The EMBO Journal Vol. 19 No. 14 pp. 3556–3564, 2000

Vacuolar uptake of host components, and a role for cholesterol and sphingomyelin in malarial infection

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Erythrocytes, which are incapable of endocytosis or phagocytosis, can be infected by the malaria parasite *Plasmodium falciparum*. We find that a transmembrane protein (Duffy), glycosylphosphatidylinositol (GPI)-anchored and cytoplasmic proteins, associated with detergent-resistant membranes (DRMs) that are characteristic of microdomains in host cell membranes, are internalized by vacuolar parasites, while the major integral membrane and cytoskeletal proteins are not. The internalized host proteins and a plasmodial transmembrane resident parasitophorous vacuolar membrane (PVM) protein are detected in DRMs associated with vacuolar parasites. This is the first report of a host transmembrane protein being recruited into an apicomplexan vacuole and of the presence of vacuolar DRMs; it establishes that integral association does not preclude protein internalization into the *P.falciparum* vacuole. Rather, as shown for Duffy, intracellular accumulation occurs at the same rate as that seen for a DRM-associated GPI-anchored protein. Furthermore, novel mechanisms regulated by the DRM lipids, sphingomyelin and cholesterol, mediate (i) the uptake of host DRM proteins and (ii) maintenance of the intracellular vacuole in the non-endocytic red cell, which may have implications for intracellular parasitism and pathogenesis.

Keywords: erythrocytes/microdomains/*Plasmodium falciparum*/trafficking

Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins associate with cellular membranes by virtue of their phosphatidylinositol anchor. Recent evidence indicates that they are components of membrane microdomains or 'rafts' on cellular plasma membranes (Sheets *et al*., 1997; Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Microdomains/rafts were originally proposed by Simons and co-workers to explain sphingolipid-based sorting properties in cellular membranes (Simons and van Meer, 1988), and later proposed to explain cholesterol-based microheterogeneities in the membrane (Simons and Ikonen, 1997). They can be isolated as detergent-resistant membranes (DRMs) from cells (Brown and Rose, 1992; Brown and London, 1998b). A characteristic feature is that depletion of cholesterol leads to disruption of microdomains and DRMs (Brown and London, 1998a; Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Despite earlier concerns, recent studies suggest that DRMs do not artifactually create domains from previously homogeneous bilayers or recruit non-raft proteins and lipids into rafts during DRM isolation (Brown and London, 1998b). Rather, DRMs reflect at least a subset of raft-sorting properties in the membrane (Brown and London, 1998b) and have been very useful tools for studying rafts. Microdomains are dispersed over the surface of the plasma membrane and it has been proposed that cross-linking of GPI-anchored proteins by antibodies results in their enrichment in specialized plasma membrane domains away from their native glycerophospholipid milieu, leading to endocytosis-independent internalization (Harder and Simons, 1997). However, results obtained when antibody cross-linking was absent or prevented (Mayor *et al*., 1998) suggest that GPI-anchored proteins and microdomains are not independently sorted for internalization but rather undergo endocytosis at cellular plasma membranes.

Microdomains are presumably present in all cells including mature human erythrocytes. These cells are known to be terminally differentiated, devoid of all intracellular organelles, lacking in endocytic machinery and incapable of *de novo* protein or lipid synthesis (Chasis *et al*., 1989). Yet, red cells are infected by a number of bacterial and protozoan pathogens. One of these, the protozoan *Plasmodium falciparum* causes the most virulent form of human malaria, a disease that afflicts 200–300 million people worldwide and kills over a million children each year. Infection begins when the extracellular 'merozoite' enters and develops in a parasitophorous vacuolar membrane (PVM) through 'ring' (0–24 h), 'trophozoite' (24–36 h) and 'schizont' stages (36–48 h). During ring to trophozoite development a tubovesicular membrane (TVM) network buds from the PVM to the periphery of the red cell (Elford and Ferguson, 1993; Elmendorf and Haldar, 1994; Haldar, 1998). The infected red cell lyses at the end of schizogony to release 8–16 daughter merozoites that re-invade new red cells and maintain the asexual cycle.

It is known that in the course of infection, the parasite expresses adherence antigens and induces numerous transport changes in the red cell (Coppel *et al*., 1998; Haldar, 1998). However, integral and cytoskeletal proteins of the red cell have not been detected in the PVM or the TVM (Gratzer and Dluzewski, 1993) and there is no endocytosis in the uninfected or infected red cell membrane (Haldar, 1998). Thus, it has been proposed that integral membrane proteins cannot access vacuolar junctions during invasion or intracellular growth (Gratzer and Dluzewski, 1993), and the mechanisms of protein trafficking from the red cell to the PVM–TVM remain largely unknown. Recent studies in a related apicomplexan *Toxoplasma gondii* suggest that during invasion of fibroblasts, GPI-anchored but not integral proteins can enter the parasite's vacuole, suggesting that the exclusion of host membrane proteins from the vacuole is on the basis of their membrane anchoring (Mordue *et al*., 1999).

Here we show that in *P.falciparum*-infected red cells, the host transmembrane, heptahelical Duffy antigen, as well as GPI-anchored and cytoplasmic proteins that associate with microdomains, can access the parasite's vacuole. A parasite-encoded, transmembrane, resident PVM protein, PfEXP1, is also in DRMs in the vacuole. Thus, we find that DRM components independent of their membrane anchors enter this apicomplexan vacuole and we show that in the absence of endocytosis, microdomain lipids, namely sphingomyelin and cholesterol, regulate novel membrane properties of the vacuole.

