Transport of Fluorescent Phospholipid Analogues from the Erythrocyte Membrane to the Parasite in *Plasmodium falciparum*-infected Cells

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Abstract. The asexual development of the human malaria parasite *Plasmodium falciparum* is largely intraerythrocytic. When 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazole-4-yl)amino]caproyl] phosphatidylcholine (NBD-PC) was incorporated into infected and uninfected erythrocyte membranes at 0°C, it remained at the cell surface. At 10°C, the lipid was rapidly internalized in infected erythrocytes at all stages of parasite growth. Our results indicate that the internalization of NBD-PC was not because of endocytosis but rapid transbilayer lipid flip-flop at the infected erythrocyte membrane, followed by monomer diffusion to the parasite. Internalization of the lipid was inhibited by (*a*) depleting cellular ATP levels; (*b*) pretreating the cells with *N*-ethyl maleimide or diethylpyrocarbonate; and (*c*) 10 mM L-α-glycerophosphorylcholine. The evidence suggests protein-mediated and energy de-pendent transmembrane movement of the PC analogue.

The conditions for the internalization of another phospholipid analogue *N*-4-nitrobenzo-2-oxa-1,3-diazoledipalmitoyl phosphatidylethanolamine (*N*-NBD-PE) were distinct from that of NBD-PC and suggest the presence of additional mechanism(s) of parasite-mediated lipid transport in the infected host membrane. In spite of the lack of bulk, constitutive endocytosis at the red cell membrane, the uptake of Lucifer yellow by mature infected cells suggests that microdomains of pinocytotic activity are induced by the intracellular parasite. The results indicate the presence of parasite-induced mechanisms of lipid transport in infected erythrocyte membranes that modify host membrane properties and may have important implications on phospholipid asymmetry in these membranes.

D URING its intraerythrocytic development, the human malaria parasite *Plasmodium falciparum* induces changes in both lipid and protein components of the infected erythrocyte membrane. Several parasite proteins are introduced into the erythrocyte membrane (4, 11, 12, 18, 24, 31, 36, 38); its morphology, rigidity, and permeability are markedly altered (5, 8, 17); and there is increased disorder in phospholipid packing of the bilayer (33). The distribution of phospholipids in *P. falciparum* and *P. knowlesi*-infected erythrocyte membranes may be altered in contrast to unin-fected cells (9, 26), and an increased uptake and exchange-ability of phospholipids is reported between *P. knowlesi*-in-fected erythrocyte membranes and synthetic phospholipids (20, 37). However, little is known about the molecular basis of these changes in parasitized erythrocytes.

Membrane phospholipid asymmetry is an intrinsic proper-ty of cellular membranes. Altering the distribution of phos-pholipids across the plasma membrane (for example, in-creasing phosphatidylserine on the exoplasmic leaflet of the

plasma membrane) can lead to clearance of the cell from cir-culation (25, 32). Rapid transbilayer phospholipid movement across biological membranes appears to be protein-mediated and energy dependent, and important for maintaining lipid asymmetry. Transbilayer movement of phosphatidylcholine (PC)[1] observed in rat liver microsomes, has been ascribed to a PC transporter in these membranes (2). In the nonendo-cytic red cell, protein-mediated transport of phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS) to the cyto-plasmic leaflet is an energy requiring process (6, 34, 40) that appears to serve as the primary mechanism for maintaining lipid asymmetry at the plasma membrane. Band 7 has been

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1. *Abbreviations used in this paper:* diI, 3,3-dihexadecylindocarbocyanine; DPPC, dipalmitoylphosphatidylcholine; DTAF, 5-(4,6-dichlorotriazinyl)-aminofluorescein; GPC, glycerophosphorylcholine; GPE, glycerophospho-rylethanolamine; GPS, glycerophosphorylserine; LY, Lucifer yellow; NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazole-4-yl)amino]caproyl] PC; NBD-PE, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazole-4-yl)amino]-caproyl] PE; NBD-PS, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazole-4-yl)amino]caproyl] PS; *N*-NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazoledipal-mitoyl PE; *N*-Rh-PE, *N*-Rhodamine PE; NEM, *N*-ethyl maleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidyl-serine; TNBS, trinitrobenzene sulfonic acid.

identified as the putative aminophospholipid translocator in human erythrocytes [3]. Human erythrocyte membranes do not contain a PC transporter, and PC transbilayer movement in these membranes is extremely slow [21].

We have investigated the intracellular translocation of the fluorescent lipid probes, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazole-4-yl)amino]caproyl] phosphatidylcholine (NBD-PC) and N-4-nitrobenzo-2-oxa-1,3-diazoledipalmitoyl phosphatidylethanolamine (N-NBD-PE), in malaria-infected erythrocytes. Analogues of PC, inserted into the erythrocyte membrane, remain in the exoplasmic leaflet of uninfected cells [21, 27]. N-NBD-PE is also inserted in the exoplasmic leaflet of the uninfected erythrocyte membrane. Its transport to the cytoplasmic face by the red cell aminophospholipid translocase appears to be hindered because of the derivatized ethanolamine head group [23]. Our results indicate that, in plasmodium-infected red cells, both probes are translocated across the erythrocyte membrane to the parasite probably by distinct mechanisms of parasite-mediated lipid transport. Evidence is presented for protein-mediated PC translocation and pinocytosis of the fluid phase marker, lucifer yellow, at the infected erythrocyte membrane. However, 3,3-dihexadecylindocarbocyanine (diI) and fluorescent band 3 were not internalized from the infected erythrocyte membrane, indicating that constitutive bulk phase endocytosis was not induced in the host bilayer.

Materials and Methods

Materials

NBD-PC was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). N-Rhodamine phosphatidylethanolamine (N-Rh-PE), N-NBD-PE, diI, and 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF) were purchased from Molecular Probes Inc. (Junction City, OR). Lecithin was from Avanti Polar Lipids, Inc. or Sigma Chemical Co. (St. Louis, MO). D- and L-α-dipalmitoylphosphatidylcholine (DPPC), lucifer yellow (LY), L-α-glycerophosphorylcholine (GPC), L-α-glycerophosphorylethanolamine (GPE), L-α-glycerophosphorylserine (GPS), phosphorylcholine, trinitrobenzene sulfonic acid (TNBS), 4 4'-diisothiocyanostilbene-2 2'-disulfonic acid, diethyl pyrocarbonate, N-ethyl maleimide (NEM), luciferin, luciferase, dimyristoyl phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and phosphatidylinositol, were from Sigma Chemical Co. Si 60 TLC plates were from E. Merck (Darmstadt, FRG). Hoechst 33258 was from Sigma Chemical Co. RPMI 1640 was from Gibco Laboratories (Grand Island, NY). All other chemicals were reagent or higher grade.

Preparation of D-α-glycerophosphorylcholine

D-α-dipalmitoylphosphatidylcholine (DPPC) was deacylated by base hydrolysis and the water soluble fraction was neutralized and concentrated as described [14]. Deacylation of the phospholipid was confirmed by the mobility of the water soluble products on chromatograms. Yields of 80–105% of the D-glycerophosphorylcholine (GPC) were obtained [14].

