Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites

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Accepted 1 February 2006
Journal of Cell Science 119, 1992-2002 Published by The Company of Biologists 2006
doi:10.1242/jcs.02911

Summary

Tetraspanins constitute a family of widely expressed integral membrane proteins that associate extensively with one another and with other membrane proteins to form specific membrane microdomains distinct from conventional lipid rafts. So far, because of the lack of appropriate tools, the functionality of these microdomains has remained largely unknown. Here, using a new monoclonal antibody that only binds to the tetraspanin CD81 associated with other tetraspanins, we show that membrane cholesterol contributes to the organization of tetraspanin microdomains on the surface of live cells. Furthermore, our data demonstrate involvement of host membrane cholesterol during infection by *Plasmodium yoelii* and *Plasmodium falciparum* sporozoites, which both depend on host CD81 expression for invasion, but not during CD81-independent infection by *Plasmodium berghei* sporozoites. Our results unravel a functional link between CD81 and cholesterol during infection by malaria parasites, and illustrate that tetraspanin microdomains constitute a novel type of membrane microdomains that could be used by pathogens for infection.

Key words: Tetraspanins, Microdomains, Cholesterol, Malaria, *Plasmodium*

Introduction

Eukaryotic cell membranes contain functional microdomains of particular lipid and protein composition, the most characterized of these microdomains being commonly referred to as lipid rafts (Munro, 2003; Simons and Ehehalt, 2002; Simons and Ikonen, 1997). Lipid rafts differ from other parts of the plasma membrane by their high content of cholesterol and sphingolipids, and are thought to act as platforms coordinating signal transduction events. Membrane cholesterol plays a crucial role during infection by some intracellular pathogens, including bacteria, viruses, protozoans and non-conventional infectious agents, and studies have indicated an involvement of lipid rafts in infection by some of these pathogens (reviewed by Manes et al., 2003; Simons and Ehehalt, 2002). It is now clear that cell membranes contain microdomains distinct from conventional lipid rafts, including a particular type of microdomains enriched in tetraspanins. Tetraspanins are integral membrane proteins sharing a common structure with four transmembrane domains delimiting two extracellular domains of unequal size and three short intracellular regions. Tetraspanins have been implicated in various biological processes such as cell adhesion, migration, cell fusion, co-stimulation, signal transduction and differentiation (reviewed by Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005), yet their precise function remains unknown. A current model is that tetraspanins function as molecular organizers of membrane multi-molecular complexes, collectively referred to as the tetraspanin web (Rubinstein et al., 1996). Within this network of interactions, tetraspanins form primary complexes with a limited number of proteins called tetraspanin partners. These tetraspanin-partner interactions are direct and highly specific. For example, integrins $\alpha6\beta1$ and $\alpha3\beta1$ directly associate with CD151 but not with other tetraspanins (Serru et al., 1999; Yauch et al., 1998), whereas CD9P-1 and EWI-2 associate only with CD9 and CD81 (Charrin et al., 2003a; Charrin et al., 2001; Clark et al., 2001; Stipp et al., 2001a; Stipp et al., 2001b). These primary interactions resist detergents such as digitonin (and in some cases Triton X-100), and typically occur at a high stoichiometry (Charrin et al., 2003a; Charrin et al., 2001; Serru et al., 1999; Stipp et al., 2001a; Stipp et al., 2001b; Yauch et al., 1998). Tetraspanins also interact with each other, building

specific proteo-lipidic membrane microdomains to which they probably target their partner proteins (Boucheix and Rubinstein, 2001; Charrin et al., 2003b; Charrin et al., 2003c; Hemler, 2003; Levy and Shoham, 2005). In this regard, tetraspanins colocalize with gangliosides (Claas et al., 2001; Delaguillaumie et al., 2004; Odintsova et al., 2003), notably CD9, which directly interacts with GM3 (Ono et al., 2001), and several tetraspanins directly interact with membrane cholesterol (Charrin et al., 2003c). Furthermore, a substantial fraction of tetraspanin complexes can be recovered in the low density fractions of isopycnic sucrose gradients (Charrin et al., 2003b; Claas et al., 2001; Delaguillaumie et al., 2004). Tetraspanin microdomains are typically disrupted by Triton X-100, but are retained in less hydrophobic detergents such as Brij 97 (Charrin et al., 2003b). Several studies have documented key differences between tetraspanin microdomains and lipids rafts (Charrin et al., 2003b; Charrin et al., 2003c; Claas et al., 2001; Hemler, 2003).

There are some lines of evidence indicating a role of tetraspanins during infection by pathogens. For instance, infection by some viruses can be specifically blocked by antibodies to certain tetraspanins, including human T cell leukemia virus 1 by anti-CD81 and anti-CD82 antibodies (Imai and Yoshie, 1993), canine distemper virus and feline immunodeficiency virus by anti-CD9 antibodies (Loffler et al., 1997; Willett et al., 1997), and human immunodeficiency virus 1 by anti-CD63 antibodies (von Lindern et al., 2003). Additionally, the tetraspanin CD81 is required for infection of hepatocytes by hepatitis C virus (HCV), and functions as a receptor for HCV envelope glycoprotein E2, at least for certain viral strains (Bartosch et al., 2003; Pileri et al., 1998; Zhang et al., 2004). Recently, we reported that CD81 is also required for infection by *Plasmodium*, the parasite responsible for malaria. *Plasmodium* sporozoites are transmitted by infected *Anopheles* mosquitoes, and first infect the host hepatocytes by forming a parasitophorous vacuole where they further differentiate into a replicative exo-erythrocytic form (EEF). We have shown that CD81 is required for invasion of hepatocytes by sporozoites of human malaria *Plasmodium falciparum* and rodent malaria *Plasmodium yoelii* parasites (Silvie et al., 2003). Indeed, *P. yoelii* sporozoites fail to infect CD81-deficient mouse hepatocytes, and anti-CD81 antibodies inhibit *P. yoelii* and *P. falciparum* sporozoite invasion of hepatocytes. However, we found no evidence that CD81 interacts directly with *Plasmodium* sporozoites, suggesting that CD81 does not act as a receptor for the parasite but, rather, is involved indirectly (Silvie et al., 2003).

