Luciferase, When Fused to an N-terminal Signal Peptide, Is Secreted from Transfected Plasmodium falciparum and Transported to the Cytosol of Infected Erythrocytes*

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Plasmodium falciparum, a unicellular parasite that causes human malaria, infects erythrocytes where it develops within a vacuole. The vacuolar membrane separates the parasite from the erythrocyte cytosol. Some secreted parasite proteins remain inside the vacuole, and others are transported across the vacuolar membrane. To identify the protein sequences responsible for this distribution we investigated the suitability of the green fluorescent protein and luciferase as reporters in transiently transfected parasites. Because of the higher sensitivity of the enzymatic assay, luciferase was quantified 3 days after transfection, whereas reliable detection of green fluorescent protein required prolonged drug selection. Luciferase was confined to the parasite cytosol in subcellular fractions of infected erythrocytes. When parasites were transfected with a hybrid gene coding for the cleavable N-terminal signal peptide of a secreted parasite protein fused to luciferase, the reporter protein was secreted. It was recovered with the vacuolar content and the erythrocyte cytosol. The results suggest that no specific protein sequences are required for translocation across the vacuolar membrane. The high local concentration of luciferase within the vacuole argues against free diffusion, and thus transport into the erythrocyte cytosol must involve a rate-limiting step.

Plasmodium falciparum, the parasite that causes the most severe form of malaria, spends part of its life cycle in human erythrocytes. Here it resides within the so-called parasitophorous vacuole, which is bound by the parasitophorous vacuolar membrane (PVM). The vacuole constitutes a separate compartment in the infected red blood cell (iRBC) that is distinct from the cytosol of the parasite and from the cytosol of the erythrocyte, respectively (1). Most proteins secreted from P. falciparum are not released into an extracellular space but are transported to various destinations within the iRBC: the parasitophorous vacuole or the erythrocyte cytosol. Membrane-bound proteins are found in the PVM and erythrocyte plasma membrane (2, 3). It is completely unknown which information within a polypeptide chain determines whether a protein is transported across the PVM. Recently we described experimental evidence for a transport pathway that involves the release of secreted parasite proteins into the vacuolar space and translocation across the PVM in a subsequent step (4). This model infers that a sorting mechanism must operate within the vacuole that discriminates between vacuolar resident proteins and proteins destined for a location beyond the PVM (5). Sorting could involve two different principles: the retention of vacuolar proteins or, alternatively, the recognition of protein signals that mediate translocation across the PVM.

A widely used experimental approach for the identification of protein targeting and sorting signals in eukaryotic cells is the fusion of putative signal sequences to reporter proteins and their subsequent localization in the transfected cell. Although transfection of P. falciparum blood stages has been established during the last 5 years (6, 7), the extremely low transfection efficiency has remained a major obstacle to a systematic analysis of putative signal sequences in this organism. Because the amount of a recombinant protein in a culture of transfected parasites is minute, either a very sensitive detection assay is required or parasite lines have to be selected for several months that stably express the reporter protein (8, 9). Furthermore, it is essential that the reporter protein can be localized reliably and reproducibly in each compartment of the iRBC and that its concentration can be precisely quantified therein. In this study we compare the green fluorescent protein (GFP) of Aequorea victoria, the luciferase of Photinus pyralis, and an epitope of the human c-myc oncogene (10–12) for their suitability as reporters for protein transport in P. falciparum. GFP and the c-Myc epitope are detected by fluorescence microscopy of transfected cells. The enzymatic activity of luciferase is determined in cell lysates; hence, a selective fractionation of iRBCs is essential. Previously we used the treatment of iRBCs with saponin and the bacterial pore-forming protein streptolysin O (SLO) to investigate the distribution of parasite proteins within the erythrocyte cytosol and vacuolar space (4). Thus the use of an enzymatically active reporter in combination with the fractionation of iRBCs should allow for the quantification of luciferase activity in different compartments of the iRBC, namely the parasite, vacuolar space, and erythrocyte cytosol. When luciferase was fused to the previously characterized N-terminal secretory signal sequence of the plasmodial exported protein 1 (EXP1), it was secreted from the parasite and recovered in the vacuolar space and erythrocyte cytosol. This suggests that translocation across the PVM does not involve protein sequences specific for secreted parasite proteins.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Luciferase and GFP were expressed from plasmids pHLH1 or pHRP-GFPm2, kindly provided by T. E. Wellems and K. Haldar, respectively (6, 13). The plasmid pDTTg23 coding for dihydrofolate reductase-thymidylate synthase of Toxoplasma gondii...
that confers pyrithione resistance to *P. falciparum* was provided by Y. Wu and T. E. Wellesms. All these plasmids contain the same control elements for transcription: the 5′-flanking regions of the gene encoding the histidine-rich protein 3 and the 3′-flanking region of the gene encoding the histidine-rich protein 2, respectively. Plasmid pHpH1, which encodes the c-Myc tag riBCs transfected with the plasmid pHpH1, was constructed by polymerase chain reaction as follows: using the primers 5′-GCT GAA TTC GACATA and 5′-GCC CCA AGC TTA AAA ATC TTC CTC ACT TAT TAA TTT CTG TTC AGA TCT CAA TTT GGA GTC TCC GTT GG and pHpH1 as template, a 345-base pair DNA fragment was synthesized that comprises the region of the luciferase gene downstream of the endogenous Clal restriction site, the sequence coding for the epitope EQKLISEEDL, and a HindIII site. Restriction sites are underlined. After digestion with PstI and NahI the polymerase chain reaction product was ligated into the naive site of pHpH1.