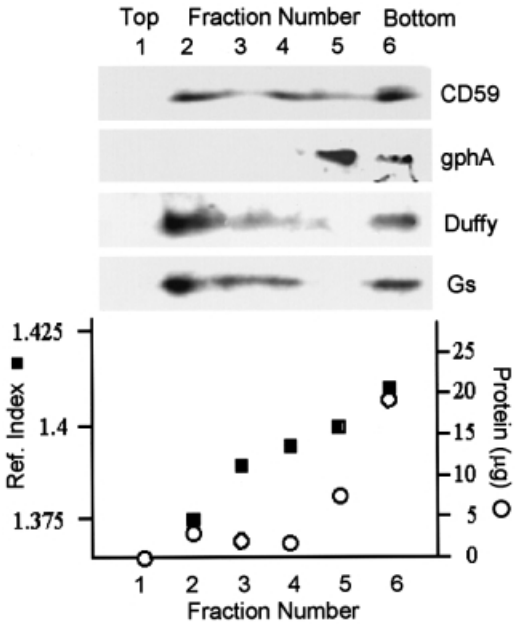

Fig. 1. Density gradient detection of DRMs from uninfected erythrocytes. Cold Triton X-100 extracts were prepared, subjected to sucrose density gradients, and the resulting fractions were analyzed by western blotting and densitometric scanning (using Molecular Dynamics ImageQuant 5.0 software) for the distribution of indicated markers (see Materials and methods). Data from one representative experiment are shown. Floating fractions 2–4 indicate DRMs.

Results

Detection of DRM proteins in the red cell membrane

There is one report on the presence of GPI-anchored proteins in DRMs isolated from mature human red cells but the association of transmembrane proteins such as the Duffy receptor and signaling molecules with these complexes was not examined (Civenni *et al*., 1998). To isolate DRMs from red cells, we prepared ghosts, extracted them in cold 1% Triton X-100 in Tris-buffered saline (TBS), and analyzed them on sucrose gradients for DRMs (see Materials and methods) that float in light membrane fractions away from bulk cellular proteins. As shown in Figure 1, despite loading the sample at the bottom of the gradient (in fraction 6) we find that 55% of a GPI-anchored protein like CD59 floats to fractions 2–4 of the gradient, with ~25% in fraction 2. (Since microdomains are dynamic only a fraction of their proteins are found in DRMs.) Fraction 2 corresponds to the 5%/35% sucrose interface, and is expected to contain erythrocyte DRMs (eDRMs). That we also detect DRM components in fractions 3 and 4 suggests that DRMs may be heterogeneous. Nonetheless, these numbers are consistent with those reported for CD59 by Civenni *et al*. (1998).

In addition to GPI-anchored proteins, we find that a heptahelical receptor such as Duffy and heterotrimeric Gαs (associated with the cytoplasmic domain of the red cell membrane) are also found in DRMs. Seventy percent of Duffy and 66% of Gαs are in fractions 2–4, indicating that like CD59 substantial fractions of these proteins are DRM associated. In contrast, integral proteins such as glycophorin A (gph A) do not float, but remain in the loading zone (fractions 5 and 6). Additionally, the integral protein band 3 and host cytoskeletal proteins (spectrin, actin, 4.1; not shown) do not float. These data indicate that eDRMs contain some transmembrane proteins while others are excluded. They also contain signaling proteins, analogous to DRMs isolated from other cells (Li *et al*., 1995). Furthermore, the data in Figure 1 indicate that DRMs constitute a small fraction of the total protein of the red cell membrane, consistent with observations in other cells.

Host DRM proteins are internalized and found in association with vacuolar parasites

The movement of host DRM-associated proteins into the vacuole of *P.falciparum* has not previously been investigated. Of the GPI-anchored proteins, CD59 is the most abundant, present at an average of 30 000 copies/cell and accounts for ~75% of all red cell GPI proteins. CD55 and CD58 are less abundant but nonetheless detectable. As shown in Figure 2ii and iii, CD59 is recruited to the vacuolar parasite from ring (ii) to schizont (iii) stages. It partially overlaps with PfEXP1 (as indicated by the orange–yellow merge in Figure 2 iv), a plasmodial protein that is known to be localized in the PVM (Kara *et al*., 1988). The lack of complete overlap between these proteins may be due to the presence of multiple, juxtaposed protein domains in the PVM. Low levels of CD59 are also seen associated with the TVM (Figure 2v, asterisk), as determined by its presence on intra-erythrocytic membranes labeled with BODIPY-ceramide (a fluorescent lipid analog that can be used to visualize the PVM–TVM network in trophozoite-infected red cells). However, the data suggest that the major site of CD59