Culturing P. falciparum FCR-3/A₂

The parasites were grown in vitro in RPMI 1640 containing 10–20% A⁺ human serum, at 3.5% hematocrit, according to a modification of the method of Trager and Jensen [35]. Asynchronous cultures of 10% parasitemia were used in the microscopic analyses of the uptake of fluorescent lipids. Cultures with 50% parasitemia (used in the lipid extraction studies) were grown at 1% hematocrit in 20% human serum and fed twice daily. Cultures with higher parasitemia, containing late rings and early trophozoites, were obtained by incubating late stage schizonts (>42 h) with uninfected erythrocytes in RPMI 1640 containing 20% human serum, at 0.5% hematocrit and 20–25% parasitemia. Cultures were fed 3, 6, and 12 h later. Late rings and early trophozoites at 60–80% parasitemia were harvested ~24 h after the time of the initial incubation. Human erythrocytes and serum (from a known donor pool) previously known to support high multiplicities of invasion and parasitemia, were used to obtain the high parasitemias. Early schizonts were purified from synchronized cultures by density centrifugation over Percoll [11].

ATP Measurements

7.5×10^7 cells from infected cultures were incubated at 7.5×10^6 parasites/5 ml of RPMI 1640, for 6 min at 37°C. Cells were depleted of ATP by including 1 mM sodium azide or 1 mM potassium cyanide in the incubation. The cells were subsequently chilled on ice, washed three times in 50 ml of RPMI 1640 at 0°C. Uninfected erythrocytes were simultaneously incubated in the same volumes of RPMI 1640 and processed identically. Cellular ATP levels were determined by a modification of the method of Kimmich et al. [15]. 7.5×10^7 cells were lysed in 0.75 ml (corresponding to 1:100 vol/vol dilution) of boiling distilled water for 1 min to inactivate the endogenous ATPase activities [6]. The lysates were spun for 30 min at 16,000 g to remove precipitated material. The supernatants were diluted 1:100 or 1:300 in 50 mM glycine dilution buffer (50 mM glycine, 1 mM Tris-HCl, 5 mM magnesium sulfate, 0.5 mM EDTA, 1 mg/ml BSA, 0.1 mg/ml sodium azide, pH 8.0). 5 μl of sample were diluted 1:10 in the luciferin extract (F-4755; Sigma Chemical Co.; 0.15 mM luciferin) prepared in glycine diluent. 50 μl of diluted samples were mixed with 50 μl of glycine diluent containing 5 μg of luciferase (L-5256; Sigma Chemical Co.) and the peak flash height was recorded. The standard curve was linear over range of 5×10^{-9} to 5×10^{-12} M ATP. The ATP levels of the diluted supernatants for all samples were found to be in this range.

Labeling and Analysis of Cellular Fluorescence

Cells were labeled with the appropriate marker, as described below, and viewed using either an epi-fluorescence EF-D microscope with a microflex UFX photomicrographic adapter (Optiphot Biological Microflex; Nikon Inc., Garden City, NY), or an epi-fluorescence microscope with a PM-10AD photomicrographic adapter (BO71; Olympus Corporation of America, New Hyde Park, NY). Cells labeled with NBD-PC, N-NBD-PE, DTAF, and LY were viewed with filters used for fluorescein. DiI and bisbenzimide fluorescence (Hoechst 33258) were monitored with filter settings for rhodamine and UV, respectively. Fluorescence micrographs of a time course of lipid internalization were taken at identical exposures, at 1600 ASA, with color print film (Fuji Photofilm USA Inc., New York, NY) or ektachrome 800/1600 ASA (Eastman Kodak Co., Rochester, NY).

NBD-PC Labeling. Cells from a parasite culture were collected by centrifugation at 550 g, washed free of serum, and resuspended in 1 ml of RPMI 1640 at 0.75% hematocrit at 0–2°C. For the experiments described in Fig. 1, various amounts of NBD-PC in 2 μl of ethanol were added to the cell suspension and incubated for 15 min on ice. For all other experiments, 2 μl of 1 mg/ml NBD-PC in ethanol was added. The cells were subsequently
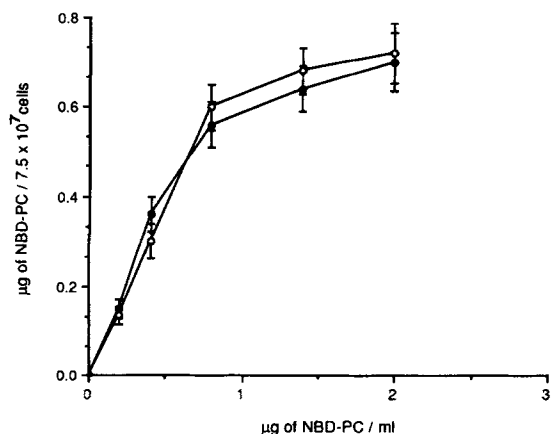

Figure 1. Transfer of NBD-PC to uninfected and infected erythrocyte membranes. 7.5×10^7 cells were incubated in 1 ml of RPMI 1640, at 0°C, with varying amounts of NBD-PC, as described in Materials and Methods. The cell-associated fluorescence in 1% Triton X-100 was determined spectrophotometrically and was quantitated using standard curves of NBD-PC containing equivalent numbers of cells as the samples: (○) uninfected cells; (●) 63.2% ring- and trophozoite-infected cells.

washed twice in RPMI 1640, followed by two more in RPMI 1640, at 0–2°C.

N-NBD-PC Labeling. Infected and uninfected cells were washed and resuspended in 10 ml of RPMI 1640 containing 1 mM sodium azide at 0.5% hematocrit at 37°C for 20 min. For the experiments in Fig. 6, varying amounts of label were added to the cell suspension. For all other experiments, 10 µl of 1 mg/ml *N*-NBD-PE in ethanol was added to the cells. The labeled cells were subsequently chilled to 0°C and washed on ice with RPMI 1640 as described above. Uninfected cells were labeled with *N*-Rh-PE under the same conditions.

diI Labeling. Varying amounts of diI (Fig. 4), or 3 µl of 1 mg/ml diI stock solution in ethanol, were added to 10 ml of parasitized cells at 0.5% hematocrit in RPMI 1640, at 0°C for 15 min. The cells were washed as described above, and resuspended in RPMI 1640 at 10°C and 37°C, for up to 2 h. The amount of cell-associated label was measured in a spectrofluorometer. NBD (λex = 470 nm, λem = 530 nm), and diI (λex = 540 nm, λem = 556 nm) fluorescence was determined in PBS (8.5 g/l sodium chloride, 0.8 g/l disodium monohydrogen phosphate, 0.1 g/l potassium dihydrogen phosphate, pH 7.4) in the presence or absence of 1% Triton X-100.

DTAF Labeling. Cells from a parasite culture were labeled with DTAF by a method of Sheetz et al. (28).

Hoechst Staining. 1–5 µg/ml of Hoechst 33258 in RPMI was added to cell suspensions being labeled with NBD-PC or *N*-NBD-PE.