**P. falciparum Culture and Transfection**—*The P. falciparum isolate FCBR was cultured in RPMI 1640 medium (Life Technologies, Inc.) containing 10% human plasma and human erythrocytes of blood group A+ (Marburg Blood Bank) following standard procedures (15). Ring-stage parasites of a parasitemia of 10–15% were electroporated with the polymerase chain reaction product was ligated into the NsiI site of the plasmid pHpH1, and luciferase-encoding plasmid pHpH1 was transfected to the *P. falciparum* isolate HLB1. For the localization of proteins inside the parasite, fractionation of iRBCs was performed as described previously (4). Parasites were released from host cells by treatment with 0.1% saponin (grade pure, Serva) in phosphate-buffered saline for 10 min on ice, and then centrifuged to remove unbound saponin. The supernatant was centrifuged at 10,000 g for 5 min. The supernatant was centrifuged at 10,000 g for 5 min. After centrifugation, the supernatant was removed, and the pelleted parasites were washed twice with phosphate-buffered saline, frozen, and then thawed in 2 volumes of parasite lysis buffer (25 mM Tris-phosphate, pH 7.5, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100) containing Complete protease inhibitor mixture prepared according to manufacturer instructions (Sigma).

*Antisera*—Antibodies to plasmodial aldolase and the vacuolar resident SERP, the endoplasmic reticulum resident PfBiP, and cytosolic parasite aldolase in different fractions obtained after SLO or saponin treatment of iRBCs, aliquots of the supernatant and pellet fraction, each corresponding to 1 × 10⁸ iRBCs for aldolase, 3 × 10⁶ iRBCs for PfBiP, or 1 × 10⁷ iRBCs for SERP, were dissolved in SDS sample buffer, electrophoresed through 10% SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with specific rabbit antisera at a dilution of 1:500 and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG adsorbed with human serum proteins (Sigma). Blots were developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) following standard procedures (18).