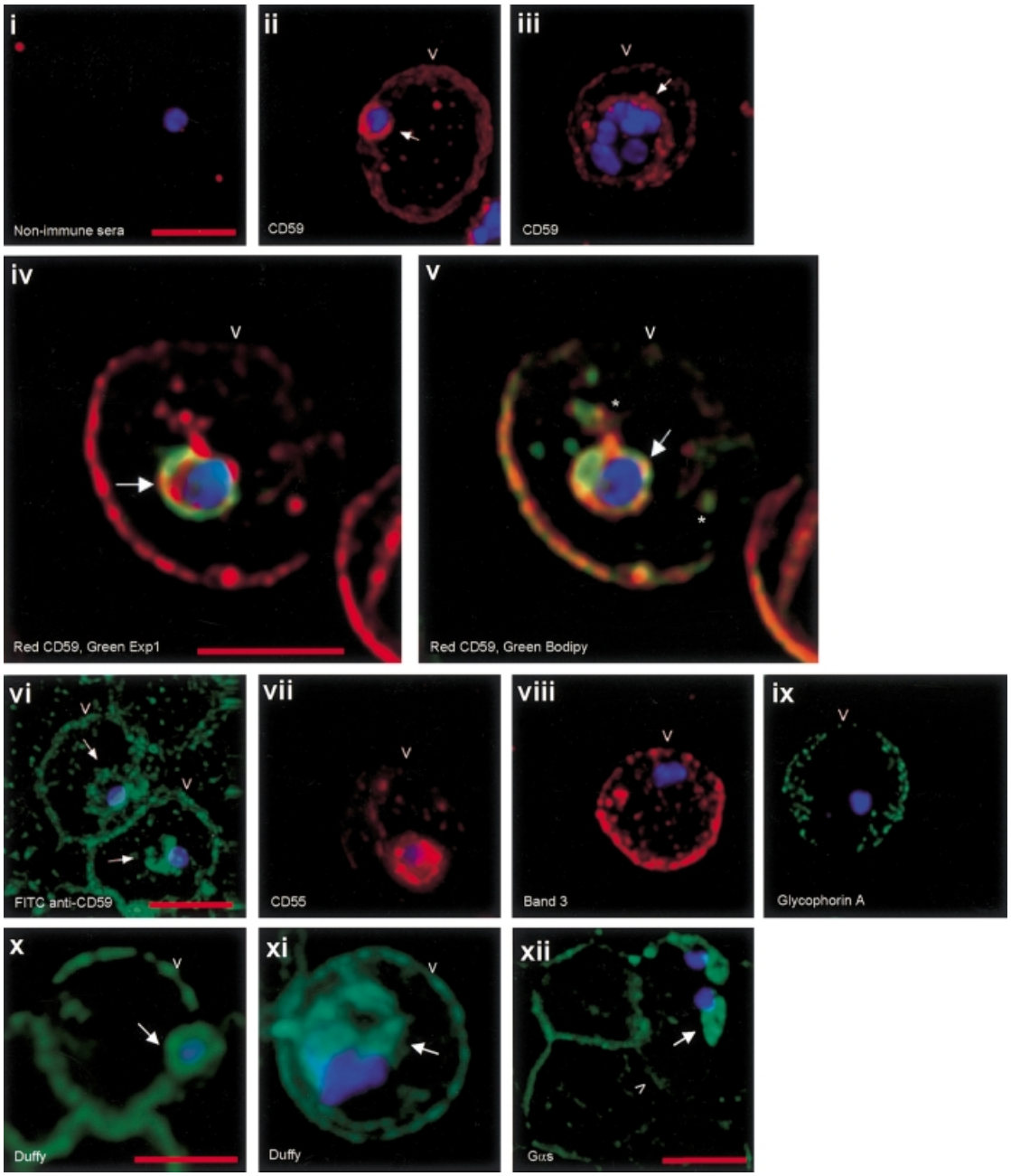

Fig. 2. Internalization of host DRM proteins in infected erythrocytes. Infected red cells were probed with primary and relevant secondary antibodies in i–v and vii–xii to detect the indicated markers. (i) Non-immune; (ii–v) CD59, red; (iv) PfEXP1, green; (v) BODIPY-ceramide, green; (vi) FITC–anti-CD59, green; (vii) CD55; (viii) band 3; (ix) glycophorin A; (x and xi) Duffy, green; (xii) Gαs, green. No internalization of CD59 was seen in uninfected red cells and western blots confirmed that the antibody recognizes a single ~19 kDa protein present at equal levels in both infected and uninfected cells (data not shown). No internalization of Duffy or Gαs proteins was seen in uninfected cells and antibodies to these proteins recognized only their respective host polypeptides in both infected and uninfected cells (data not shown). In (vi), infected red cells were labeled with anti-CD59 antibody that was directly conjugated to FITC. In all images, the nucleus (blue) is stained with Hoechst, v indicates the periphery of the red cell, arrows indicate vacuolar parasite, asterisks indicate TVM and the scale bar is 5 μm.

accumulation is directly around the parasite, in the vacuole. The internalization of CD59 could also be detected in cells probed with anti-CD59 antibody that was directly coupled to FITC (Figure 2vi; as well as Fab fragments; not shown), and in cells permeabilized with either cold Triton X-100 or saponin (not shown). Hence, the internalization is not due to cross-linking of CD59 by double antibody complexes, nor is it dependent on the detergent used to permeabilize cells. In addition to CD59, CD55 (Figure 2vii) and CD58 (not shown) also appear around the vacuolar parasite. However, as previously reported (Dluzewski *et al*., 1989; Gratzer and Dluzewski, 1993), major membrane proteins such as band 3 and gph A (Figure 2viii and ix) as well as erythrocyte cytoskeletal components such as spectrin and actin (not shown) are not internalized and remain exclusively at the host cell membrane. Thus, the internalization of GPI-anchored proteins, CD59, CD55 and CD58, occurs by a selective

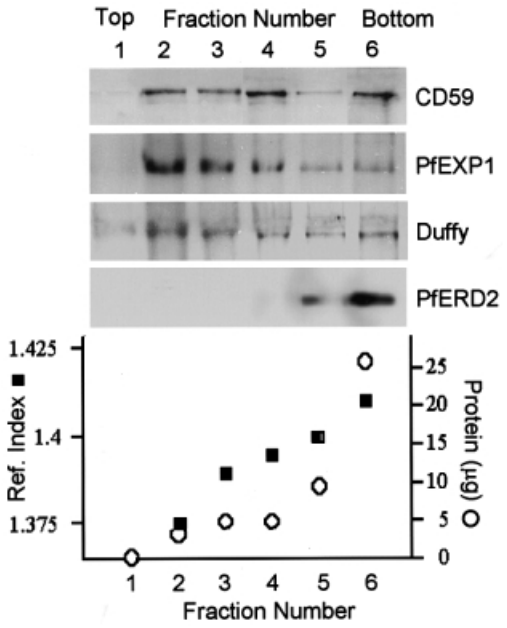

Fig. 3. Density gradient detection of DRMs from isolated vacuolar parasites. Vacuolar parasites were isolated and the level of red cell contamination judged by band 3 and gph A staining was <5% (see Materials and methods). Fractions were obtained (as described in Materials and methods and Figure 1) and analyzed for the distribution of indicated markers. Data from one experiment are shown. Floating fractions 2–4 indicate DRMs.

