding an N-terminal fragment of Exp1 linked to the reporter protein, GFP. The construct was successfully used to prepare stably transfected \textit{P. falciparum}. The transfectants were readily maintained in culture and continued to express the fluorescent protein even under very low drug pressure. This suggests that the expression of the GFP chimera did not compromise the growth of the parasites. Western analysis indicated that part of the population of Exp1-(1–35)-GFP was processed to a 28-kDa product, presumably by removal of the N-terminal signal by a signal peptidase in the ER, while part of the population was further processed to a 26-kDa species.

Using confocal fluorescence microscopy, we have shown that the Exp1-(1–35)-GFP chimera is present in the parasite PV and in extensions of the PV as well as in a compartment within the parasite cytoplasm. There appears to be little or no GFP released into the erythrocyte cytosol. To confirm the location of the chimera, we have subfractioned the parasitized erythrocytes using selective permeabilization protocols. The pore-forming toxin, SLO, inserts into the erythrocyte membrane and releases the soluble erythrocyte components but leaves the PV intact (21, 28, 29). By contrast, saponin, a detergent that interacts with cholesterol, disrupts the erythrocyte membrane and the PVM but leaves the PPM intact (21, 47). Saponin lysis of the transfectants released the 28-kDa form of the GFP chimera but did not release the 26-kDa form. This indicates that the 28-kDa product is present in the PV but that the 26-kDa product is located in a site within the confines of the PPM. When the fractionation data are taken together with the fluorescence microscopy data (which show that some of the GFP is localized near the hemoglobin crystal) and with previous electron microscopy (EM) studies (19), the data indicate that some of the chimera is correctly processed and trafficked to the PV, while a proportion of the population is diverted to (or endocytosed into) the PV, where it is further degraded. Previous studies have shown that the core of GFP forms a highly stable \(\beta\)-barrel structure that may protect proteolytic degradation (48). Western analysis of the relative levels of the 26- and 28-kDa species indicates that a significant proportion of the GFP chimera (~50%) is present in the PV. However, the fluorescence from this compartment was usually less than that from the PV. This may reflect the larger volume of the PV and/or be due to the acidic environment of the PV (49) quenching the fluorescence of the GFP (50).

Our data show that the PV is the default destination for export of proteins from the malaria parasite. Taken together with the work of Wickham \textit{et al.} (19), the data indicate that both classical and recessed N-terminal signal sequences of malaria proteins contain information only for entry into the ER (and hence default secretion to the PV), whereas additional sequence information is needed to direct proteins past this point. This is in direct contrast to the data of Burghaus and Lingelbach (22), who reported that an Exp1-luciferase chimera was largely directed to the erythrocyte cytosol. This discrepancy may be explained by the different approach taken by Burghaus and Lingelbach (22). For example, they used a transient transfection system and used luciferase as a reporter. Since protocols for the transfection of \textit{P. falciparum} are very inefficient (about 1 in 100,000 for transient transfection and 1 in a million for stable transfection (see Refs. 51 and 52), Burghaus and Lingelbach (22) relied on a cell fractionation protocol and the very high sensitivity of the luciferase reporter to detect the chimeric product in parasites 3 days after the transfection protocol. In our study, we used a dual cassette transfection construct that, in addition to the transgene, encodes human DHFR, which confers resistance to WR99210. We cultured the parasites for several months in the presence of drug and thus obtained a transfectant stably expressing the chimeric protein from an episteme. It is possible that, in the transient transfection system, the electroporation protocols used to introduce the plasmid or initial high copy numbers of the plasmid in some cells compromised the integrity of the parasite membranes, allowing release of the fusion protein into the erythrocyte cytosol. Also, it is interesting to note that luciferase carries a SKL sequence at its C terminus that functions as a peroxisomal import sequence in higher eukaryotes (53). Thus, it is possible that this motif or another cryptic motif within the luciferase reporter is responsible for translocation of the chimera into the erythrocyte cytosol.

The export of Exp1-(1–35)-GFP is sensitive to brefeldin A. This indicates that the protein transits through the ER before secretion into the PV. The Western blot analysis and the selective permeabilization studies are consistent with the suggestion that the N-terminal signal has been removed during trafficking of Exp1-(1–35)-GFP through the ER. Since the Exp1-GFP chimeric construct lacks the transmembrane domain, it is likely that it exists as a soluble protein in the lumen of the PV. Nonetheless, the GFP chimera often appeared to be restricted to subdomains of the PV. For example, the fusion protein often appeared to reside in bead-like subcompartments forming a “necklace” around the parasite. To examine the nature of the PV subcompartments, we have used BODIPY-TR-ceramide, which appears to preferentially label membrane components of the PV/TVN. The dual labeling of live cells showed that some regions and extensions of the PV/TVN contained both GFP and the BODIPY-TR lipid probe; however, in other regions there appeared to be a segregation of the lipid and protein probes.