Vesicle Preparation and "Back-Extractions" of NBD-PC from Infected and Uninfected Cells

Commercially available egg lecithin was dried from chloroform/methanol, 2:1, under a stream of nitrogen, and vesicles were prepared by mild sonication of a 2 mg/ml lipid suspension in PBS/glucose, in a bath sonicator, for 30 min. The vesicle preparation was centrifuged at 600 g, and the supernatant was stored at 0°C and used to perform back-extractions within 12 h. NBD-PC-labeled cells from parasite cultures were incubated in RPMI 1640 at 7.5 × 10^6 parasites/5 ml, at 0°C or 10°C for 30 min. Control uninfected cells were incubated in the same volume of buffer under identical conditions. The labeled cells were collected by centrifugation and were incubated with 1 ml of PBS containing 20 mM glucose and 2 mg/ml PC vesicles, at 0°C, for 10 min (back-extraction). The cells were collected by centrifugation at 550 g (which did not bring down the vesicles), back-extracted a second time, washed once in PBS/glucose, and the cell-associated fluorescence, in 1% Triton X-100 in PBS, was quantitated spectrophotometrically. Ca^{++}, Mg^{++}, and EDTA had no effect on the back-extraction procedure. Non-back-extracted cells were processed in the same way, but incubated in PBS/glucose not containing the lipid vesicles. The fluorescence of a sample was normalized to the total amount of heme in the sample, which was determined spectrophotometrically.

Uptake of LY by Parasitized Erythrocytes

Parasitized erythrocytes washed free of serum were resuspended in RPMI 1640 at 3.5% hematocrit, 10–20% parasitemia with 0.5 mg/ml of LY, at the desired temperature (37°C ± sodium azide, or 10°C) for 30 min. At the end of the incubation, the cells were washed to remove excess dye and resuspended in fresh RPMI and viewed with filter settings for fluorescein. Ring-stage parasites were detected by Hoechst 33258 staining.

Extraction and Analysis of Lipids by TLC and Spectrophotometry

NBD-PC and *N*-NBD-PE-labeled parasitized erythrocytes (50% parasitemia) were extracted twice in acidified chloroform/methanol, 2:1. Lipids from equivalent numbers of cells in the infected and uninfected samples were applied to an HPTLC Si 60 plate and chromatographed in CHCl$_3$/CH$_3$OH/28% NH$_4$OH, 65:25:5. Lipid standards were detected by staining in iodine. For qualitative analysis, the fluorescent lipids were detected by UV and photographed. For quantitative analysis, the extracted TLC-purified cell-associated fluorescent lipid spot was scraped, the lipid was eluted in ethanol and quantitated spectrophotometrically.

Results

Transfer of NBD-PC to the Erythrocyte Membrane of Uninfected and Parasitized Erythrocytes

When parasite cultures were incubated with NBD-PC on ice

for 15 min, the lipid analogue was incorporated into the erythrocyte membrane. To compare the amount of fluorescent lipid transferred to uninfected and parasitized cells, we incubated normal red cells and cultures at ∼50% parasitemia, with different amounts of NBD-PC at 0°C. Because of technical limitations in culturing human malaria parasites, it was not possible to obtain a population of 100% viable parasitized erythrocytes on a routine basis. Parasitemias ∼50% were obtained and routinely used for our experiments. However, whenever possible and available, higher parasitemias were employed. The labeled cells were lysed in Triton X-100 and the fluorescence was quantitated spectrophotometrically. Heme in cell lysates resulted in partial quenching of NBD-PC fluorescence. Consequently, standard curves for NBD fluorescence, containing equivalent numbers of uninfected cells and those from parasite cultures, were constructed. The degree of quenching by equal numbers of cells from each group was the same. This might be expected, since the total amount of hemin that can be extracted from cells appears to be constant for infected and uninfected erythrocytes (1). As shown in Fig. 1, at 7.5 × 10^7 cells/ml, the same amount of label is transferred to uninfected red cells and those at 63.2% parasitemia, over a range of 0–2 µg/ml of NBD-PC added to the medium. At these concentrations of NBD-PC, the amount of label transferred to the erythrocyte membrane did not vary with the degree of parasitism of the cell population (not shown). At 2 µg/ml of NBD-PC ∼30% of the added label was maximally associated with the erythrocyte membranes of both infected and uninfected cells. The cell-associated label approached saturation at concentrations beyond 2 µg/ml.

Internalization of NBD-PC in Parasitized Erythrocytes

As shown in Fig. 2 *A*, when cells were incubated with NBD-PC at 0°C, both infected and uninfected erythrocytes displayed a cell surface labeling pattern that could be maintained for 2 h at 0–2°C. When the cells were warmed to 10°C for 15 min, a distinctly different pattern of fluorescence was observed (Fig. 2 *B*). In parasitized erythrocytes, a considerable amount of the cell surface fluorescence was transported to the intraerythrocytic parasite. Erythrocyte membrane fluorescence was still associated with both infected and uninfected cells. TLC analysis of lipids, from parasitized erythrocytes (50% parasitemia) and uninfected erythrocytes labeled with NBD-PC at 0°C and incubated for 1 h at 10°C, indicated that over 99% of the label in both infected and uninfected cells was NBD-PC (not shown). Consequently, the observed cell-associated fluorescence in Fig. 2 was because of the NBD-PC moiety originally inserted in the erythrocyte membrane. The internalization of fluorescent lipid was observed at all stages of intraerythrocytic parasite growth. To facilitate the handling and detection of unfixed parasitized erythrocytes by light microscopy, our measurements were made with asynchronous cultures containing predominantly late ring and early trophozoite stages (15–30 h in a 48-h developmental cycle).

We investigated the quenching of NBD-PC in parasitized erythrocytes, after various times of incubation at 10°C, to determine whether the probe was accumulated at high concentrations in an intracellular compartment, as a consequence of which it was self quenched and not detected in Fig. 2. Upon insertion into the erythrocyte membrane of cells from
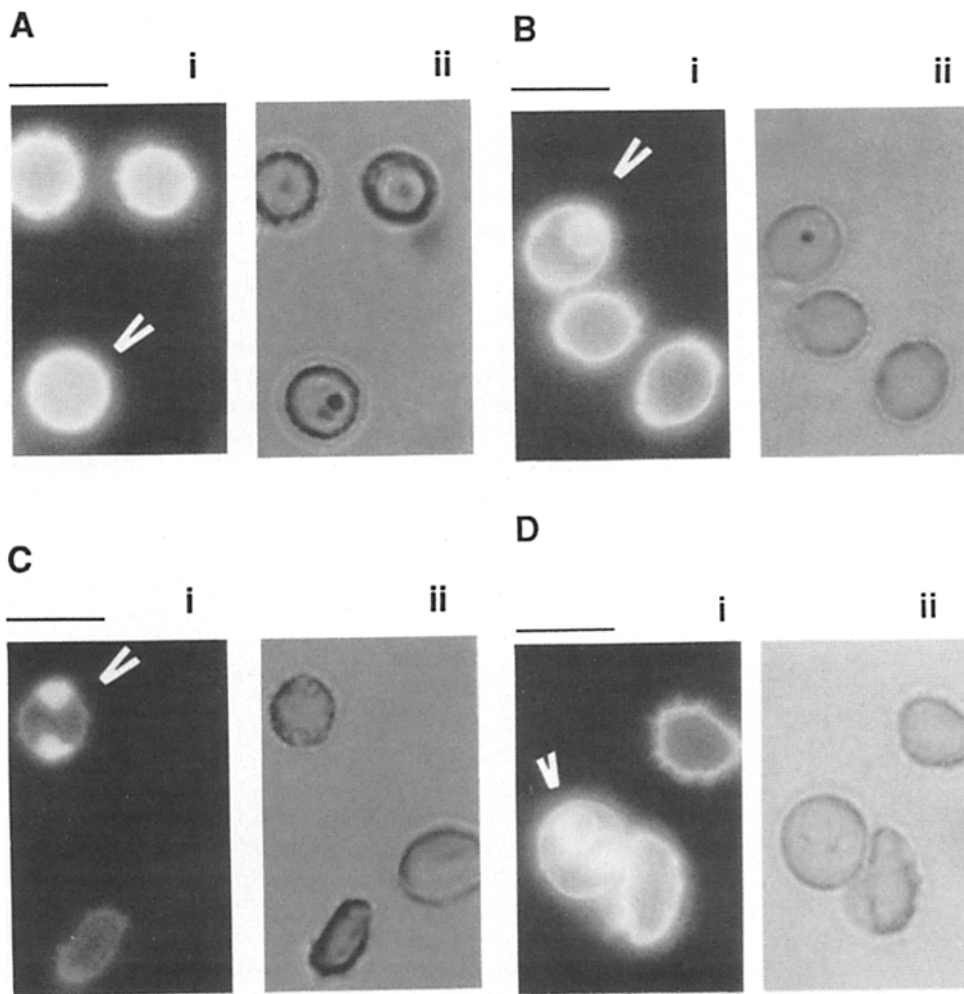
Figure 2. Fluorescence micrographs of uninfected and parasitized erythrocytes incubated with NBD-PC. Cells from parasite cultures (10% parasitemia) were labeled with NBD-PC. (*i*, fluorescence micrographs; *ii*, light micrographs.) *A*, Trophozoite incubated at 0–2°C for 15 min; at these temperatures the observed cell surface fluorescence remained unchanged for up to 2 h; *B*, late ring/early trophozoite incubated at 10°C for 15 min; *C*, double infected late rings incubated at 10°C for 30 min and back-extracted; *D*, non–back-extracted cells from *C*; i.e., double infected late rings incubated at 10°C for 30 min. Bar, 10 μm.