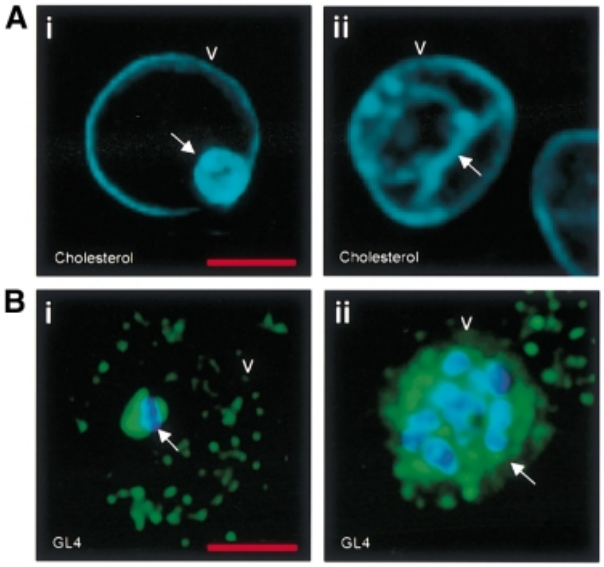

Fig. 4. Internalization of host DRM lipids in infected red cells. (**A**) Cholesterol in a (i) ring and (ii) trophozoite as detected by filipin staining (Haldar *et al*., 1991). (**B**) GL4 in (i) rings and (ii) schizont as detected in an indirect immunofluorescence assay using anti-GL4 and FITC-conjugated secondary antibody.

transport process, and these molecules are the first host cell protein markers detected in the plasmodial vacuole.

GPI-anchored proteins differ from gph A and band 3 by the absence of transmembrane and cytosolic domains as well as their ability to associate in DRMs (see Figure 1). To determine whether the difference in membrane anchor alone could explain vacuolar uptake, we investigated the presence of the heptahelical Duffy antigen (which is DRM associated) in infected red cells. As shown in Figure 2, we find that the Duffy protein is internalized in ring- (Figure 2x) and trophozoite-infected (Figure 2xi) red cells. Thus, integral proteins can also be internalized in malaria-infected red cells. Moreover, Gαs, peripherally attached to the cytoplasmic face of the red cell membrane and also a DRM component, were found associated with the vacuolar parasite (Figure 2xii). Thus, it appears that DRM components independent of their membrane anchor can be selectively drawn to the vacuole in infected red cells.

Host DRM proteins Duffy and CD59 are found in DRMs from isolated vacuolar parasites, which also contain an integral PVM marker PfEXP1

If the property to exist in DRMs results in internalization of CD59 and Duffy, we reasoned that DRMs might be detected in the parasite's vacuolar membrane as vacuolar DRMs (vDRMS). To examine this, we isolated parasites that contained the PVM (and to some degree the TVM) but were free of the red cell membrane (see Materials and methods), extracted them in 1% Triton X-100 in TBS, and analyzed them on sucrose gradients for DRMs (see Materials and methods). As shown in Figure 3, the integral PVM marker PfEXP1 floats to fractions 2–4 and densitometric analysis indicates that 75% of this marker is in these fractions. Thirty-five percent of PfEXP1 is found in fraction 2, providing strong evidence for the presence of DRMs in the PVM. Sixty percent of vacuolar CD59 and Duffy, respectively, also float to fractions 2–4, and fraction 2 contains 25% of each. Since contamination from the erythrocyte membrane is <5% (see Materials and methods), the CD59 and Duffy signals detected in Figure 3 are primarily from vDRMs. PfERD2, a secretory membrane marker of the parasite's Golgi complex, did not float but was found in fractions 5 and 6; fraction 5 includes the interface between 35%/40% sucrose as well as the loading zone of 40% sucrose. Approximately 80% of the total membrane protein also fails to float. That we also detect DRM components in fractions 3 and 4, and there are small variations in the relative distribution of CD59, Duffy and PfEXP1 in these fractions, suggests that vDRMs may be heterogeneous (this may also explain the lack of complete overlap between CD59 and PfEXP1 in the immunolocalization data in Figure 2iv). Thus, our data show that Duffy and CD59 assemble in DRMs isolated from vacuolar parasites, and provide the first evidence for the presence of DRMs or rafts in an apicomplexan vacuole. Furthermore, as in other cells, DRM/raft proteins constitute a very small fraction of the total cell-associated proteins.

Detection of host DRM lipids with vacuolar parasites

In virtually all cell types examined, cholesterol and glycosphingolipids have been found to be components of DRMs and rafts (Brown and London, 1998a). Consistent with this expectation, we find that both lipids are detected around the vacuolar parasite and its attached TVM (see Figure 4A and B, respectively). Plasmodia do not synthesize cholesterol *de novo* (Vial *et al*., 1990) and thus all detected cholesterol comes from the host. The antibody used to detect the glycosphingolipid globoside (GL4) in Figure 4B does not recognize other erythrocyte glycolipids.

Inhibition of glycosphingolipid synthesis (with fumonisin) in *P.falciparum* had no effect on the vacuolar accumulation of GL4, CD59, Duffy or cholesterol (not shown). In conjunction with the results from Figures 1–3, these data suggest that DRM proteins and lipids can access the malarial vacuole and exist as DRMs or rafts in the malarial vacuole.

Duffy and CD59 are internalized at the same rate into infected red cells and intracellular accumulation of both proteins is dependent on sphingomyelin biosynthesis in the PVM–TVM

To investigate further whether their distinct membrane anchors resulted in a different or the same mechanism of translocation, we investigated the relative kinetics of CD59 and Duffy uptake during *P.falciparum* infection. To do this we examined the levels of each protein associated with the vacuolar parasite at different stages of asexual growth (see Materials and methods). To detect newly infected rings immediately after invasion, cultures containing high levels of schizonts that were in the process of rupturing and releasing infectious progeny were sampled several times over 3–4 h. Detailed methods for quantitative projections are provided in Materials and methods.