The aim of this study was to investigate the mechanisms underlying tetraspanin microdomain assembly and to determine the functionality of these microdomains with respect to *Plasmodium* sporozoite invasion. So far, these aspects have remained poorly understood, primarily because of a lack of appropriate tools, including mAbs specific for microdomain-associated tetraspanins and reagents able to disrupt tetraspanin microdomains in live cells.

Results

Generation of two new anti-mouse CD81 mAbs

In an effort to develop new tools to characterize tetraspanin microdomains, we generated rat mAbs against mouse tetraspanin complexes, and screened the hybridomas by indirect immunofluorescence and co-immunoprecipitation using Brij 97 lysates of biotin-labeled cells. Because lysis in Brij 97 preserves tetraspanin-tetraspanin interactions, any anti-tetraspanin mAb can co-immunoprecipitate the entire set of proteins present in tetraspanin microdomains, yielding complex and very similar patterns of co-immunoprecipitated proteins. The tetraspanin pattern obtained with the mouse hepatoma cell line Hepa1-6 using a CD9 mAb for immunoprecipitation is shown in Fig. 1. The major proteins co-immunoprecipitated with CD9 have an apparent molecular mass consistent with that of α integrin, CD9P-1 and EWI2. We selected two mAbs, MT81 and MT81w (for mouse tetraspanin CD81 'web'), which gave a pattern very similar to that of CD9, and clearly different from the pattern obtained with a mAb to α5 integrin, which does not associate with tetraspanins (Fig. 1). This pattern strongly suggested that MT81 and MT81w were anti-tetraspanin antibodies, and additional experiments demonstrated that MT81 and MT81w are both anti-mouse CD81 antibodies. First, MT81 and MT81w labeled the surface of embryonic fibroblasts from wild-type and CD9-deficient mice, but not from CD81-deficient mice (Fig. 2, upper panels). Second, MT81 and MT81w labeling of Hepa1-6 could be specifically knocked-down by siRNA-mediated silencing of CD81 (Fig. 2, middle panels). We were concerned that MT81 and MT81w could recognize a mouse molecule that associates with CD81 and the expression of which depends on the expression of CD81, as described for CD19 in B lymphoid cells (Shoham et al., 2003). To rule out this possibility, we tested MT81 and MT81w reactivity on human HeLa cells transfected with a mouse CD81 cDNA construct, in comparison with control HeLa cells, which express only the endogenous human CD81. Both MT81 and MT81w bound to mouse CD81-transfected HeLa but not to mock-transfected HeLa cells (Fig. 2, lower panels), implying that these mAbs do not recognize human CD81 or a human CD81-associated protein, but are actual anti-mouse CD81 antibodies. Additional experiments revealed that MT81 but not MT81w could bind to a soluble form of mouse CD81 large extracellular domain in ELISA (data not shown), and react with CD81 in western blotting conditions (Fig. 4 and data not shown).

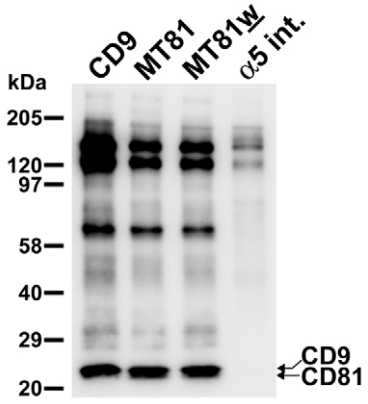


Fig. 1. Generation of new mAbs against mouse tetraspanin complexes. Biotin-labeled Hepa1-6 cells were lysed in 1% Brij 97 before immunoprecipitation with the anti-mouse CD9 mAb 4.1F12 (CD9), with the mAbs MT81 and MT81w, or with an anti-α5 integrin mAb (α5-int.).

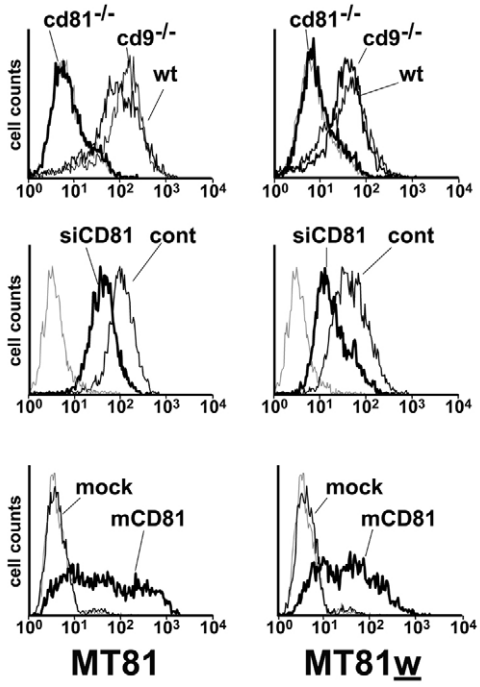


Fig. 2. MT81 and MT81w are anti-mouse CD81 mAbs. Flow cytometry analysis of different cell types stained with the mAbs MT81 (left panels) and MT81w (right panels). Upper panels: Embryonic fibroblasts from wild-type (wt), CD81 knockout (*Cd81*$^{-/-}$, thick line) or CD9 knockout (*Cd9*$^{-/-}$) mice. The labeling of *Cd81*$^{-/-}$ cells with mAbs MT81 and MT81w was the same as the control staining. Middle panels: Hepa1-6 cells transfected with a siRNA oligonucleotide targeting CD81 (siCD81, thick line) or with a control siRNA (cont, thin line). Lower panels: HeLa cells transfected with a mouse CD81 cDNA (mCD81, thick line) or with the vector alone (mock, thin line). The thin gray line represents the control staining.