an infected culture (50.5% parasitemia), NBD-PC was quenched, compared to its fluorescence in a Triton X-100 cell lysate. However this ratio of fluorescence (0.48 ± 0.07) was essentially unaltered during its incubation at 10°C. The results indicate that the probe did not move to an intracellular compartment where it was subsequently relatively quenched or enhanced, and the distribution of fluorescence in Fig. 2 appeared to be representative of the relative distribution of the probe in the cell. NBD-PC that was inserted into normal erythrocyte membranes suffered slightly more quenching (0.42 ± 0.07; Fig. 3), in contrast to its fluorescence in a Triton X-100 lysate. The degree of quenching remained unchanged upon incubation at 10°C for 1 h. The data suggest that the quenching observed upon insertion of the probe in both infected and uninfected cells was due, not to self quenching, but probably to the high localized concentration of heme in intact cells. The difference in the degree of quenching in the infected and uninfected cells fell within the error of our measurements.

To determine the amount of NBD-PC internalized, parasitized erythrocytes were back-extracted at 0°C with an excess of unlabeled PC vesicles (Materials and Methods). Most of the erythrocyte membrane fluorescence of both infected and
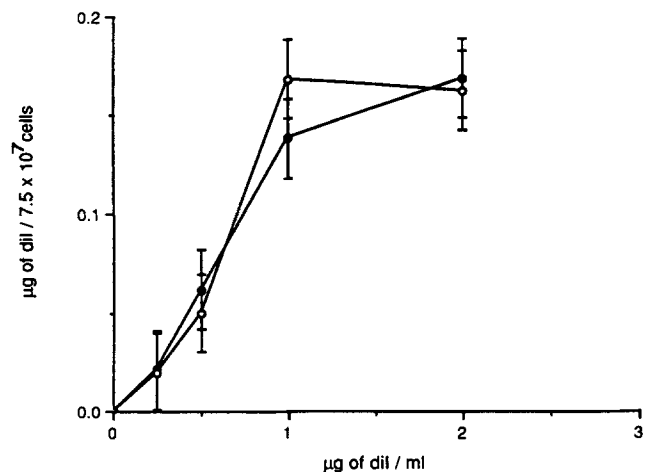

Figure 3. Transfer of diI to parasitized erythrocytes. Cells were incubated with varying amounts of diI at 0°C. The cell-associated label was quantitated spectrophotometrically and corrected for quenching due to hemoglobin, as described in Fig. 1 and Materials and Methods. (○) uninfected erythrocytes; (●) 47.8% infected erythrocytes.

Table I. Internalization of NBD-PC by Parasitized Erythrocytes

Incubation (temperature; time)	% Label resistant to back-extraction
Uninfected red cells	
0°C, 10°C; 0 min	25.6 ± 4.3
0°C; 30 min	28.2 ± 5.2
10°C; 30 min	26.8 ± 5.6
+ Azide, 10°C; 30 min	26.6 ± 6.3
+ Cyanide, 10°C; 30 min	27.9 ± 7.3
Ring- and trophozoite-infected cells	
0°C, 10°C; 0 min (49% parasitemia)	26.2 ± 6.2
0°C; 30 min (49% parasitemia)	27.3 ± 9.1
10°C; 30 min (49% parasitemia)	53.2 ± 10.8
+ Azide, 10°C; 30 min (49% parasitemia)	27.7 ± 10.2
+ Cyanide, 10°C; 30 min (49% parasitemia)	29.0 ± 10.4
0°C, 10°C; 0 min (60% parasitemia)	26.1 ± 6.8
0°C; 30 min (60% parasitemia)	30.1 ± 8.1
10°C; 30 min (60% parasitemia)	67.4 ± 12.4
+ Azide, 10°C; 30 min (60% parasitemia)	28.4 ± 11.4
+ Cyanide, 10°C; 30 min (60% parasitemia)	31.6 ± 11.1

Infected and uninfected cells were labeled with NBD-PC and incubated as described in Materials and Methods at the indicated times and temperatures. For each batch of cells (i.e., uninfected, 49% parasitemia and 60% parasitemia), the labeling was repeated three times, in triplicate. Consequently, the numbers reflect three independent labeling experiments and are an average of nine measurements.

uninfected red cells was removed. The intracellular parasite-associated fluorescence was resistant to back-extraction (Fig. 2 *C*). TLC analysis indicated that the parasite-associated label was NBD-PC (not shown). As shown in Table I, both labeled uninfected and infected erythrocytes at 0°C, 0 and 30 min, were back-extracted twice, with ~25–32% of the label remaining cell associated. After three to four back-extraction steps, 15–5% of the label remained cell associated with uninfected erythrocytes. However, because of the relative fragility of trophozoite-infected erythrocyte membranes to repeated washings and centrifugation steps, three to four back-extractions resulted in significant loss of parasitized cells from the samples. Cells were lost in both control and back-extracted samples, indicating that the back-extraction with lecithin vesicles per se, was not destabilizing the cell. Consequently, two back-extraction steps were found to be optimal in estimating the relative differences of cell-associated label in infected and uninfected cells. When labeled infected cultures at 48 and 60% parasitemia were incubated for 30 min at 10°C and back-extracted, 53 and 67% of label remained cell associated. In contrast, only 25–30% of the label remained associated with control uninfected erythrocytes. Judging from Fig. 2, *C* and *D*, the depleted fluorescence appears to correspond to the erythrocyte membrane of both infected and uninfected cells. If the results were extrapolated to 100% parasitemia, ~70–75% of the label in parasitized cells would be resistant to back-extraction after 30 min, compared to ~25% in normal erythrocytes. Shown in Fig. 2 *D* are the corresponding non–back-extracted cells of Fig. 2 *C*. The fluorescence intensity in the erythrocyte membrane appears to be similar for both infected and uninfected cells, although our calculations would predict a 50% relative decrease of label in the infected erythrocyte membrane. The observed uniform distribution is probably because of rapid monomer