Every ring stage parasite detected showed the presence of both markers, and in the youngest rings (corresponding to 0 h of infection; Figure 5A), ~25% of both CD59 and Duffy were found to be vacuole associated, and this probably reflects internalization during invasion. This suggests that both markers were internalized during invasion. During subsequent intracellular growth, there was continued uptake of both proteins until the terminal stages of intracellular development (Figure 5A). The rates of internalization were the same for CD59 and Duffy, strongly supporting the possibility that they were internalized by a common mechanism and pathway of transport. This uptake was blocked by inhibiting (parasite-induced) sphingolipid synthesis in the PVM–TVM of ring- and/or trophozoite-infected cells (see Figure 5B). This block was effected by treating cells with low (1–5 μM) concentrations of DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and its dodecyl analog (PDMP), which we have previously shown to arrest tubular membrane development specifically between the PVM and the red cell (Lauer *et al*., 1995). It should be noted that for the time course of the experiments, PPMP is not toxic for general metabolic functions, does not block maturation and its effects are completely reversible (Lauer *et al*., 1995, 1997). Hence the induced block observed in intra-erythrocytic uptake of Duffy (or CD59) is not due to reduced viability of these cells. Rather, the synthesis of sphingomyelin (a DRM lipid) regulates accumulation of DRM components in the vacuole during intracellular growth.

Thus, in summary, despite differences in their membrane anchors, CD59 and Duffy are internalized with similar kinetics during malarial infection and the synthesis of a DRM lipid in the PVM–TVM can influence the uptake process for both proteins to the same degree, consistent with the notion that their transport is due to shared DRM properties. This uptake is not due to lipid diffusion because, as indicated earlier, even low levels of band 3 and gph A are not detected in the vacuole, arguing that the non-

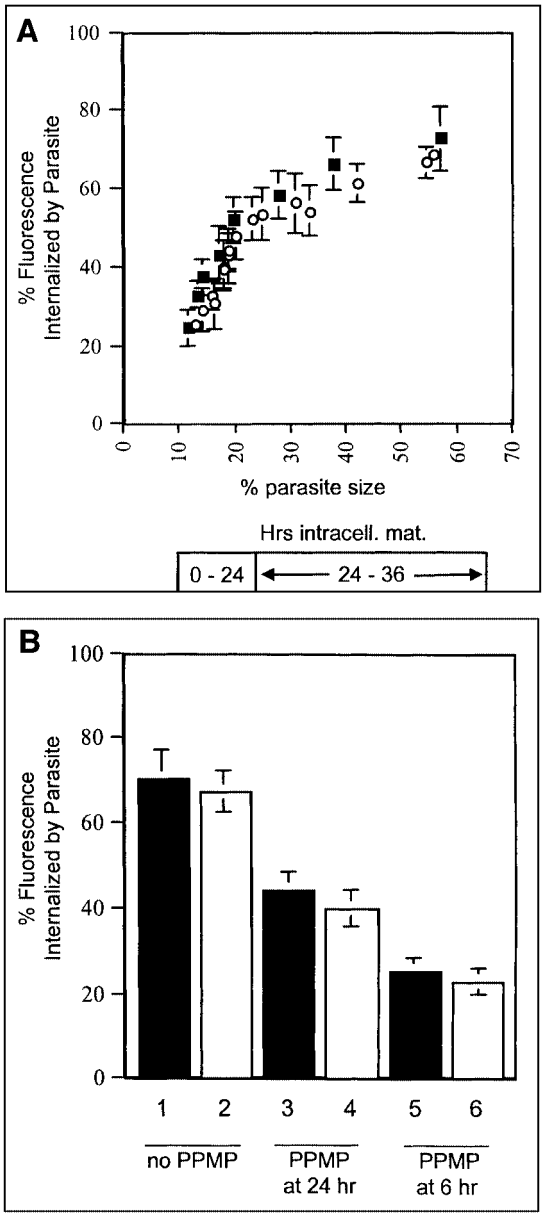

Fig. 5. Quantitative uptake of CD59 and Duffy and their inhibition by PPMP in infected red cells. (**A**) Amount of cell-associated CD59 and Duffy detected in association with the vacuolar parasite as a function of size and corresponding hours of intracellular parasite development. CD59 (filled squares) and Duffy (open circles) fluorescence associated with the parasite and red cell were quantitated as described in Materials and methods. Each point represents an average of 10 *P.falciparum*-infected red cells. (**B**) Vacuolar accumulation of CD59 (black bars) and Duffy (open bars) in: (lanes 1 and 2) mock-treated cells at 36 h of development; (lanes 3 and 4) 24 h trophozoites incubated with 5 μM PPMP for a subsequent 12 h in culture; (lanes 5 and 6) 0–6 h rings exposed to 5 μM PPMP for the next 30 h in culture.

skeleton-bound fraction of these proteins does not enter the vacuole. Nor is it due to endocytosis, since a non-exchangeable lipid marker such as DiC16 inserted into the red cell membrane fails to be internalized during intracellular development and lucifer yellow (LY)–dextran is not internalized by infected cells (data not shown and Haldar and Uyetake, 1992). Finally, our biochemical analysis indicates that DRM proteins represent a small fraction of total red cell membrane proteins and hence

their quantitative uptake during malarial infection suggests a specialized pathway of transport. Since PPMP completely inhibits tubular development of the TVM (during ring to trophozoite development), it is possible that TVM tubules may facilitate 'capture' of DRM components from the host membrane at sites of close juxtaposition with the red cell.