MT81w recognizes a subset of CD81 molecules associated with tetraspanin microdomains

As shown by flow cytometry analysis, the intensity of labeling with MT81w only reached 40-60%, depending on the experiment, of the intensity of labeling with MT81 on Hepa1-6 cells (Fig. 3), suggesting that MT81w may recognize only a subset of CD81 molecules. To further characterize this subset, we analyzed the ability of MT81w to immunoprecipitate CD81 from cell lysates made under different detergent conditions, using MT81 as a control anti-CD81 antibody. Western blot analysis of immunoprecipitates showed that MT81 could precipitate CD81 under all detergent conditions tested (Fig. 4A). By sharp contrast, MT81w was able to precipitate CD81 only from Brij 97 and CHAPS lysates, both detergents preserving tetraspanin-tetraspanin interactions, but not from digitonin and Triton X-100 lysates (Fig. 4A). These latter conditions prevent tetraspanin-tetraspanin interactions, as confirmed by the absence of CD81 in the CD9 immunoprecipitate, and reciprocally by the absence of CD9 in the CD81 immunoprecipitate. These results suggest that MT81w binds only to CD81 associated with other tetraspanins. To provide further evidence in favor of this hypothesis, we performed experiments where cells were lysed in 1% Brij 97 (to preserve tetraspanin-tetraspanin interactions) followed by

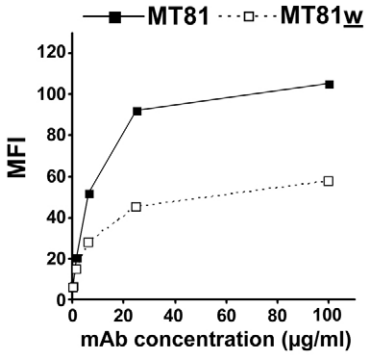


Fig. 3. Binding of MT81 and MT81w mAbs to Hepa1-6 cells. Hepa1-6 cells labeled with increasing concentrations of mAbs MT81 (solid squares) and MT81w (open squares) were analyzed by flow cytometry. Results are expressed as the mean fluorescence intensity (MFI) as a function of mAb concentration.

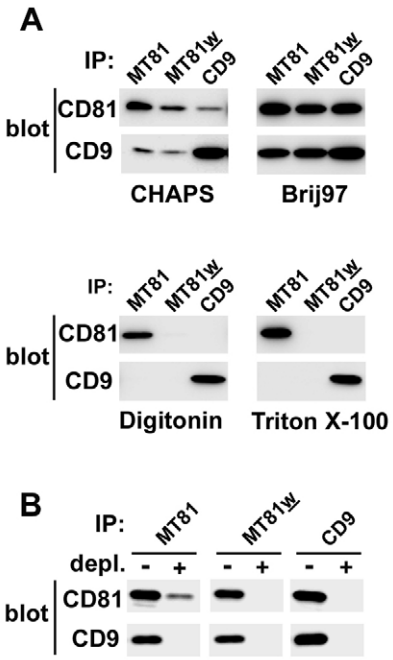


Fig. 4. MT81w only binds to CD81 associated with tetraspanins. (A) Hepa1-6 cells were lysed in the presence of CHAPS, Brij 97, digitonin or Triton X-100 before immunoprecipitation with the anti-CD81 mAbs MT81 and MT81w or with the anti-CD9 mAb 4.1F12. Immunoprecipitates were analyzed by western blotting using biotin-labeled anti-CD81 (MT81) and anti-CD9 (4.1F12) mAbs. Note that MT81w precipitates both CD81 and CD9 in CHAPS and Brij 97, but not in digitonin or Triton X-100. (B) Hepa1-6 cells were lysed in 1% Brij 97 before five rounds of immunoprecipitation with anti-CD9 mAb 4.1F12 to deplete CD9 (depl. +) or with beads alone as a control (depl. –). The supernatants were then used for immunoprecipitations as in A. The membranes in each panel were given the same exposure.

depletion of tetraspanin complexes by successive rounds of pre-clearing with the anti-CD9 mAb 4.1F12. Immunoprecipitation with MT81, MT81w and 4.1F12 were then performed and analyzed by western blot. Complete CD9 depletion was confirmed by the absence of CD9 in the lysates

(Fig. 4B). Strikingly, CD9 depletion induced a dramatic reduction (~85%, as determined by densitometry) of CD81 recovered by MT81 (Fig. 4B, upper left panel). This indicates that in Hepa1-6 cells a major fraction of the CD81 molecules is engaged in tetraspanin microdomains. Moreover, CD9 depletion completely removed the pool of CD81 molecules recognized by MT81w (Fig. 4B, upper middle panel), confirming that MT81w essentially recognizes CD81 associated with tetraspanins in tetraspanin microdomains.

CD81 localization into cell surface tetraspanin microdomains depends on cholesterol.

Since MT81w binds only to CD81 associated with other tetraspanins, this mAb constitutes a valuable tool not only to evaluate the level of association of CD81 on live cells, but also to dissect the mechanisms underlying tetraspanin-tetraspanin interactions. We have previously shown that tetraspanins interact with cholesterol, as indicated by the labeling of the tetraspanins CD9, CD81 and CD82 with photoactivatable cholesterol and by precipitation of tetraspanins by digitonin, which forms insoluble complexes with cholesterol (Charrin et al., 2003c). We performed digitonin-precipitation experiments in Hepa1-6 cells, using MT81w as a marker for CD81 association with tetraspanins. Treatment of Brij 97 lysates with digitonin resulted in a dramatic reduction in the amount of CD81 that could be recovered by MT81w in the soluble fraction, as compared with MT81, whereas immunoprecipitation of α5 integrin (used as a control) was not affected by digitonin precipitation (Fig. 5). This result, together with the fact that digitonin precipitates tetraspanins only under conditions preserving their mutual interaction (Charrin et al., 2003c), shows that digitonin preferentially precipitates tetraspanins associated with each other, and supports the idea that association of tetraspanins with cholesterol is concomitant with the formation of tetraspanin-tetraspanin complexes.