**RESULTS**

**Comparison of GFP and Luciferase as Reporter Proteins in Transiently Transfected Parasites**—The DNA constructs analyzed in this study are shown schematically in Fig. 1. In initial experiments we compared the detectability of GFP and luciferase as reporters in transiently transfected parasites. *P. falciparum* was transfected with the plasmid pHpP-GFPM2 or pHpH1, and parasites were analyzed for the expression of the recombinant genes 24–72 h after transfection. Analysis of iRBCs transfected with the plasmid pHpP-GFPM2 revealed no detectable expression of the reporter protein, while 100% of iRBCs transfected with the plasmid pHpH1 revealed detectable levels of GFP. Transfections were conducted in parallel with iRBCs transfected with the plasmid pHpH1, and luciferase activity was detected in parasite lysates, which were harvested at 72 h after treatment of iRBCs with saponin. Reproducibly, luciferase activity was detectable in transfected but not in mock-transfected parasites. When transfctions were carried out using either larger amounts of DNA or larger numbers of iRBCs, chemiluminescence consistently increased, at most 5-fold. In subsequent experiments we routinely used 75 μg of DNA and 2 × 10⁸ iRBCs.
exported protein 1 (30). N-terminal amino acids of the precursor form of the epitope EQKLISEEDL (17), and the EXP1 signal comprises the 26 N-terminal amino acids of the precursor form of the P. falciparum exported protein 1 (30).

iRBCs. It is noteworthy that both genes contained an identical 5'- and 3'-flanking region including the transcriptional control elements.

To elucidate the reason for the failure to detect GFP in transiently transfected parasites, we intended to produce stable transfectants by co-transfection of iRBCs with either pHHRP-GFPM2 or pHHL1 and pDTTg23, a plasmid that encodes *T. gondii* dihydrofolate reductase-thymidylate synthase and thus confers resistance to pyrimethamine (8). From day 5, pyrimethamine was added to the cultures, and daily blood smears confirmed elimination of the parasites by the drug. 20 days after infection, parasitemia began to rise again, and cultures were maintained like wild-type parasites. As shown in Fig. 2, 35 days after transfection, luciferase activity in the parasite lysate was three orders of magnitude higher than after 3 days. After this prolonged period of selection, GFP was also detectable by fluorescence microscopy in parasites transfected with pHHRP-GFPM2. Bright green parasites of all developmental stages were clearly visible, amounting to a rate of 24% of the infected erythrocytes at day 30. When pyrimethamine pressure was applied further, the expression of both reporter genes declined. Most likely, the episomal replication of pDTTg23, the plasmid that carries the selectable marker, was favored. Alternatively, it is possible that spontaneous mutations in the endogenous plasmodial dihydrofolate reductase gene may have occurred, thus conferring resistance to pyrimethamine (22). For about 3 weeks, the amount of recombinant protein produced was sufficient for analytical purposes. Considering that the regions flanking the luciferase and gfp genes are identical and that transfections were carried out in parallel, the most plausible explanation for the early detection of recombinant luciferase remains that the enzymatic assay is far more sensitive than the visual analysis of GFP by fluorescence microscopy. This interpretation was corroborated using a chimeric construct in which the c-Myc epitope was fused to the C-terminus of the luciferase. Although luciferase activity was detectable in transfected parasites after 3 days, no signal was obtained in the immunofluorescence analysis using a specific monoclonal antibody to the c-Myc tag (data not shown).