diffusion of NBD-PC from one erythrocyte membrane to the next. When the uninfected erythrocytes are dual labeled with *N*-Rh-PE and NBD-PC, and incubated with unlabeled parasitized erythrocytes in RPMI 1640 at 10°C for 30 min, the infected erythrocytes are labeled by the NBD-PC (not shown). *N*-Rh-PE is not exchanged at these temperatures, indicating that the transfer of NBD-PC is not because of fusion of infected and uninfected cells. 1 mM Ca^{++} and Mg^{++} have no effect on label transfer. Other reports indicate that C6-NBD analogues of D- and L-αPS, when microinjected in Chinese hamster fibroblasts, rapidly label intracellular membranes by monomer diffusion (19). Our results suggest that NBD-PC inserted in plasmodium-infected erythrocyte membranes is rapidly "flipped" to the cytoplasmic face of the membrane, whence it is transferred to the parasite, probably by monomer diffusion.

Transport of diI, DTAF-labeled Band 3, and LY in Parasitized Erythrocytes

We used a fluorescent lipophillic carbocyanine dye, diI (C16), as a nonspecific marker for the lipid bilayer of the erythrocyte membrane. The dye was inserted into the erythrocyte membrane of cells from a parasite culture, at 0°C. As shown in Figs. 3 and 4 *A*, infected and uninfected membranes were labeled with equal amounts of diI. As is common with many lipophillic dyes, there was heterogeneity in the labeling of individual cells. However, the same degree of heterogeneity appeared to exist in a population of cells independent of parasitism. Surface fluorescence was observed for all cells. At 10°C and under conditions which cause internalization of NBD-PC, no internalization of diI was observed, indicating that intracellular transport of NBD-PC was not because of endocytosis. Even after 60 min at 37°C, there was no polarization or internalization of the cell surface fluorescence (Fig. 4 *A*). The initial quenching of diI infected erythrocytes at 50% parasitemia was small and the ratio of fluorescence between intact and triton-solubilized cells was constant for up to 60 min at 37°C. This fluorescence ratio was also constant 10°C (not shown). Consequently, the probe did not appear to suffer different extents of self quenching during the incubation, and relative distribution of fluorescence in the micrographs is indicative of the real distribution of the probe in the cells. The fluorescence pattern of both parasitized and nonparasitized erythrocytes remained unchanged and entirely with the erythrocyte membrane for several hours. No internalization of lipid was observed even when the cells were labeled with 0.1 μg of diI/7.5 × 10^7 cells, (~0.03 μg of dI were delivered to 7.5 × 10^7 cells in Fig. 4 *A*). The data indicate that constitutive endovesiculation of the lipid bilayer in general does not occur in the infected erythrocyte membrane, even at 37°C. When cells from a parasite culture were labeled with the membrane-impermeable amino-reactive reagent DTAF, under conditions where over 90% of the label becomes associated with Band 3 (28), a uniform cell surface fluorescence was observed for both parasitized and non parasitized erythrocytes. The intracellular parasite was not labeled. A mature trophozoite, surface-labeled with DTAF and incubated in RPMI 1640 for 60 min at 37°C, is shown in Fig. 4 *B*. This uniform surface label remained essentially unchanged over the entire intraerythrocytic life cycle.
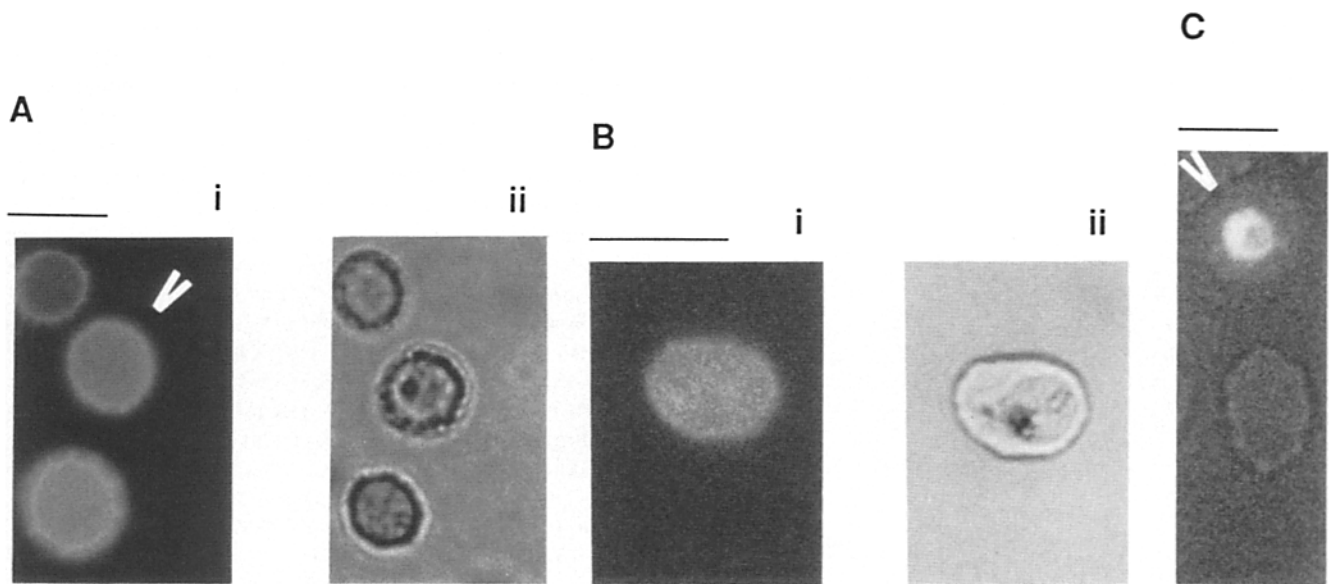
The fluid phase marker, LY, was used to characterize con-

Figure 4. Fluorescence micrograph of parasitized erythrocytes incubated with diI and surface-labeled with DTAF. *A,* Cells labeled with diI at 0°C were incubated in RPMI 1640 for at 37°C for 60 min (*i*, diI fluorescence micrograph; *ii*, light micrograph). *B,* Mature trophozoite, surface-labeled with DTAF and incubated in RPMI 1640 at 37°C for 60 min (*i*), DTAF label; *ii*, light micrograph). *C,* Cells from an asynchronous parasitized culture were incubated with 0.5 mg/ml LY at 37°C in RPMI 1640 for 60 min. Only mature infected cells (*arrowhead*) internalized the label. Bar, 10 μm.

ditions for pinocytosis in parasitized erythrocytes. Uptake of LY was observed in mature infected cells at 37°C, suggesting that their erythrocyte membranes were capable of pinocytosis (Fig. 4 *C*). This pinocytotic uptake was blocked in the presence of 1 mM sodium azide or potassium cyanide and also not observed at 10°C or in uninfected and ring-infected erythrocytes. The results confirm that the uptake of NBD-PC observed at 10°C and at all stages of parasite growth, was independent of endocytosis.