Photobleaching studies allow an analysis of the organization and dynamics of fluorescent components in living cells (42). In this work, we have used photobleaching protocols to examine the diffusional properties of GFP in the PV. Theoretical considerations predict that unrestricted diffusion of soluble Exp1-(1–35)-GFP molecules would be sufficiently rapid to allow complete equilibration of the protein throughout the PV compartment within the 1-s bleach time employed in this study. This would result in an even loss of fluorescence throughout a continuous compartment the size of the PV (42). The observation that a bleach pulse at the PV produces a local loss of fluorescence that subsequently recovers shows that the diffusion of the GFP chimera in the PV is considerably slower than in free solution. In GFP-labeled cells that exhibited a smooth PV appearance, these slowly diffusing GFP molecules were free to equilibrate throughout the entire PV lumen. This was not the case in GFP-labeled cells that exhibited a PV with a beaded morphology. In these cells, it appeared that the Exp1-GFP was located in subcompartments within the PV and that there was little or no diffusion between these subcompartments on the time scale of the measurements. This provides a direct demonstration of unlinked subcompartments within the PV. We propose that the barrier to GFP diffusion arises from a constriction of the PV lumenal space due to the close apposition of the PPM and PVM.
in some regions (Fig. 6E). These constrictions are presumably responsible for the "string of beads" appearance of the PV.

Part of the population of chimeric GFP molecules appears to be directed to the PV. This may represent an overflow pathway for proteins en route through the secretory system. Alternatively, it is possible that the GFP fusion proteins are taken up during the parasite's feeding process. The parasite uses a cytosome (mouth) to ingest small packets of hemoglobin from the host cytoplasm. These endocytic compartments are surrounded by a double membrane originating from the PPM and the PVM and thus are likely to contain proteins from the PV as well as the erythrocyte cytoplasm (Fig. 6E). These vesicles are transported to and fuse with the PV, which will deliver the fusion proteins to this compartment. In some cells, bleaching of the PV-located GFP chimera revealed the presence of other structures within the parasite cytosol; these may represent endocytic or secretory vesicles.

In more mature stage parasites, the GFP chimera was often present in looped extensions of the PV. Bleaching of a PV extension was often followed by gradual recovery of the signal, which indicates that these protrusions remain connected to the PV. However, some of the evaginations appeared to be closed compartments; photoobleaching of these blebs did not cause a loss of fluorescence signal throughout the PV, and no recovery was observed. This indicates a physical barrier to diffusion of the chimera between the evaginated region and the PV. In some cases, the GFP-Exp1 chimera is packaged into double membrane-bound compartments that are released from the PV. Membrane-bound structures containing endogenous Exp1 have been observed in the erythrocyte cytosol using immunofluorescence microscopy (14, 15). These budded double membrane-bound compartments were quite frequently observed in trophozoite stage parasites; however, they did not connect or fuse with the erythrocyte membrane. This suggests that these PV lumen-containing structures probably do not play a direct role in delivery of proteins to the erythrocyte membrane. This is in agreement with previous studies (19, 36). However, we also observed regions of the PV and protrusions emanating from the PV that were labeled with the BODIPY-TR-ceramide probe but that excluded the GFP chimera. These regions may represent sites for sorting of PV-resident proteins from proteins destined for transport to the erythrocyte membrane. PV resident-free structures may bud from the PV as vesicles or tubules and mature to form the Maurer's clefts, which are presumably incorporated into vesicles or tubules that bud from specialized regions of the PV that exclude PV-resident proteins. The PTEMP1-containing structures are presumably trafficked to (or mature to become) the Maurer's clefts, and then PTEMP1 is transferred to the erythrocyte membrane. The Maurer's clefts contain homologues of components of the classical secretory pathway such as Sar1p (13, 19), Sec31p (26), and a component SNARE-mediated fusion process, NSF (56), which may be involved in the sorting of proteins destined for the erythrocyte membrane. Therefore, it appears likely that trafficking between these compartments is vesicle-mediated. Indeed, vesicle-like structures that may be involved in the trafficking of proteins from the PVM (44) and the Maurer's clefts could have been identified at the EM level.