The results described above clearly demonstrate that luciferase is the preferable reporter when it is required to analyze a large number of different DNA constructs, which is necessary for the identification of protein targeting and sorting signals. Apart from being detectable at low concentrations, the enzymatic activity can be quantified precisely. Luciferase carries at its C-terminus the sequence SKL, which functions as a peroxisomal import sequence in higher eukaryotes. Therefore we first investigated whether the recombinant protein in transfected parasites segregated from a characterized cytosolic plasmodial protein, parasite lactate dehydrogenase, that can also be quantified by an enzymatic assay. Infected erythrocytes, 3 days after transfection, were treated with increasing concentrations of saponin and separated into a pellet fraction and a supernatant. Both fractions were analyzed for the activity of parasite lactate dehydrogenase and luciferase (Fig. 3). Consistent with previous reports (4, 23), treatment of iRBCs with low saponin concentrations resulted in almost complete hemolysis, but up to a concentration of 0.15% saponin less than a 10% release of parasite lactate dehydrogenase and luciferase from the parasite was observed. At these concentrations more than 95% of the erythrocyte hemoglobins were released, demonstrating almost complete lysis of the infected host cell. At higher concentrations of saponin, the release of enzymatic activity shows essentially the same profile for both proteins, suggesting that the majority of luciferase was localized cytosolically (Fig. 3). In the following experiments, the distribution of luciferase in transfected parasites that had been selected for high expression by prolonged treatment with pyrimethamine (Fig. 2) was
determined. In these analyses an independent cytosolic protein, parasite aldolase, and a marker for a compartmentalized protein, PfBiP, were included. Infected erythrocytes were treated with different saponin concentrations applying a more narrow range of 0.2–0.8% saponin. Parasite aldolase and PfBiP were detected by immunoblotting, and recombinant luciferase was quantified by enzymatic activity (Fig. 4). Release of aldolase was detectable after the treatment of iRBCs with 0.2% saponin, and complete release was observed at 0.5% saponin. In contrast, release of low amounts of PfBiP required saponin concentrations of >0.5%. The amounts of the respective proteins in the pellet fractions decreased accordingly (data not shown). The segregation of luciferase resembled that of aldolase and PfBiP were detected by immunoblotting, and recombinant luciferase was quantified by enzymatic activity (Fig. 4). Release of aldolase was detectable after the treatment of iRBCs with 0.2% saponin, and complete release was observed at 0.5% saponin. In contrast, release of low amounts of PfBiP required saponin concentrations of >0.5%. The amounts of the respective proteins in the pellet fractions decreased accordingly (data not shown). The segregation of luciferase resembled that of aldolase. After lysis with 0.2% saponin, a significant amount of luciferase activity was released, and a steep increase of activity was detected at the next higher concentrations of saponin. Activity in the pellets showed a sharp decline from 0.2 to 0.6% saponin. In conclusion, these results argue against a localization of the recombinant protein within a subcellular compartment of the parasite.

Luciferase, When Fused to a Secretory Signal Sequence, Is Transported into the Infected Erythrocyte—The detection of luciferase as a reporter protein in various compartments of the infected erythrocyte, namely within the parasite, the vacuolar space and the erythrocyte cytosol depends on (i) an accurate subcellular fractionation and (ii) an accurate quantification of luciferase activity that takes into account possible quenching effects in different subcellular fractions. In fact, considerable quenching of luciferase activity by hemoglobin has been reported recently (24).

An experimental approach that allows a selective fractionation of iRBCs is based on the different lytic properties of SLO and saponin (4). SLO forms pores of 30 nm in diameter within the erythrocyte plasma membrane, but it does not insert into the PVM. After the treatment of iRBCs with SLO and subse-
quent centrifugation, the supernatant contains soluble proteins of the erythrocyte cytosol only. Saponin disintegrates both the erythrocyte plasma membrane and PVM. Soluble proteins contained in both compartments are released into the supernatant fraction. Routinely, complete disintegration of the erythrocyte plasma membrane is determined by the complete release of hemoglobin. The integrity of the parasite plasma membrane and the PVM is determined by the segregation of marker proteins contained within the parasite cytosol and vacuolar space, respectively.

Infected erythrocytes were transfected with either pHx1luc, which encodes luciferase, or pHx11uc, which codes for a chimeric luciferase that contains the N-terminal secretory signal sequence of the parasite protein EXP1. Routinely, transfections with a particular plasmid were carried out in triplicate and processed individually in parallel. The cells were harvested 72 h after transfection and fractionated by treatment with either saponin or SLO. In each sample, complete disintegration of the erythrocyte plasma membrane was monitored by quantification of hemoglobin in the supernatant and the respective pellet fractions. Consistently more than 97% of total hemoglobin was released upon treatment. To assess the integrity of the vacuolar membrane and the parasite plasma membrane in each sample, the segregation of two parasite marker proteins was determined in each experiment. Parasite aldolase is a cytosolic protein within the parasite, and SERP is localized within the vacuolar space. Fig. 5 shows exemplary results of these controls from two separate experiments conducted with iRBCs transfected with pHx1luc. After permeabilization with SLO, SERP was found predominantly in the pellet fraction (Fig. 5A, lanes 1 and 2) with only minor traces in the supernatant (Fig. 5A, lanes 3 and 4). Treatment with saponin resulted in an opposite distribution of the vacuolar protein, most of it being present in the supernatant (Fig. 5B). The segregation of aldolase into the pellet fraction after saponin treatment and its extremely low abundance in the supernatant fraction (Fig. 5C) clearly demonstrate that saponin treatment had not affected the integrity of the parasite plasma membrane.