We then used MT81w as a tool to directly evaluate, through a non-biochemical approach, the role of cholesterol in the organization of tetraspanin microdomains on intact cells. We used methyl-β cyclodextrin (MβCD), a cyclic oligosaccharide

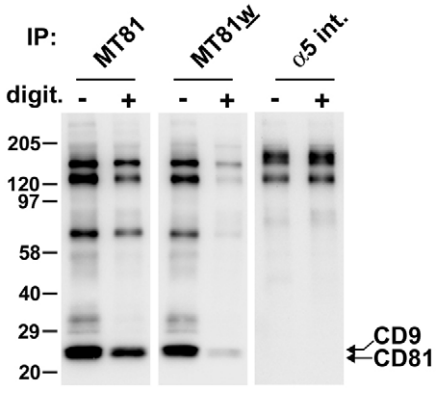


Fig. 5. Precipitation of tetraspanins by digitonin. Biotin-labeled Hepa1-6 cells were lysed in 1% Brij 97 and the lysates were treated with digitonin (digit. +) or with vehicle alone as a control (–). After elimination of the precipitated material, the supernatants were used for immunoprecipitation using anti-CD81 mAbs MT81 and MT81w or anti-α5 integrin (α5-int.). The panels are from the same membranes and were given the same exposure.

that selectively removes cholesterol from the cell plasma membrane without incorporating into the membrane (Yancey et al., 1996). Hepa1-6 cells were treated with MβCD before analysis of CD81 and CD9 expression by immunofluorescence on fixed monolayers. Treatment of Hepa1-6 cells with MβCD decreased the cell cholesterol content by 44±5%, consistent with previously described levels of cholesterol depletion (Friedrichson and Kurzchalia, 1998; Green et al., 1999; Keller and Simons, 1998; Sheets et al., 1999; Varma and Mayor, 1998). MβCD had no effect on CD81 and CD9 expression, as determined using mAbs 4.1F12 and MT81, but strongly reduced MT81w labeling (Fig. 6A). We further quantified this effect by flow cytometry analysis. Whereas MβCD treatment had no effect on CD81 and CD9 surface expression, cholesterol depletion induced a significant reduction (40-50%) in MT81w binding (Fig. 6B, upper panels). To test whether this was specifically due to the removal of cholesterol, we analyzed the effects of preformed MβCD-cholesterol complexes, which are known to replenish cells with cholesterol (Christian et al., 1997). Cholesterol replenishment of cholesterol-depleted Hepa1-6 cells reversed the effect of MβCD on MT81w binding, thereby confirming the specificity of the MβCD effects (Fig. 6B, middle panels). Furthermore, an increase in MT81w binding was observed when cells were treated with MβCD-cholesterol without prior cholesterol depletion (Fig. 6B, lower panels). Taken together, these results demonstrate a role of cholesterol in the organization of tetraspanin microdomains.

CD81 in tetraspanin-enriched microdomains supports infection by *P. yoelii* sporozoites

We have previously reported that hepatocyte CD81 is required for infection by *P. falciparum* and *P. yoelii* sporozoites (Silvie et al., 2003). As shown in Fig. 7A, MT81w efficiently inhibited *P. yoelii* invasion of Hepa1-6 cells, which normally support *P. yoelii* sporozoite invasion and exoerythrocytic development (Mota and Rodriguez, 2000), in a concentration-dependent manner (Fig. 7A). This shows that the fraction of CD81 associated with tetraspanin-enriched microdomains contributes to infection by *P. yoelii* sporozoites. However, MT81w was not as efficient as MT81 in blocking infection. MT81 could almost completely block sporozoite invasion (>95% inhibition), whereas MT81w induced a maximal inhibition of 60-70%. The blocking efficiency of MT81w was increased when cells were pretreated with MβCD-cholesterol complexes (Fig. 7B). Importantly, both mAbs also inhibited *P. yoelii* sporozoite invasion of primary mouse hepatocytes, with a similar efficiency to that seen with Hepa1-6 cells (data not shown).

The molecular determinants responsible for interactions between tetraspanins, and thus for their localization into tetraspanin microdomains, still remain elusive. However, it has been shown that palmitoylation-deficient mutants of CD9 and CD151 associate to a lower extent with other tetraspanins as compared to wild-type molecules (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002). To determine whether CD81 palmitoylation is required for its ability to support *P. yoelii* sporozoite infection, Hepa1-6 cells were transfected with wild-type human CD81 (hCD81) or palmitoylation-defective human CD81 (hCD81plm) constructs, and infected with *P. yoelii* sporozoites in the presence of MT81 mAb to block endogenous mouse CD81. As shown in Fig. 8, expression of

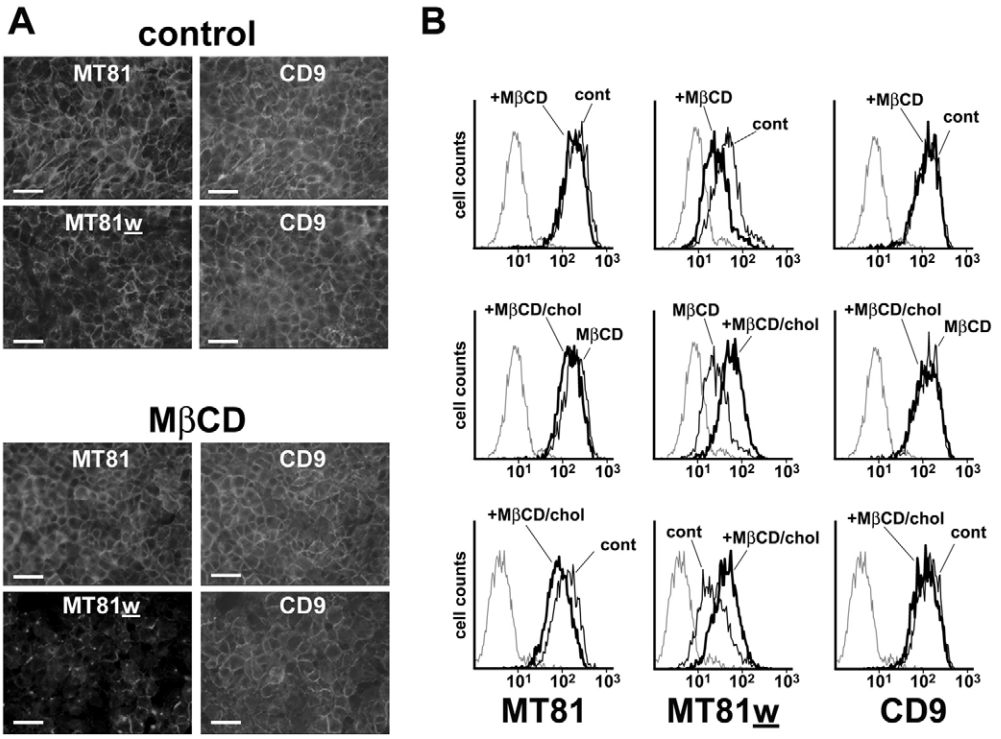
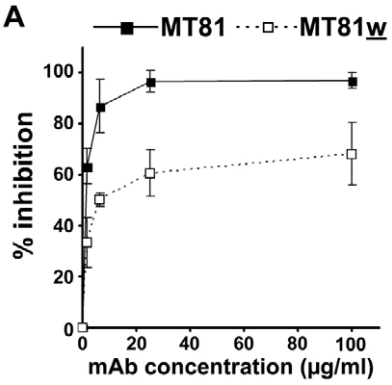