Energy Depletion in Parasitized Erythrocytes

The steady-state ATP levels of control 80% parasitized erythrocytes containing late rings and early trophozoites, was 54.6% of uninfected erythrocytes. The ATP level of a population of ∼80% ring- and trophozoite-infected erythrocytes pretreated with 1 mM sodium azide or 1 mM sodium cyanide for 6 min at 37°C, was depleted by 67 and 62.3% respectively, compared to the control. The ATP level of a population of 77% mature trophozoite/early schizont–infected cells was 35–40% of uninfected cells. Preincubating the mature infected cells for 6 min at 37°C with 1 mM azide or cyanide, depleted their ATP by 70.6 and 45.1%, respectively. Malaria parasites depend primarily on fermentation reactions. Although they do not appear to possess the complete citric acid cycle, they are very sensitive to cyanide and other mitochondrial inhibitors, and possess cytochrome oxidase (7). Pretreating uninfected erythrocytes with sodium azide or sodium cyanide for 6 min did not affect their ATP levels. Low levels of cyanide inhibits the luciferin–luciferase assay used to measure ATP. To determine that the diminished ATP levels observed on treating the parasitized erythrocytes with sodium cyanide were not because of contaminating traces of the anion, uninfected erythrocytes incubated with 1 mM sodium cyanide were simultaneously processed with the infected erythrocyte samples.

Pretreating parasitized erythrocytes with 1 mM sodium azide or 1 mM potassium cyanide significantly inhibited the internalization of cell surface NBD-PC (Table II). Over 95% of the infected erythrocytes contained no detectable internalized label. The amount of label inserted in the erythrocyte membrane of cyanide or azide treated infected cells remained unchanged compared to control cells, and the amount of label available for back exchange after incubation of azide- or cyanide-treated parasites at 10°C for 30 min, was similar to that for uninfected red cells (Table I). The data indicate that the ATP level of ring- and trophozoite-infected cells was substantially lower than uninfected erythrocytes, but sufficient to support the rapid transmembrane movement of NBD-PC. Further depletion of ATP in parasitized cells, to levels that were 33–38% of parasitized erythrocytes and only 15–18% to that of uninfected cells, inhibited internalization of NBD-PC. In early schizont–infected cells, preincubation with 1 mM azide or cyanide depleted cellular ATP levels to 10–20% of that of uninfected erythrocytes, blocking the transmembrane movement of NBD-PC at 10°C (Table II) and the uptake of LY at 37°C.

Effects of Chemical Modification and Structural Analogues on the Uptake of NBD-PC by Parasitized Erythrocytes

Preincubating cells with NEM at 0°C, before labeling with NBD-PC, significantly inhibited lipid internalization in parasitized erythrocytes (Table II). At 1 mM NEM, over 90% of the parasitized cells did not accumulate intracellular fluorescence. In contrast, 100% of control parasitized cells internalized the fluorescent lipid. TLC analysis of cellular lipids indicated that the pretreatment of parasitized or uninfected cells with NEM did not modify the structure of the fluorescent probe (not shown). When the cells were labeled with NBD-PC at 0°C and subsequently treated with NEM, signif-

Table II. The Effects of ATP Depletion, Chemical Modification, and Structural Analogues on the Internalization of NBD-PC in Parasitized Erythrocytes

Pretreatment	Preincubation (temperature; time)	Inhibition of NBD-PC internalization
Energy inhibitors		
Control	37°C; 6 min	−
1 mM azide	37°C; 6 min	+
1 mM cyanide	37°C; 6 min	+
Chemical modification		
Control	0–2°C; 30 min	−
1 mM NEM	0–2°C; 30 min	+
2.5 mM NEM	37°C; 6 min	+
0.5 mM DEPC	0–2°C; 30 min	+
1 mM TNBS	0–2°C; TNBS	−
1 mM DIDS	0–2°C; 30 min	−
Structural analogues		
10 mM L-GPE	0–2°C; 30 min	−
10 mM L-GPS	0–2°C; 30 min	−
10 mM L-GPC	0–2°C; 30 min	+
10 mM D-GPC	0–2°C; 30 min	−
10 mM phosphorylcholine	0–2°C; 30 min	−

Parasitized erythrocytes were depleted of ATP by incubating in 1 mM sodium azide or sodium cyanide, as described in Materials and Methods. The cells were subsequently labeled with NBD-PC, incubated at 10°C for 15 min, and viewed in the microscope, in the continuing presence of the energy inhibitors. Control incubations were identically processed in the absence of the inhibitors. Parasitized erythrocytes at 0.75% hematocrit in RPMI 1640 were chemically modified with the indicated reagents. The cells were subsequently washed three times in excess RPMI 1640 at 0°C and labeled, incubated at 10°C for 15 min, and examined in the microscope. In all cases, NBD-PC incorporated in the cells was not chemically modified because of the pretreatment (not shown). Parasitized cells in RPMI 1640 at 0.75% hematocrit, were incubated at 0°C with different analogues, before labeling with NBD-PC. The analogues were also present in all subsequent washing steps and the 15-min incubation at 10°C. Internalization of label was monitored in the microscope. 10 mM phosphorylcholine brings down a precipitate in RPMI 1640. Consequently, in studying the effect of phosphorylcholine, PBS/glucose was used instead of RPMI 1640. A corresponding control experiment, lacking the analogue, was also performed in PBS/glucose. The same amount of NBD-PC appeared to be transferred to the cells in PBS/glucose as that transferred to the cells in RPMI 1640 (not shown).

icant internalization of fluorescent lipid was observed. The data indicate the presence of free sulphydryl sites important for the lipid translocation in the membrane, and suggest that some of these sites may be protected from NEM inactivation in the presence of NBD-PC.

As shown in Table II, 0.25 mM 4 4'-diisothiocyanostilbene-2 2'-disulfonic acid which inactivates band 3, had no effect on lipid internalization. This is consistent with reports that modification of erythrocytes with 4 4'-diisothiocyanostilbene-2 2'-disulfonic acid at pH 7.4 does not affect parasite invasion or subsequent intraerythrocytic development (16). 1 mM TNBS also had no effect on probe internalization in the infected cells. Higher concentrations of TNBS led to rapid lysis of infected cells and could not be employed in these studies. In contrast, treating parasitized cells with 0.5 mM diethylpyrocarbonate, a mild esterifying reagent, inhibited the internalization of NBD-PC. The data indicate that chemical modifications of some infected erythrocyte proteins, and more importantly those of sulphydryl residues, can inhibit the parasite lipid translocating activity, thus supporting its proteinaceous character.

We investigated the effects of structural analogues of phospholipid head groups on the internalization of NBD-PC. Infected cells were incubated with the different compounds before and during labeling with NBD-PC. The cells were subsequently warmed to 10°C for 15 min in the presence of the analogues. As shown in Table II and Fig. 5, when parasitized erythrocytes were incubated with 10 mM L-α-GPC, the internalization of label was inhibited. D-α-GPC had no effect on internalization, suggesting that the L-stereoisomer competitively displaced NBD-PC from its translocase in the membrane, while the D-isomer did not. 10 mM phosphorylcholine did not inhibit internalization of the fluorescence (Table II), confirming that the choline group alone was insufficient to cause competitive displacement of NBD-PC. Similarly L-GPS and L-α-GPE did not appear to significantly inhibit the internalization of NBD-PC in the infected cells. In the presence of GPE, 75–80% of the infected cells contained internalized label, while 90–100% internalization was observed in control incubations. This difference between control and GPE-treated cells was no larger than two standard deviations of error, and its significance is unclear. The glycerophosphoryl compounds did not inhibit uptake of LY by mature-infected cells at 37°C (data not shown). The data indicate that the import of NBD-PC in parasitized erythrocytes appeared to be head group- and stereospecific and lend support to the presence of a protein mediated flip–flop mechanism for PC translocation in infected erythrocyte membranes.