In the initial experiments, defined amounts of recombinant luciferase (Promega) were added to parasite lysis buffer, supernatant fractions obtained either after SLO or saponin treatment of erythrocytes infected with nontransfected parasites, and the respective pellet fractions. Chemiluminescence was significantly lower in fractions that contained hemoglobin than in parasite lysis buffer alone or in fractions that consisted of parasite lysate devoid of hemoglobin (data not shown). Because luciferase activity remained constant in samples over a period of at least 3 h, significant proteolysis can be excluded. In accordance with previous reports (24), hemoglobin apparently affects the chemiluminescence of luciferase. Consequently, it was necessary to generate standard curves for each set of samples that were transfected and processed in parallel to allow a comparison of luciferase content in subcellular fractions of transfected iRBCs. Standard curves were generated as follows. First, the absorbance at 412 nm was measured in the supernatant of each sample, and the mean value was determined. Variations in the absorbance readings between different samples were less than 5%. A corresponding supernatant fraction from nontransfected iRBCs was adjusted to the same absorbance at 412 nm. Defined amounts of recombinant luciferase (Promega) were added to this control supernatant and parasite lysis buffer. Chemiluminescence was plotted versus luciferase protein concentration. An example for standard curves in the presence and absence of hemoglobin is shown in Fig. 6. Both curves cover the range of chemiluminescence measured in corresponding samples from transfected iRBCs.

Fig. 5. Distribution of marker proteins after fractionation of infected erythrocytes. Erythrocytes infected with transfected parasites were treated with either saponin or streptolysin O and separated into pellet (P) and supernatant (SNT) fractions. Samples corresponding to equivalent numbers of infected erythrocytes were solubilized in SDS sample buffer under reducing conditions and electrophoresed through 10% SDS-polyacrylamide gels. Proteins were blotted onto nitrocellulose filters and probed with antisera specific for the vacuolar resident serine-rich protein of \textit{P. falciparum} and aldolase (ALD), a marker protein of the parasite cytosol. Lanes 1 and 2 and lanes 3 and 4 are two representative examples from different experiments described in Table I, respectively. The molecular mass markers are indicated on the left.

Table I summarizes results from six independent experiments (A–F). In each experiment three independent cultures of iRBCs were transfected with the same plasmid and processed individually (see above). Transfected iRBCs treated with either saponin or SLO were fractionated into supernatant and pellet fractions. The fractionation was monitored as described for Fig. 5. The chemiluminescence of three 10-μl aliquots from each pellet and supernatant fraction was determined as relative light units. The values presented in column 5 are the mean values from nine measurements obtained from three independent cultures. The amount of luciferase present in each aliquot was extrapolated from the respective standard curve, and the total amount of luciferase contained in each fraction was calculated; the mean value is presented in column 6. None of the individual measurements deviated from the mean value by more than 10%. The mean values calculated for each fraction differed between different experiments, e.g. compare experiments E and F. We found that variations were caused by variables in the parasitemias of iRBCs, developmental stages of the parasite, batches of host erythrocytes, and the storage time of the luciferase assay reagents. When the distribution of
luciferase in different compartments was expressed as a percentage of total activity recovered from iRBCs in each experiment, results obtained from different experiments were comparable (for example, compare experiments C and D and E and F). Consistent with the results shown in Figs. 3 and 4, most of the luciferase expressed from plasmid pHLH1 was recovered inside the parasite, whereas most of the luciferase expressed as a chimeric construct from plasmid pHx1luc was detected outside the parasite, i.e. in the supernatant fractions obtained either after SLO permeabilization or treatment with saponin. Saponin treatment resulted in almost complete release of luciferase. The low activity recovered in the pellet after saponin treatment may either result from incomplete lysis of the PVM or luciferase en route to the parasite plasma membrane. After SLO treatment, the pellet contained considerable enzyme activity that therefore must have been located within the vacuolar space. Surprisingly, however, approximately two thirds of the secreted activity were detected in the fraction containing the erythrocyte cytosol. These results demonstrate that translocation of luciferase across the PVM had occurred.