Fig. 6. CD81 localization in tetraspanin-enriched microdomains depends on cholesterol. (A) Hepa1-6 cell monolayers were treated with 10 mM MβCD for 15 minutes at 37°C (or left untreated as a control) before fixation and double labeling with anti-CD9 mAb 4.1F12 (right panel) and anti-CD81 mAbs MT81 or MT81w (left panel). Bars, 40 μm. (B) Flow cytometry analysis of Hepa1-6 cells stained with the anti-CD81 mAbs MT81 and MT81w or the anti-CD9 mAb 4.1F12. Upper panels: cells were treated with MβCD (thick black line) or left untreated (thin black line). Middle panels: cells were treated with MβCD (thin black line) followed by MβCD/cholesterol (thick black line). Lower panels: cells were treated with MβCD/cholesterol (thick black line) or left untreated (thin black line).



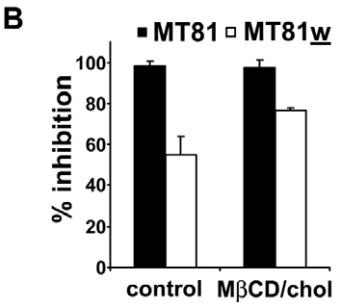


Fig. 7. Microdomain-associated CD81 supports *P. yoelii* sporozoite infection. (A) Effects of increasing concentrations of mAbs MT81 (solid squares) and MT81w (open squares) on *P. yoelii* infection of Hepa1-6 cells, expressed as the mean percentage of inhibition in triplicate wells (± s.d.). (B) Untreated (control) and MβCD/cholesterol treated Hepa1-6 cells were infected with *P. yoelii* sporozoites in the presence of MT81 and MT81w mAbs. Results are expressed as the mean percentage inhibition in triplicate wells (± s.d.), as compared to wells without mAbs.

both wild-type and palmitoylation-defective CD81 restored infection of MT81-treated cells, demonstrating that CD81 palmitoylation is dispensable during *P. yoelii* sporozoite infection. The infection of fewer cells with hCD81plm as compared to hCD81 was probably due to a lower surface expression in transfected cells, as shown by FACS analysis after labeling with the anti-CD81 mAb TS81 (MFI 56 versus 154 with hCD81plm and hCD81, respectively). Similar results were obtained in HepG2 cells (data not shown).

Host cell membrane cholesterol is necessary for *P. yoelii* and *P. falciparum* sporozoite invasion

If CD81 localization in tetraspanin microdomains is important for its function during hepatocyte infection by sporozoites,

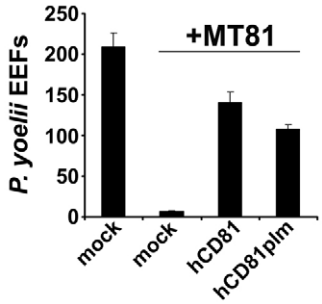


Fig. 8. CD81 palmitoylation is dispensable for *P. yoelii* sporozoite infection. Hepa1-6 cells were transfected with plasmids coding for wild-type human CD81 (hCD81) or palmitoylation-defective human CD81 (hCD81plm), or with the vector alone (mock), 24 hours before infection with *P. yoelii* sporozoites in the presence of MT81 as indicated. The number of exo-erythrocytic form (EEF)-infected cells (mean ± s.d.) was determined as described in the Materials and Methods section.

then cholesterol depletion should inhibit, to some extent, the level of infection, since as shown above it affects the organization of surface tetraspanin microdomains. Hepa1-6 cells were treated with MβCD prior to inoculation with *P. yoelii* sporozoites. MβCD treatment resulted in a reduction in the number of EEF-infected cells by 40-60% depending on the experiment (Fig. 9A). Interestingly, this inhibition was of the same magnitude as the reduction of MT81w binding following treatment with MβCD (Fig. 6B). Inhibition of infection was specifically due to cholesterol removal from the cell surface since it was completely reversed by cholesterol replenishment before sporozoite inoculation. Cholesterol removal was effective in preventing *P. yoelii* infection because of inhibition of sporozoite entry into MβCD-treated Hepa1-6 cells, as evidenced by counting intracellular sporozoites 3 hours after parasite inoculation (Fig. 9B). Interestingly, treatment of cells with MβCD-cholesterol complexes without prior cholesterol depletion enhanced infection (Fig. 9C). Furthermore, treatment of Hepa1-6 cells with MβCD or MβCD-cholesterol after completion of sporozoite invasion had no effect on infection (Fig. 9C), confirming that the host membrane cholesterol is required at the invasion step, and clearly demonstrating that MβCD is not toxic under the experimental conditions used here. We also tested the role of cholesterol during infection of primary mouse hepatocytes by *P. yoelii* sporozoites. As shown in Fig. 9D, cholesterol depletion by MβCD inhibited hepatocyte infection by *P. yoelii* sporozoites, and as seen with Hepa1-6 cells this effect was completely reversed by cholesterol replenishment before sporozoite inoculation.