Effects of cholesterol depletion on eDRMs and malarial infection

Cholesterol is the only resident component known to be essential for microdomain/DRM formation and its depletion disrupts DRMs in cells (Brown and London, 1998a). Cholesterol also retards recycling of GPI-anchored proteins to the plasma membrane during endocytosis of rafts (Mayor *et al*., 1998) and its depletion disrupts caveolae, which are invaginations seen on mammalian plasma membranes (Parton and Simons, 1995). To determine the effects of cholesterol depletion in infected erythrocytes, we incubated parasite cultures (containing infected and uninfected red cells) with the cholesterol-depleting agent methyl-β-cyclodextrin (MBCD). As shown in Figure 6A, lane 3, incubations of trophozoite-infected red cells (~24–30 h) with MBCD (5 mM) released 50–70% of parasites into the extracellular medium, without rupturing host cells. Figure 6B indicates filipin staining of an infected and uninfected erythrocyte as well as a released parasite. MBCD acts by intercalating cholesterol in large inclusion complexes without permeating the bilayer. The released parasites were 95% viable as measured by exclusion of Evans blue (see Materials and methods; and remained viable for ~24 h, under standard conditions of culture; data not shown) but did not mature to schizogony or re-infect red cells. This suggests that escape from the red cell is not a stress response that is advantageous to the trophozoite and is thus distinct from ion-mediated release of *T.gondii* or other apicomplexans that infect nucleated mammalian cells (Stommel *et al*., 1997).

Released trophozoites contain the parasite plasma membrane (PPM) marker PfMSP1 (Figure 6C, panel ii, green stain) around their periphery. However, the PVM marker PfEXP1 (red) is lost or found trailing one end of the parasite (in a punctate distribution, shown by arrows; Figure 6C). Importantly there is little or no co-localization of the two markers in treated cells. Regions of PfEXP1 staining may reflect isolated vacuoles or those remaining behind in red cell ghosts. In panels (iii) and (iv), preparations stained for band 3 (a red cell marker; green) and PfEXP1 (red) show an erythrocyte ghost (arrowhead) closely apposed to a released parasite (blue nuclear stain) with PfEXP1 (arrow) localized between the two. This was frequently observed, as were clusters of released parasites depleted of PfEXP1 and band 3 (Figure 6C, panel iv). In contrast, in control incubations (Figure 6C, panel i) there was (almost continuous) overlap between the PVM (red) and PPM (green) markers.

Thus, after MBCD treatment, both red cell- and PVM-associated components are separated away from the PPM, suggesting that the parasites are expelled from the vacuole and the red cell, as outlined in the first line of the schematic in Figure 6D. Other treatments such as detergents (saponin or digitonin), antibiotics such as filipin and nystatin (at <0.1 μg/ml), mechanical homogenization, dipeptide or sorbitol treatment leave the vacuolar membrane tightly associated with the PPM (Figure 6D). In contrast to trophozoites, rings are not released by MBCD treatment (not shown). One explanation for these effects is that once the intracellular vacuole forms (around a ring) it is a relatively stable entity, until the trophozoite stage where high levels of DRM accumulation necessitate increased cholesterol to stabilize the vacuole.

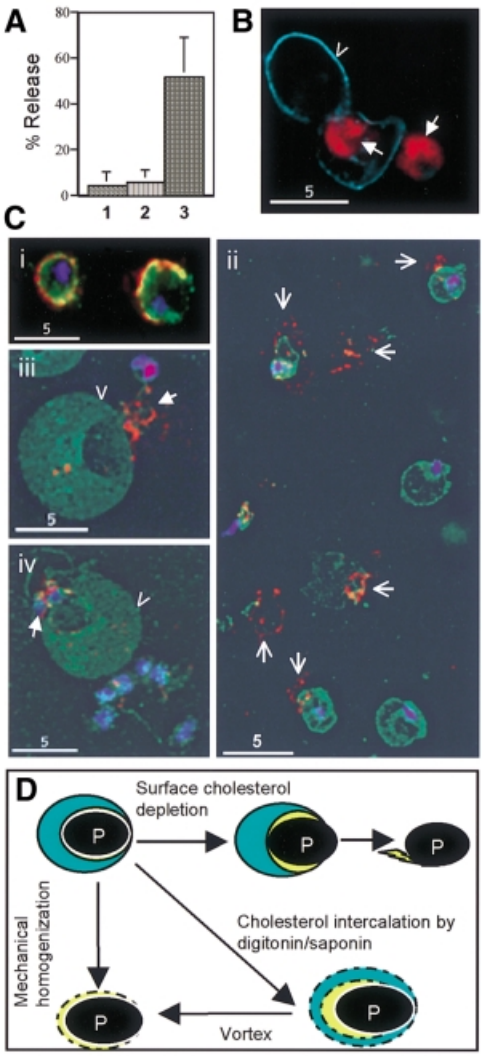

Fig. 6. Effects of cholesterol depletion on intracellular trophozoite release. (**A**) Fraction of parasites released when cells were treated with (1) RPMI, (2) RPMI + cyclodextrin–cholesterol complex and (3) RPMI + cyclodextrin. (**B**) Cyclodextrin-treated cells were fixed in 1% glutaraldehyde and stained with filipin (blue) and ethidium bromide (red) and viewed by fluorescence microscopy. v indicates an uninfected cell; arrows indicate parasites. Scale bar is 5 μm. (**C**) Indirect immunofluorescence assay of (i) mock- and (ii–iv) cyclodextrin-treated cells. In (i) and (ii), cells were stained for the PPM marker PfMSP1 (green) and the PVM marker PfEXP1 (red). Arrows indicate 'spots' of PfEXP1 detected at one end of the parasite or in association with red cell ghosts (negative contrast not shown). In (iii) and (iv), cells were labeled with a red cell marker band 3 (green) and the PVM marker PfEXP1 (red). Blue indicates Hoechst-stained nuclei. Scale bar indicates 5 μm. (**D**) Summary of the association of the PVM and the PPM under different conditions of parasite release from red cells. Depletion of erythrocyte surface cholesterol releases parasites freed of their surrounding vacuolar membrane. Other known methods of parasite release, such as permeabilization with digitonin/saponin or mechanical homogenization, fail to separate vacuole from the parasite.