**DISCUSSION**

The intracellular parasite *P. falciparum* develops within a host cell that is deficient in *de novo* protein synthesis and lacks the complex machinery required for protein transport. Thus the distribution of parasite proteins within the infected cell represents a biological phenomenon without precedence. The transport of secreted parasite proteins can be envisaged as a multistep process that includes secretion from the parasite cell and translocation across the PVM. In the present study we investigated to what extent specific targeting and sorting signals may be involved in these two steps. A major methodological obstacle to a systematic and reliable screening of a large number of putative targeting and signal sequences fused to reporter proteins is the extremely low transfection efficiency that necessitates long term selection to obtain analytical amounts of recombinant protein (25). Therefore we sought a reporter protein that was (i) detectable at very low levels and thus in transiently transfected cells and (ii) that could be quantified in various compartments of iRBCs. Three reporter proteins were compared: (i) the green fluorescent protein, which has been used extensively for morphological studies, and also in parasites because its bioluminescence can be detected in *situ*, without further processing of recombinant cells (13, 26–28), (ii) the c-Myc tag, a 10-amino acid epitope fused to the C terminus of luciferase that can be analyzed by immunofluorescence of a fixed cell after reaction with a specific monoclonal antibody, and (iii) luciferase, an enzyme that emits light when it catalyzes the reaction of luciferin with ATP. This chemiluminescence can be quantified precisely at very low enzyme concentrations (29). In contrast to morphologically detectable reporters, detection of luciferase activity generally requires lysis of cells and fractionation for its localization.

**TABLE I**

Luciferase activity in different subcellular fractions of infected erythrocytes

| Experiment | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------------|---|---|---|---|---|---|---|
|            | Plasmid | Lyzed with | Fraction | Fraction of iRBC | Luciferase activity | Total amount of luciferase in this fraction | Total amount of luciferase in this fraction |
| A          | pHHLH1 | saponin | SNT | V + E | 34 | 3.6 | 6 |
| B          | pHHLH1 | SLO    | E   | P | 7500 | 59.2 | 94 |
| C          | pHx1luc | saponin | SNT | V + E | 1187 | 76.6 | 96 |
| D          | pHx1luc | saponin | SNT | V + E | 500 | 2.9 | 4 |
| E          | pHx1luc | SLO    | E   | P | 1107 | 7.4 | 14 |
| F          | pHx1luc | SLO    | E   | P + V | 1296 | 5.4 | 38 |

FIG. 6. Standard curves of luciferase activity in samples of different hemoglobin content. Defined amounts of recombinant luciferase were added either to 10 μl of lysis buffer (solid line) or to 10 μl of an erythrocyte cytosolic fraction from infected erythrocytes obtained after permeabilization with streptolysin O (broken line). The hemoglobin absorbance of the cytosolic fraction was adjusted to 0.17 × 10⁻⁶. Luciferase activity was detected as RLU. Each data point represents the mean of three samples. The values did not deviate from the mean value by more than 5%.
In our experiments neither GFP nor the c-Myc epitope were visible by fluorescence microscopy in parasites 3 days after transfection, although the respective coding regions were flanked by the identical 5’- and 3’-regulatory elements as the gene encoding the readily detectable luciferase. Analysis of the hybrid pHluc-3m, composed of luciferase and the c-Myc epitope, demonstrates that at least in the case of the c-Myc epitope this discrepancy is caused by the higher sensitivity of the enzymatic assay. After prolonged pyrimethamine selection of parasites co-transfected with a plasmid carrying the dihydrofolate reductase-thymidylate synthase gene, GFP fluorescence was visible. We conclude that either the amount of GFP expressed in individual parasites 3 days after transfection was too low to be detected by fluorescence microscopy or that because of the extremely low transfection efficiency we missed positive parasites. Considering the increase of luciferase activity by several orders of magnitude during 35 days of selection, we estimate the transfection efficiency as 2.5 × 10⁻⁴, presumably for all constructs. This estimate is consistent with the report by Crabb et al. (25) and different from that of van Wye and Haldar (13). Transfection vectors have become available that carry both a selectable marker and the DNA sequence of interest (25). Although this provides a distinct advantage over the co-transfection with two separate plasmids, selection for stable recombinant parasite lines nevertheless remains time consuming and impractical for the initial screening of a large number of plasmid constructs.