Uptake of N-NBD-PE by Mature Infected Erythrocytes

Ethanolic solutions of N-linked NBD-PE were introduced into parasitized cell suspensions at 37°C and incubated for 20 min. N-NBD-PE could not be transferred to the erythrocyte membrane at 0–2°C. This may be because of steric hindrance by the N-substituted head group and is similar to the characteristics of insertion of this analogue in other eucaryotic cells. The insertion of the fluorescent lipid was restricted to the erythrocyte membrane in the presence of 1 mM azide or cyanide.

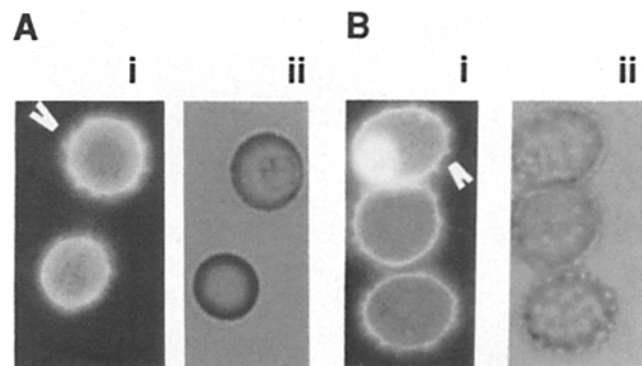As shown in Fig. 6, equal amounts of N-NBD-PE were transferred to the erythrocyte membrane of infected and

Figure 5. Effect of D-α and L-α stereoisomers of glycerophosphorylcholine on the internalization of NBD-PC. Parasite cultures were preincubated with 10 mM. (*A*) L-α-GPC and (*B*) D-α-GPC as described in Table II. The cells were subsequently labeled with NBD-PC, warmed to 10°C for 15 min in the presence of the appropriate analogue, and examined microscopically.
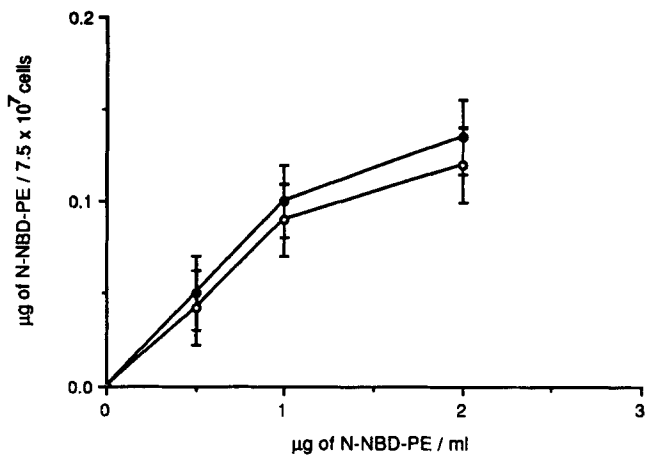
Figure 6. Transfer of N-NBD-PE to parasitized erythrocytes. Cells were incubated with varying amounts of N-NBD-PE at 37°C, in the presence of 1 mM sodium azide. The cells were washed to remove the azide, and the cell-associated label was quantitated spectrophotometrically and corrected for quenching because of hemoglobin, as described in Fig. 1 and Materials and Methods. (○) uninfected erythrocytes; (●) 38.6% infected erythrocytes.

uninfected cells. A time course of lipid internalization by a trophozoite-infected erythrocyte, incubated in RPMI 1640 in the absence of sodium azide, at 37°C, is shown in Fig. 7 A. In 15 min, substantial amounts of fluorescent lipid accumulated in the intracellular parasite, and, in 60 min, there was considerable depletion of erythrocyte membrane fluorescence. The probe does not suffer self quenching in an intracellular compartment, during the internalization at 37°C. The ratio of fluorescence of intact to Triton-lysed cells was similar for both infected and uninfected cells. As observed for NBD-PC, a lower fluorescence signal measured in intact

cells versus Triton-lysed extracts, is probably because of the high local concentration of heme in the red cells. To correct for quenching because of heme in the triton lysates, standard curves containing equal numbers of cells were prepared (not shown). The predominant fluorescent lipid extracted from both 50% parasitized erythrocytes and uninfected erythrocytes, incubated with N-NBD-PE for 1 h at 37°C, comigrated with authentic standard on TLC, indicating that no new fluorescent lipid species was synthesized because of parasite metabolism (not shown). This internalization of N-NBD-PE lipid was not observed at 10°C (Table II). Using 4-h synchronized cultures it was possible to determine that N-NBD-PE was not internalized in parasites <27–30 h in the life cycle. As shown in Fig. 7 B, ring stage parasites detected by staining with Hoechst dye (Fig. 7 B, ii) did not internalize the probe, indicating that the mechanism of uptake of N-NBD-PE is induced at the trophozoite stage (27 h) of the life cycle, and is distinct from that of NBD-PC.

The lack of internalization of N-NBD-PE at 37°C in the presence of 1 mM sodium azide suggests that the process is energy dependent. The temperature and energy requirements for the internalization of N-NBD-PE are similar to those required for pinocytosis of LY in infected erythrocytes and other eucaryotic cells, suggesting endocytotic uptake of the N-linked probe. However, the lack of internalization of diI indicates that constitutive pinocytosis is not a general property of infected erythrocyte membrane and endovesiculation must either be restricted to small domains in the membrane (which exclude diI), or induced via a specific stimulus, such as receptor-mediated endocytosis.

Discussion

We have used fluorescent phospholipid probes to investigate mechanisms of lipid transport in malaria-infected erythrocytes. A variety of these fluorescent lipid analogues have
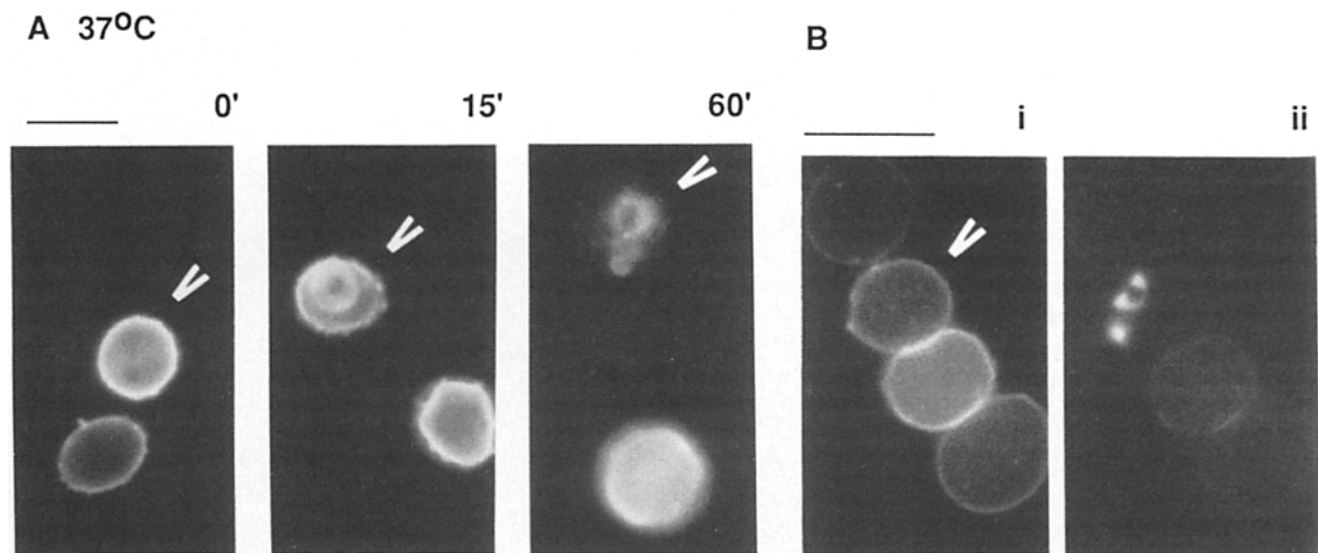