In summary, we have shown that by tagging a parasite protein of interest at the gene level with GFP it is possible to decipher the signals that target proteins to specific compartments of the infected erythrocyte. In addition, the GFP transfectants were used to monitor the location and organization of the labeled component in live parasites during the course of the cell cycle. Use of photobleaching techniques enabled us to identify novel subcompartments within the PV. This has allowed an increased understanding of the nature of the pathway for protein trafficking in parasitized erythrocytes. The unusual characteristics of the external sector of this pathway make it a potential target for new antimalarial strategies.

Acknowledgments—Expert technical assistance was provided by Emma Fox. We thank Prof. Robin Anders and Prof. Klaus Lingelbach for providing antibodies and for useful discussions.

REFERENCES

1. Blobel, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496–1500
2. von Heijne, G. (1985) J. Mol. Biol. 184, 99–105
3. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
4. Martoglio, F. and Dobberstein, B. (1988) Trends Cell Biol. 8, 410–415
5. Hegle, R. S., and Lingappa, V. R. (1999) Trends Cell Biol. 9, 132–137
6. DelRocker, A., Hagen, C. B., Frohlich, J. E., Feagin, J. E., and Parsons, M. (2000) J. Cell Sci. 113, 3909–3977
7. Schwartzbach, S. D., Osaftune, T., and Loffeldhart, W. (1998) Plant Mol. Biol. 38, 247–263
8. Walter, R. F., Reed, M. B., Cowman, A. F., and McFadden, G. I. (2000) EMBO J. 19, 1794–1802
9. Lingelbach, K. R. (1995) Exp. Parasitol. 76, 318–327
10. Foley, M., and Tilley, L. (1998) Int. J. Parasitol. 28, 1671–1680
11. van Dooren, G. G., Waller, R. P., Joiner, K. A., Ross, D. S., and McFadden, G. I. (2000) Parasitol. Today 16, 421–427
12. von Heijne, G. (1986) Nucleic Acids Res. 14, 4863–4869
13. Albano, F. R., Berman, A., La Greca, N., Hibbs, A. R., Wickham, M., Foley, M., and Tilley, L. (1999) Eur. J. Cell Biol. 78, 453–462
14. Gunther, K., Tummeler, M., Arnold, H. H., Rieley, R., Goman, M., Scaife, J. G., and Lingelbach, K. (1990) Mol. Biochem. Parasitol. 46, 149–158
15. Kara, U., Murray, B., Pam, C., Lahnstein, J., Gould, H., Kidson, C., and Saul, A. (1990) Mol. Biochem. Parasitol. 38, 19–23
16. Mattei, D., Berry, L., Couffin, S., and Richard, O. (1990) Novartis Found. Symp. 226, 215–230
17. Kilejian, A., Sharma, Y. D., Karoui, H., and Naslund, L. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7903–7914
18. Hinterberg, K., Scherf, A., Gynin, J., Tsyosmikha, T., Aikawa, M., Mazy, J. C., Dasilva, L. P., and Mattei, D. (1994) Exp. Parasitol. 79, 279–291
19. Wickham, M. E., Rug, M., Ralph, S. A., Klonis, N., McFadden, G. I., Tilley, L., and Cowman, A. F. (2001) Exp. Parasitol. 97, 568–5649
20. Reed, M. B., Saliba, K. J., Carannuca, S. R., Kirk, K., and Cowman, A. F. (2000) Nature 403, 906–909
21. Altfeld, I., Belling, J., Bhakdi, S., and Lingelbach, K. (1996) Biochem. J. 315, 307–314
22. Burghaus, P. A., and Lingelbach, K. (2001) J. Biol. Chem. 276, 26838–26845
23. Simmons, D., Wootlett, G., Bergan-Cartwright, M., Kay, D., and Scaife, J. (1987) EMBO J. 6, 485–491
24. Hope, I. A., Mackay, M., Hyde, J. E., Goman, M., and Scaife, J. (1985) Nucleic Acids Res. 13, 369–379
25. Fridek, D. A., and Wellemans, T. E. (1997) Proc. Natl. Acad. Sci. USA 94, 10931–10936
26. Adisa, A., Albano, P. R., Reeder, J., Foley, M., and Tilley, L. (2001) J. Cell Sci. 114, 3377–3386
27. Aley, S. B., Sherwood, T. A., Marsh, D., Eidelman, O., and Howard, R. J. (1986) Parasitology 92, 511–525
28. Brummeister, S., Paproka, K., Bhakdi, S., and Lingelbach, K. (2001) Mol. Biochem. Parasitol. 112, 133–137