Another consideration is the reliable quantification of low amounts of the reporter in various compartments, in this case the parasite cell, vacuolar space, and erythrocyte cytosol. The volume of the erythrocyte cytosol is considerably larger than the volume of the vacuole (see below). Thus, the fluorescence intensity may drop below detection limits when a protein is transported beyond the PVM and diluted into the host cell cytosol. If a soluble reporter protein such as GFP distributes between the vacuole and the erythrocyte cytosol, a vacuolar staining will be identified easily because of the high local concentration of the protein, whereas a cytosolic staining may be missed. In contrast, luciferase activity can be quantified in subcellular fractions at very low concentrations. In addition, the option to detect low levels of the reporter protein reduces the possibility of mistargeting as a result of overexpression.

To investigate whether transport across the PVM requires sequences specific to exported parasite proteins, luciferase was fused to the 26 N-terminal amino acids of the precursor of EXP1, a protein that is anchored within the PVM via a constitutive secretory pathway in both the parasite and a reconstituted heterologous system (14) and (ii) N-terminal sequences of the mature protein had revealed a signal peptide cleavage site at Ala-22–Glu-23 (31). Thus, the N-terminal signal sequence is removed from the mature protein in the parasite endoplasmic reticulum where signal peptide cleavage occurs. After fractionation of transfected iRBCs, luciferase activity was detected within the vacuolar space and erythrocyte cytosol. After correcting for the strong quenching of the chemiluminescence by hemoglobin, approximately two thirds of the entire luciferase activity were detected in the erythrocyte cytosol, whereas the vacuolar resident protein SERP was almost undetectable in this fraction. The most conceivable explanation for a location of luciferase in these two compartments is that transport occurs via a two-step process in which the vacuolar space is a transit compartment (5). The presence of luciferase in the erythrocyte cytosol argues against the existence of parasite-specific signal sequences required for protein translocation across the PVM, and it strongly suggests that the default pathway for secreted parasite proteins is transport to the erythrocyte cytosol. Although the respective volumes of the erythrocyte cytosol and vacuolar space have not been determined precisely, it is obvious from morphological data (32, 33) that the PVM tightly encloses the parasite and that the vacuolar space is small. At the trophozoite stage we estimate the ratio of vacuolar space/erythrocyte cytosol to be at least 1:10,000. In other words, the local concentration of luciferase is much higher within the vacuole than within the host cell cytosol. Therefore, the distribution of luciferase between the two compartments cannot be the result of free diffusion. It is more likely that luciferase is transported to the parasite plasma membrane via the constitutive secretory pathway of the parasite, which is completed within a few minutes (4), and that subsequent translocation across the PVM is the rate-limiting step. Hence, the protein accumulates in the vacuolar space. Although the levels of luciferase in transfected parasites are too low to perform pulse-chase experiments and to detect metabolically labeled protein directly, this view is supported by a study on the parasite-encoded glycoporphin-binding protein, which has a similar distribution to that of luciferase. This protein is transported to the erythrocyte cytosol in a two-step process (4). Translocation across the PVM requires ATP, and hence regulatory elements may be involved.

Our data demonstrate that parasite-specific signals are not required for the translocation of proteins across the PVM. Nevertheless, the glycoporphin-binding protein, the function of which is unknown (34), and luciferase, which is a foreign reporter protein, accumulate at high local concentrations within the vacuolar space. It remains an interesting possibility that other parasite proteins, for example those that modify the host cell plasma membrane (3), are transported to their final destination more efficiently.

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