Figure 7. Fluorescence micrograph of uninfected and parasitized erythrocytes incubated with N-NBD-PE and LY. Infected and uninfected erythrocytes were labeled for 20 min in RPMI 1640 at 37°C in 1 mM sodium azide. The azide was removed, and the cells were resuspended in RPMI 1640. A, mature trophozoite (arrowhead) incubated at 37°C for 0, 15, and 60 min; B, Ring-stage parasites incubated for 30 min at 37°C: i, NBD fluorescence; ii, Hoechst 33258 fluorescence. Bar, 10 μm.

been exploited to probe mechanisms maintaining phospholipid asymmetry across membranes. NBD-PC and the corresponding 1-palmitoyl-C_6 analogues of PE (NBD-PE) and PS (NBD-PS) exhibit rapid monomer diffusion between membranes, but do not undergo spontaneous flip-flop across lipid bilayers. It has been shown that, upon insertion into the plasma membrane of Chinese hamster fibroblasts, rapid flip-flop of NBD-PE and NBD-PS occurs via a stereospecific protein-mediated mechanism (19, 30). In contrast, NBD-PC is not transported to the cytoplasmic face, and is taken up into the cell only by endocytosis (29), suggesting a phospholipid distribution similar to that of normal erythrocytes.

Our studies suggest that NBD-PC undergoes parasite-induced transbilayer flip-flop in the infected erythrocyte membrane, possibly via a plasmodial PC transporter. The evidence supporting this is the following: (a) rapid transport of C6-NBD-PC from the erythrocyte membrane to the intracellular malaria parasite occurs at temperatures well below those required for endocytosis and at all stages of parasite growth, even those that do not exhibit pinocytosis at the infected erythrocyte membrane; (b) bulk membrane endocytosis does not occur at the infected erythrocyte membrane; (c) L-α-GPC inhibits internalization of the label while D-α-GPC does not, indicating a stereospecific, protein-mediated step in the translocation of NBD-PC from erythrocyte membrane to the parasite; (d) transport of NBD-PC can be inhibited by chemical modification of cellular proteins; and (e) depleting cellular ATP inhibits internalization of NBD-PC. The ATP levels of infected cells are lower than their uninfected counterparts. Depleting erythrocyte ATP to 10% of normal levels, increases the $t_{1/2}$ of translocation of PC in uninfected cells to ~3.5 h (21). However, the rate of PC translocation in ATP-depleted uninfected erythrocytes is too slow to account for the observed internalization of NBD-PC in parasitized erythrocytes. The ATP levels of infected erythrocytes are 40–60% of uninfected red cells. In azide- or cyanide-treated infected cells, when the ATP levels are decreased to ~10% of that of uninfected erythrocytes, NBD-PC translocation is not observed. Gupta et al. (9, 10) indicated that erythrocyte phospholipid organization was altered in both infected and uninfected cells from monkeys infected with *P. knowlesi*, with PS exposed on the outer leaflet, and PC tranported to the exoplasmic face of the cell. A subsequent report (13) indicated that the observed changes in the phospholipid asymmetry of uninfected erythrocytes was not a consistent change because of infection, and was limited to cells obtained from chronically infected splenectomized animals. It is unclear whether this is a consequence of direct action of the spleen on the phospholipid asymmetry of red cells, or selective clearance of cells containing exofacial PS in the nonsplenectomized animals. The role of the degree of infection on the phospholipid asymmetry of uninfected erythrocytes is not understood and these results are contrary to those of van der Schaft et al. (37), which indicate that the phospholipid organization remains unaltered despite infection. The discrepancies in these data remain unresolved. In our studies, the NBD-PC label both of uninfected erythrocytes from an infected culture and of those that had never encountered parasites was equally susceptible to back-extraction at 0°C. Since resistance to back-extraction (rather than transport of the label) to the parasite is the measure of lipid flip-flop, our results indicate that protein-mediated transmembrane movement of PC is restricted to infected erythrocyte membranes and does not occur in uninfected erythrocytes. *P. falciparum* is grown in vitro culture and we have not investigated the effects of host factors like the spleen on cell surface phospholipid asymmetry.

The mechanism of uptake of another phospholipid analogue N-NBD-PE in infected cells appears to be distinct from that of NBD-PC. This might be expected since a PC translocator would likely not recognize the N-NBD-PE head group. The results suggest that mature infected erythrocytes possess additional mechanisms for the uptake of phospholipids from the erythrocyte membrane, although the precise mechanism of internalization of N-NBD-PE remains unclear. Studies investigating the effects of structural analogues and chemical modification of infected cell proteins on the internalization of N-NBD-PE, are under way. The parasite's uptake of the analogue from the erythrocyte membrane results in the decrease of peripheral cell surface fluorescence. Even after 60 min, there was no evidence of fluorescent lipid flow from the parasite back to the erythrocyte membrane. However, this time may be too short for the fluorescent lipids to reach equilibrium distribution in the cell. We are investigating the transport of phospholipids from the parasite to the erythrocyte membrane. Plasmodia engage in active phospholipid biosynthesis in the ring and trophozoite stages (39), although they are incapable of chain elongation. Parasite proteins of the erythrocyte membrane (4, 11, 12, 18, 24, 31, 36, 38) are presumably transported via vesicular traffic, which must provide a route of exocytotic lipid flow.

The uninfected erythrocyte does not constitutively pinocytose. Evidence suggests that plasmodium-infected erythrocytes endocytose human transferrin (11, 22, 24). The results of Rodriguez-Lopez and Jungery (24) suggest this is receptor-mediated uptake since ferro but not apotransferrin is internalized by the infected cells. Little is known about the regulation of endocytosis in the infected erythrocyte membrane. Our results indicate that, although constitutive endocytosis of the lipid phase does not occur, the pinocytotic marker LY is internalized by mature parasitized erythrocytes. Taken in conjunction, these observations suggest that the uptake of LY may result either from constitutive endocytosis in microdomains of the infected erythrocyte bilayer that exclude diI or by receptor-mediated endocytosis. Direct microscopic evidence for the uptake of LY in endocytotic vesicles at the infected erythrocyte membrane is not available. We are currently pursuing these studies and the kinetics of uptake and release of LY by parasite-infected erythrocytes. We suggest that, along with PC flip-flop, the parasite may induce microdomains of endocytotic activity in infected erythrocyte membranes.

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