* L. Tilley, N. Kriek, D. Ferguson, and C. Newbold, unpublished data.
29. Ansorge, I., Paprotka, K., Bhakdi, S., and Lingelbach, K. (1997) Mol. Biochem. Parasitol. 84, 259–261
30. Behari, R., and Haldar, K. (1994) Exp. Parasitol. 79, 250–259
31. Haldar, K., Uyetake, L., Ghori, N., Elmedendorf, H. G., and Li, W. L. (1991) Mol. Biochem. Parasitol. 49, 143–156
32. Wu, Y., Sifri, C. D., Lei, H. H., Su, X. Z., and Wellems, T. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 973–977
33. Coppel, R., Favaloro, J., Crewther, P., Burkot, T., Bianco, E., Stahl, H., Kemp, D., Anders, R., and Brown, G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5121–5125
34. La Greca, N., Hibbs, A. R., Riffkin, C., Foley, M., and Tilley, L. (1997) Mol. Biochem. Parasitol. 89, 283–293
35. Culvenor, J. G., and Crewther, P. E. (1990) J. Protozool. 37, 59–65
36. Elmedendorf, H. G., and Haldar, K. (1994) J. Cell Biol. 124, 449–462
37. Benting, J., Mattei, D., and Lingelbach, K. (1994) Biochem. J. 300, 821–826
38. Nacer, A., Berry, L., Slomianny, C., and Mattei, D. (2001) Int. J. Parasitol. 31, 1371–1379
39. Elford, C. B., Cowan, M. G., and Ferguson, P. J. D. (1995) Biochem. J. 308, 361–374
40. Gormley, J. A., Howard, R. J., and Taraschi, T. F. (1992) J. Cell Biol. 119, 1481–1495
41. Sam-Yellowe, T. Y., Fujioka, H., Aikawa, M., Hall, T., and Drazba, J. A. (2001) Parasitol. Res. 87, 173–185
42. Klonis, N., Rug, M., Harper, I., Wickham, M., Cowman, N., and Tilley, L. (2002) Eur. Biophys. J. 31, 36–51
43. Albano, F. R., Foley, M., and Tilley, L. (1999) Novartis Found. Symp. 226, 157–172
44. Taraschi, T. F., Trelka, D., Martinez, S., Schneider, T., and O'Donnell, M. E. (2001) Int. J. Parasitol. 31, 1381–1391
45. Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Lecch, J. H., Lew, A. M., Wellems, T. E., Rener, J., and Taylor, D. W. (1986) J. Cell Biol. 103, 1267–1277
46. Papalexis, V., Siomos, M. A., Campanale, N., Guo, X. G., Kosak, G., Foley, M., and Tilley, L. (2001) Mol. Biochem. Parasitol. 115, 77–86
47. Saliba, K. J., Horner, H. A., and Kirk, K. (1998) J. Biol. Chem. 273, 10190–10195
48. Ormos, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Science 273, 1392–1395
49. Krugstad, D. J., Schlesinger, P. H., and Gluzman, I. Y. (1985) J. Cell Biol. 101, 2301–2309
50. Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R., and Piston, D. W. (1997) Biophys. J. 73, 2782–2790
51. Crabb, B. S., Trigila, T., Waterkeyn, J. G., and Cowman, A. F. (1997) Mol. Biochem. Parasitol. 90, 131–144
52. O'Donnell, R. A., Freitas-Junior, L. H., Preiser, P. R., Williamson, D. H., McElwain, T. F., Scherf, A., Cowman, A. F., and Crabb, B. S. (2002) EMBO J. 21, 1231–1239
53. Miura, S., Kasuya-Arai, I., Mori, H., Miyazawa, S., Osumi, T., Hashimoto, T., and Fujiki, Y. (1992) J. Biol. Chem. 267, 14404–14411
54. Bannister, L. H., Hopkin, J. M., Fowler, R. E., Krishna, S., and Mitchell, G. H. (2000) Parasitol. Today 16, 427–433
55. Howard, R. J. (1988) The Biology of Parasitism (Englund, P., and Sher, A., eds) pp. 111–145, Alan R. Liss Inc., New York
56. Hayashi, M., Taniguchi, S., Ishizuka, Y., Kim, H. S., Wataya, Y., Yamamoto, A., and Moriyama, Y. (2001) J. Biol. Chem. 276, 15249–15255
The Signal Sequence of Exported Protein-1 Directs the Green Fluorescent Protein to the Parasitophorous Vacuole of Transfected Malaria Parasites
Akinola Adisa, Melanie Rug, Nectarios Klonis, Michael Foley, Alan F. Cowman and Leann Tilley

J. Biol. Chem. 2003, 278:6532-6542.
doi: 10.1074/jbc.M207039200 originally published online November 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207039200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 55 references, 19 of which can be accessed free at http://www.jbc.org/content/278/8/6532.full.html#ref-list-1