Discussion

Our data provide definitive evidence that endogenous, integral membrane proteins of the host plasma membrane can be transported to an apicomplexan vacuole. Furthermore, we find that proteins with diverse types of membrane association that share the property of complexing into DRMs or microdomains can access the malarial vacuole. In the course of preparing this manuscript, Sibley and co-workers reported that GPI-anchored proteins enter the vacuole of a related apicomplexan *T.gondii* when it infects fibroblast cells (Mordue *et al*., 1999). Replacement of the GPI anchor with a transmembrane domain alone had no effect on vacuolar recruitment but the addition of a cytoplasmic domain prevented vacuolar access, leading to the notion that GPI-anchored proteins diffuse into the *T.gondii* vacuole due to a lack of interactions with cytoplasmic components. However, movement of other transmembrane proteins predicted to be in rafts was not investigated. Our data on the internalization of Duffy indicate that integral proteins with substantial transmembrane and cytoplasmic domains can access the malarial, apicomplexan vacuole. Some integral proteins such as gph A or band 3 are indeed excluded. These proteins are linked to the cytoskeleton and it has been suggested that this might restrict the flow of integral proteins into the vacuole. However, in red cells, a significant fraction of both gph A and band 3 are not skeleton bound (Knowles *et al*., 1997). Hence, that we fail to detect internalization of even low levels of gph A or band 3 suggests that absence of linkage to the cytoskeleton is not sufficient to allow internalization of host membrane components into the vacuole. Consistent with this, Mordue *et al*. (1999) report that depolymerization of actin filaments had no significant effect on recruiting integral proteins to the *T.gondii* vacuole.

Our studies show that access of both GPI-anchored and transmembrane DRM proteins can be regulated by the synthesis of a major DRM lipid component, sphingomyelin, within the vacuolar environment, and that cholesterol is required to maintain the parasite's intracellular residence. In nucleated mammalian cells cholesterol has been shown to regulate raft association as well as endocytic transport of raft-associated proteins (Mayor *et al*., 1998). This suggests that in the absence of endocytosis, in addition to cholesterol, *de novo* synthesis of sphingomyelin may be required to regulate novel mechanisms of vacuolar uptake of raft proteins in red cells.

If cholesterol, a major DRM component, is depleted from trophozoite-infected red cells, the parasite is expelled from the vacuole, suggesting that in the course of intracellular growth, cholesterol is essential to maintain infection. It is possible that recruitment of DRM components (via the TVM) provides a major source of cholesterol for the vacuolar parasites. Further, incorporation of GPI-anchored proteins CD59 and CD55 in the vacuole leads to their depletion from the surface of infected red cells. This predicts that infection of cells leads to a decrease in protection from complement. Consistently, increased binding of membrane attack complex (MAC) (Wiesner *et al*., 1997) and lysis by complement (B.U.Samuel and K.Haldar, unpublished) are seen at the terminal stages of intra-erythrocytic parasite development. Furthermore, since levels of normal CD59 as low as 20% are sufficient to block assembly of the activated MAC complex on red cells (Wilcox *et al*., 1991), regulated depletion of CD59 (and CD55) may provide a mechanism to retain significant levels of complement resistance until the very end of the asexual life cycle. Thus, infected red cells may exploit the movement of DRM components to couple vacuolar development to the sensing of host complement. This could regulate multiple host–parasite interactions, such as the release of infectious progeny at the end of the asexual cycle as well as disease pathologies such as inflammation and splenomegaly.

In mammalian cells, plasma membrane vacuolar invaginations called caveolae have been implicated in concentrative transport of small molecules (Anderson, 1998). Membrane morphologies similar to caveolae have been described in reticulocytes and erythrocytes infected by *Plasmodium vivax* and *P.falciparum* (Barnwell, 1990; Olliaro and Castelli, 1997) and a wide range of low molecular weight nutrient solutes is transported by the PVM–TVM (Lauer *et al*., 1997). However, there is no uptake of large molecules such as antibodies into the plasmodial vacuole during intracellular parasite growth, suggesting that the connections between the PVM–TVM and the red cell membrane are strictly size restricted (Haldar and Uyetake, 1992; Lauer *et al*., 1997). It is possible that they may be novel, microdomain-based filters, which allow small gaps that exclude large molecules such as LY–dextran of 10 kDa, but import small nutrient solutes, perhaps analogous to selective, constricted, lipid-based junctions proposed at the center of immune synapses (Viola *et al*., 1999).

Materials and methods

Preparation of vacuolar parasites

Trophozoite- and schizont-infected red cells were removed from culture (Trager and Jensen, 1976), purified over a 65% Percoll cushion and incubated with 5% sorbitol (or the dipeptide Gly-Ser) for 15 min at 37°C to release the parasites (Haldar *et al*., 1994). Contaminating uninfected red cells were removed by centrifugation at 500 *g*. Released vacuolar parasites were sedimented by centrifugation at 3200 r.p.m. for 10 min and washed twice in RPMI-1640 to remove ghosts, which do not sediment under these conditions. If required, released parasites were also purified on Percoll gradients. Either procedure yields isolated vacuolar parasites of 90–95% purity as judged by staining fractions for host (band 3), PVM (PfEXP1) and PPM (PfMSP1) markers.

Extraction of DRMs and flotation gradients

Isolated vacuolar trophozoites/schizonts (1×10^9) were extracted on ice in 1 ml of 1% Triton in TBS (25 mM Tris–HCl, 150 mM NaCl pH 7.4), containing protease inhibitors and 1 mM EDTA. After 30 min at 4°C, the extract was mixed with 1 ml of 80% sucrose and then overlaid with 6 ml of 35% sucrose and 4 ml of 5% sucrose in TBS, and subjected to ultracentrifugation in an SW41 rotor at 35 000 r.p.m. for 3 h at 4°C. The top 3 ml were collected as the first fraction. Proceeding down the gradient, fractions 2–6 were collected as 2 ml aliquots, their protein concentrations were determined, if necessary the proteins were concentrated by precipitation in acetone and samples were subjected to electrophoresis by SDS–PAGE, then probed with the appropriate antibodies in western blots and analyzed by densitometric scanning (using Molecular Dynamics ImageQuant 5.0 software). For uninfected erythrocytes, ghosts were prepared by hypotonic lysis (Civenni *et al*., 1998) prior to extraction in 1% Triton X-100 and flotation on gradients. For both vacuolar parasites and uninfected red cells, DRMs (fractions 2–4) could be sedimented by centrifugation at 15 000 *g* for 30 min.