We have reported previously that invasion of hepatocytes by the human parasite *P. falciparum* can be specifically blocked by anti-CD81 but not anti-CD9 antibodies (Silvie et al., 2003). To directly assess whether expression of CD81 is essential for *P. falciparum* infection, as for *P. yoelii*, we used RNA interference to knock-down CD81 expression in primary human hepatocytes in vitro. We could achieve ~50% reduction of CD81 surface expression using a small interfering RNA (siRNA) targeting CD81 (Fig. 10A). CD9 expression was not affected by the CD81 siRNA, showing the specificity of silencing. The reduced expression of CD81 led to an inhibition of *P. falciparum* sporozoite infection of the same magnitude (Fig. 10B). As a control, a siRNA targeting CD9 had no effect

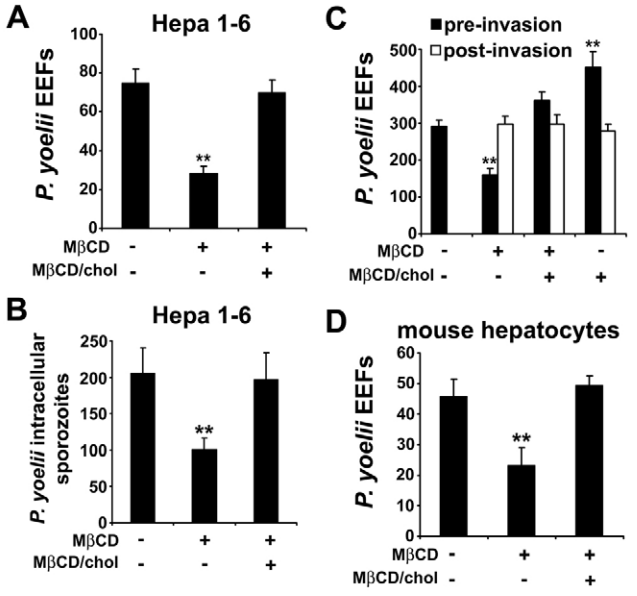


Fig. 9. Host cholesterol is required for *P. yoelii* sporozoite invasion. (A-C) Hepa1-6 cells were treated with MβCD followed by MβCD/cholesterol when indicated, before infection by *P. yoelii* sporozoites and quantification of exo-erythrocytic forms (EEFs) at 24 hours (A) or intracellular sporozoites at 3 hours (B). (C) Cells were treated either before (black bars) or 3 hours after (open bars) infection by sporozoites. (D) Primary mouse hepatocytes were treated with MβCD followed by MβCD/cholesterol when indicated before infection with *P. yoelii* sporozoites. The number of infected cells was determined as described in the Materials and Methods section. $^{**}P<0.01$, as compared to untreated control.

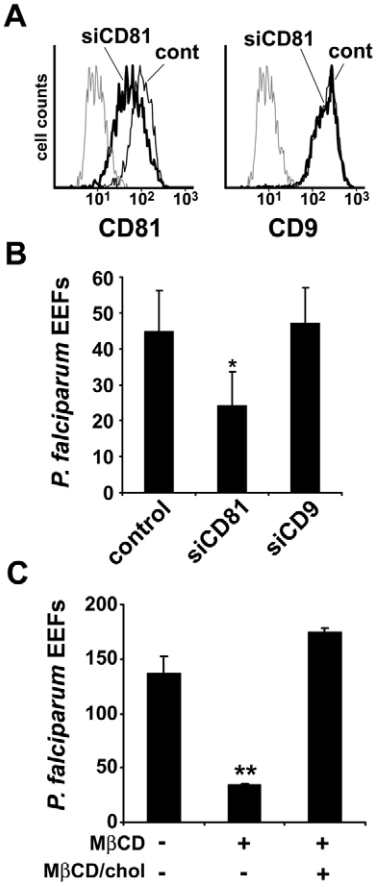


Fig. 10. CD81 and cholesterol are required for *P. falciparum* sporozoite invasion of human hepatocytes. (A) Primary human hepatocytes transfected with a siRNA oligonucleotide targeting CD81 (siCD81) or with a control siRNA (cont) were stained with anti-CD81 (mAb TS81, left panel) or anti-CD9 (mAb TS9, right panel) and analyzed by flow cytometry. (B) Quantification of *P. falciparum* exo-erythrocytic forms (EEFs; mean of triplicate wells ± s.d.) in human hepatocytes transfected with siRNA oligonucleotides targeting CD81 (siCD81) or CD9 (siCD9), or with a control siRNA (control). $^{*}P<0.05$ as compared to control. (C) Primary human hepatocytes were treated with MβCD followed by MβCD/cholesterol, when indicated, before infection by *P. falciparum* sporozoites. The number of EEF-infected cells was determined as described in the Materials and Methods section. $^{**}P<0.01$, as compared to untreated control.

on infection, although it reduced by ~40% the surface expression of CD9 (data not shown). Together, these results provide additional evidence for the specific role of CD81 during *P. falciparum* sporozoite invasion. We then analyzed the effects of MβCD-mediated cholesterol depletion on infection of primary human hepatocytes by *P. falciparum* sporozoites. As seen with *P. yoelii*, MβCD inhibited *P. falciparum* sporozoite invasion by up to 75%, and this effect was fully reversed by cholesterol replenishment of cholesterol-depleted hepatocytes (Fig. 10C), indicating a role for the host membrane cholesterol also in *P. falciparum* sporozoite infection.

Cholesterol and CD81 are functionally linked during *Plasmodium* sporozoite infection

So far, our data demonstrate that, (1) CD81 molecules associated with the tetraspanin web participate in sporozoite invasion, (2) cholesterol contributes to the organization of tetraspanin microdomains on the cell surface and (3) both CD81 and cholesterol are required on the host cell surface for sporozoite infection. Taken together these observations suggest that cholesterol is required during sporozoite invasion through the assembly of tetraspanin microdomains and that the effects of cholesterol depletion on infection are due to the disorganization of these microdomains. To obtain further evidence that the role of cholesterol is linked to CD81 during sporozoite invasion, we tested the effect of cholesterol depletion on CD81-independent infection by the rodent parasite *P. berghei*. In contrast to *P. yoelii*, sporozoites from *P. berghei* can infect CD81-deficient mouse hepatocytes (Silvie et al., 2003), as well as human hepatocarcinoma HepG2 cells (Hollingdale et al., 1983), which do not express CD81 (Berditchevski et al., 1996; Charrin et al., 2001). As shown in Fig. 11A, MβCD treatment had no effect on *P. berghei* sporozoite invasion of HepG2 cells. To rule out a low efficiency of MβCD-mediated cholesterol depletion in HepG2 cells, we repeated the experiments using HepG2 cells stably expressing CD81 (HepG2/CD81), which support *P. yoelii* sporozoite invasion and exo-erythrocytic development (Silvie et al., in press). Infection of HepG2/CD81 cells by *P. yoelii* was inhibited by MβCD treatment, showing that MβCD is efficient in depleting cholesterol (Fig. 11B). Inhibition was fully reversed by cholesterol replenishment prior to sporozoite inoculation, confirming the specificity of MβCD effects. By contrast, *P. berghei* invasion of HepG2/CD81 was not affected by MβCD-mediated cholesterol depletion, as observed with the parental CD81-negative HepG2 cells (Fig. 11C). By showing that cholesterol is required only for sporozoite invasion through the CD81-dependent pathway, our results unravel a functional link between cholesterol and CD81 during *Plasmodium* sporozoite entry.

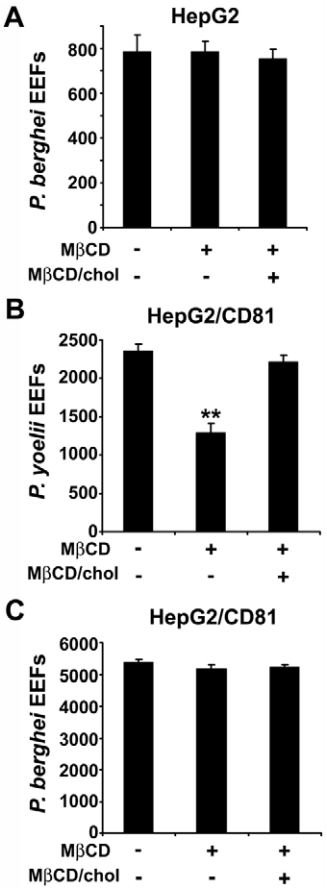


Fig. 11. Cholesterol and CD81 are functionally linked during *Plasmodium* sporozoite invasion. HepG2 (A) or HepG2/CD81 cells (B,C) were treated with MβCD followed by MβCD/cholesterol when indicated before infection by *P. berghei* (A,C) or *P. yoelii* (B) sporozoites. The number of exo-erythrocytic form (EEF)-infected cells was determined as described in the Materials and Methods section. $^{**}P<0.01$, as compared to untreated control.

Discussion

Use of mAbs to dissect the tetraspanin web in live cells

One remarkable feature of tetraspanins is their ability to act as molecular organizers of specific proteo-lipidic membrane microdomains (Charrin et al., 2003b; Charrin et al., 2003c; Hemler, 2003; Levy and Shoham, 2005). These tetraspanin microdomains display a number of critical differences that distinguish them from conventional lipid rafts, such as their disruption by Triton X-100 at 4°C and conversely their maintenance during cell lysis at 37°C in milder detergents such as Brij 97 (Charrin et al., 2003b; Claas et al., 2001). Also, the composition of tetraspanin microdomains differs from that of lipid rafts. For instance, typical raft resident proteins such as GPI-linked proteins and caveolin do not associate with tetraspanins (Berditchevski et al., 2002; Charrin et al., 2003c; Claas et al., 2001).

Recently, it became evident that antibodies recognizing subpopulations of tetraspanin molecules could be useful to analyze the tetraspanin web, notably on live cells. For example, the binding of two anti-tetraspanin antibodies is modulated by the expression of molecular partners. The anti-CD151 mAb TS151r only binds to CD151 when it is not associated with α3β1 or α6β1 integrins (Kazarov et al., 2002; Serru et al., 1999; Sterk et al., 2002), whereas the binding of the anti-CD9 mAb PAINS13 depends both on the expression of the integrin α6 subunit and on the activation of the β1 integrin subunit (Gutierrez-Lopez et al., 2003). Here, we describe a new anti-mouse CD81 mAb, named MT81w (for mouse tetraspanin CD81 'web'), which specifically recognizes CD81 associated with other tetraspanins. This is evidenced by the lack of recognition of CD81 after cell lysis with detergents that do not preserve tetraspanin-tetraspanin interactions, and by the

complete removal of the CD81 pool recognized by MT81w following immunodepletion of tetraspanin complexes. Additional studies will be required to determine the molecular basis of MT81w-restricted specificity. Even though the slope of MT81w binding curve is very similar to that of MT81, MT81w may be a low-affinity antibody that binds to CD81 bivalently, and thus would bind preferentially to CD81 molecules clustered within tetraspanin microdomains. Alternatively, MT81w may recognize an epitope on CD81 induced by CD81 localization within tetraspanin-enriched microdomains. This epitope may be induced as a consequence of the interaction with other tetraspanins or with cholesterol. It is very unlikely that MT81w could recognize CD81 associated with cholesterol outside tetraspanin-microdomains, because there is a tight correlation between association of tetraspanins with other tetraspanins and association with cholesterol (Charrin et al., 2003c), and all the CD81 fraction recognized by MT81w is associated with CD9. Whatever the molecular basis for MT81w specificity is, our data demonstrate that this antibody is a valuable tool that can be used as a marker for CD81 association with the tetraspanin web. In this regard, we have found that a major fraction of CD81 associates with the tetraspanin web in mouse hepatoma Hepa1-6 cells, and also in primary mouse hepatocytes, where MT81w labeling was at 55-75% that of MT81 (data not shown). Conversely, MT81w labeling was lower in embryonic fibroblasts (30% of MT81, Fig. 2) and much lower in mouse fibroblastic LM cells (3% of MT81, data not shown). These observations suggest that a high level of CD81 engagement into the tetraspanin web may constitute a specific feature of hepatocytic cells as compared to other cell types.