Indirect immunofluorescence and quantitative analysis

Cell preparations were washed three times in RPMI-1640 and then resuspended at 1×10^7 cells/ml, and allowed to adhere to coverslips pre-coated with poly-L-lysine for 30 min at room temperature. The cells were then fixed in 1% formaldehyde, permeabilized with 0.05% saponin, blocked with 0.2% fish skin gelatin (fsk) and probed with the relevant primary antibodies (diluted 1:100 to 1:500), secondary antibodies or Fab fragment in phosphate-buffered saline (PBS) containing 0.05% fsk. In studies on the localization of CD59, CD58 and CD55 in infected red cells, where indicated the cells were permeabilized in cold 1% Triton X-100 (Mayor and Maxfield, 1995) instead of saponin. If required, cells were labeled with 20 μM BODIPY-ceramide (to stain lipid-labeled TVM structures) prior to their attachment to poly-L-lysine-coated coverslips. For all cells, parasite nuclei (blue) were stained with 10 μg/ml Hoechst for 30 min.

Fluorescence microscopy and digital image collection were performed on an Olympus IX inverted fluorescence microscope and a Photometrix cooled CCD camera (CH350/LCCD) driven by DeltaVision software from Applied Precision Inc. (Seattle, WA). Twenty 200 nm optical sections were taken through the depth of the cell, and DeltaVision software (softWoRx) was used to deconvolve these images and construct 3D volume views. DeltaVision softWoRx uses a constrained iterative deconvolution algorithm to remove out of focus blur in fluorescence optical sections and was set for a minimum of 15 iterative cycles. The optical transfer function (OTF) used in deconvolution was computed from a measured point spread function (PSF), which in turn was obtained by optically sectioning a fluorescent bead for all of the available objective lenses (40×, 60× and 100×). For quantitative projections, the 'additive' method of data collection was used. Here, each ray collects and sums data from all the voxels in its path and scales it down to an appropriate intensity. These data can be used for comparison of intensity in various structures within the image data. The DeltaVision workstation is based on a workstation originally developed by Agard and Sedat for 3D multiple-wavelength fluorescence microscopy for structural analysis of chromosomes, microtubules and nuclear lamins (Hiraoka *et al*., 1991). For CD59 and Duffy labeling, the concentrations of the primary and secondary antibodies were optimized by titrating empirically such that the fluorescence emission was linear over a 100-fold range of emission signal, and that the fluorescence detected with the parasite as well as the red cell was well within this range. Fluorescence quantification was carried out with a low magnification objective (40× NA 1.00) as well as with 60× NA 1.4 and 100× NA 1.35. Data from at least 200 individual infected red cells of mixed stages ranging from newly infected rings to mature schizonts in the process of rupturing were analyzed and polygons were drawn to delineate the parasite and red cell. Background (or non-specific) signal was subtracted by imaging areas that had no cells. Autofluorescence and non-specific fluorescence levels were determined by viewing control samples with either primary or secondary antibody (but not both), obtained under the same illumination and exposure conditions. Total fluorescence intensity, areas and pixel densities associated with the parasite and red cell were determined in 0° projections of 3D volume views. To determine the fluorescence internalized by the vacuolar parasite, we subtracted signal associated with the red cell membrane above and below the parasite. To normalize for the variability in the amount of CD59 and Duffy found on uninfected red cells, the data are represented as a fraction of the total cell-associated fluorescence internalized by the parasite at different times of intra-erythrocytic development. A minimum of at least 10 cells centered around a given parasite size was used to compute mean values and error bars shown in Figure 5A and B.

Release of parasites by cholesterol depletion of trophozoite-infected red cells

Cells from cultures synchronized to contain trophozoite stage parasites (~24–36 h) at 20% parasitemia were washed free of serum in RPMI-1640 and then resuspended at 5×10^8 cells/ml in RPMI-1640 containing 1–10 mM MBCD at 37°C for 5–60 min. Incubations with 5 mM MBCD yielded the maximum release of parasites without lysis of red cells, which was monitored by release of hemoglobin and the yield of uninfected cells after treatments. Control treatments contained RPMI alone or 5 mM MBCD complexed to cholesterol, which was prepared as described (Klein *et al*., 1995). Released parasites, those remaining within the red cell, were scored along with uninfected cells and percentage parasite release was determined by Giemsa staining or fluorescence microscopy. Cholesterol was quantitated using the Boehringer Mannheim test kit.

To determine viability immediately after release, the freed parasites were incubated in 0.5% Evans blue dye for 15 min and the numbers of blue and colorless parasites were scored. Similar assays were carried out on free parasites that were incubated under standard culture conditions in the incubator for 24 h.

Acknowledgements

We are grateful to Drs J.McBride, C.Parker and K.Yazdanbakhsh for antibodies to PfEXP1, CD59 and anti-Fy3 (which detects both Fya and Fyb of the Duffy protein). We also thank Drs K.Simons, S.Mayor, R.Anderson, B.F.Hall and D.Brown for their helpful discussions on properties of microdomains/rafts, the dynamics of GPI-anchored proteins, caveolae, complement regulation and DRM isolation, respectively, and Dr K.Kirkegaard and members of the Haldar laboratory for careful reading of the manuscript and comments. We thank S.Palmieri for expert assistance with the DeltaVision microscope and workstation and R.Valdivia and D.Monack for help with the FACS assays. Supported by NIH grants (AI39071 to K.H. and DK 32094 to N.M.), the MacArthur Foundation (S.L.) and a Burroughs Wellcome Fund New Initiatives in Malaria Award to K.H.

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Received April 25, 2000; revised and accepted June 2, 2000