Cholesterol and tetraspanin microdomains

Although membrane cholesterol directly associates with tetraspanins (Charrin et al., 2003c), its role in the assembly of tetraspanin microdomains has remained unclear. Tetraspanin-tetraspanin interactions (notably CD81-CD9) are not readily disrupted upon MβCD treatment of intact cells (Charrin et al., 2003b; Charrin et al., 2003c; Claas et al., 2001). By contrast, cell lysis in the presence of MβCD results in a significant disruption of tetraspanin-tetraspanin interactions (Charrin et al., 2003c). Also, MβCD treatment strongly reduces the amount of tetraspanin complexes recovered in the light membrane fractions of sucrose gradients (Claas et al., 2001; Delaguillaumie et al., 2004). In one study, it was shown that this phenomenon can result from the shedding of tetraspanin microdomains from the cell membrane induced upon MβCD treatment (Claas et al., 2001). Here, using MT81w as a marker for tetraspanin-tetraspanin interactions, we provide clear evidence that the membrane cholesterol regulates the organization of cell surface tetraspanin microdomains. Indeed, the binding of MT81w to cells decreased after MβCD-mediated cholesterol depletion, and increased following cholesterol cell loading. Importantly, in our experimental conditions (using 10 mM MβCD, for 15 minutes at 37°C), there was no significant shedding of tetraspanin microdomains induced by MβCD, as evidenced by no reduction of surface expression of either CD9 or CD81. However, we noticed that increasing the concentration or the duration of MβCD treatment could eventually cause a reduction of surface expression of both CD81 and CD9 (up to 30%), which was not reversed by the back-addition of cholesterol and thus is consistent with a loss of tetraspanins from the plasma membrane.

The observation that MβCD directly affects cell surface tetraspanin microdomains, as evidenced by the reduction of MT81w labeling, without inducing major changes in tetraspanin-tetraspanin interactions observable by biochemical methods (Charrin et al., 2003c; Claas et al., 2001), may be explained by the existence of different levels of organization of tetraspanin microdomains (Claas et al., 2001). On one level, tetraspanins may interact with each other through a mechanism that is not affected by MβCD treatment of live cells. This level of interaction is likely to involve membrane cholesterol since these complexes can be disrupted with a Brij 97 lysis buffer supplemented with MβCD (Charrin et al., 2003c). We propose that on a second level these complexes cluster into higher order structures, through a mechanism affected by MβCD treatment of live cells. The existence of a MβCD-resistant level of interaction between tetraspanins would explain why the effect of MβCD on live cells is not detectable through biochemical approaches, but is uncovered using labeling with the mAb MT81w.

There are some lines of evidence suggesting that the MβCD-sensitive second level corresponds to a functional level of tetraspanin microdomain organization. In one study, MβCD treatment of B lymphoid cells was found to inhibit tyrosine phosphorylation induced upon ligation of tetraspanins, whereas, on the contrary, addition of cholesterol enhanced phosphorylation induced by tetraspanin engagement (Charrin et al., 2003c). In another study, all functional effects linked to CD82 engagement were abolished or strongly reduced in MβCD-treated Jurkat T cells (Delaguillaumie et al., 2004).

Tetraspanin microdomains and infection by intracellular pathogens

Previous studies have shown that the host membrane cholesterol is required for infection by erythrocytic stages of *Plasmodium* (Lauer et al., 2000; Samuel et al., 2001), and also by tachyzoites of *Toxoplasma gondii*, a distant relative of *Plasmodium* (Coppens and Joiner, 2003). Interestingly, the parasitophorous vacuole membrane of *Plasmodium* erythrocytic stages and also *Toxoplasma* was found to be enriched in raft components (Lauer et al., 2000; Mordue et al., 1999), suggesting that these parasite stages may use conventional lipid rafts for cell entry. Here, we show that membrane cholesterol is also necessary during host cell infection by *P. yoelii* and *P. falciparum* sporozoites, as evidenced by the inhibition of infection resulting from cholesterol depletion induced by MβCD. Membrane cholesterol is needed for sporozoite invasion, as shown by the inhibition of *P. yoelii* sporozoite entry into MβCD-treated cells and by the absence of inhibition when cells were treated with MβCD after completion of invasion.

Several elements suggest that the effect of MβCD on sporozoite invasion is the consequence of an alteration of tetraspanin-enriched microdomains. First, MβCD affects the organization of tetraspanin microdomains, as judged by the reduction of MT81w binding. Interestingly, the inhibition of *P. yoelii* infection of Hepa1-6 cells was of the same magnitude as the reduction of MT81w binding. Also, addition of cholesterol induced a significant enhancement of both infection and

MT81w binding, again showing a correlation between the level of CD81 engagement into microdomains and the level of infection. Second, our data demonstrate that CD81 in microdomains participates in infection, since the mAb MT81w, which only binds to CD81 associated with tetraspanins, inhibits *P. yoelii* infection. The lower inhibition achieved with saturating concentrations of MT81w, as compared with MT81, seems to conflict with an essential role of tetraspanin microdomains in the invasion process. However, it is noteworthy that MT81w does not recognize all the CD81 molecules present in the microdomains, as suggested by the difference between MT81w surface labeling (40-60% of total CD81 surface expression) and the level of CD81 association (~85%, as determined by the CD9 immunodepletion experiment). Therefore, the partial inhibition of infection achieved with MT81w is not inconsistent with a major, and possibly, exclusive role of microdomain-associated CD81 during sporozoite infection, since it could simply reflect the partial recognition of this fraction of CD81 by MT81w. The higher degree of inhibition of infection achieved with MT81w when cells are pretreated with MβCD-cholesterol complexes is consistent with this hypothesis. Also, the ability of palmitoylation-defective CD81 to support infection by *P. yoelii* does not preclude an essential role of tetraspanin microdomains in the invasion process since palmitoylation is not the only mechanism by which tetraspanins interact with each other (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002).

Although *P. yoelii* and *P. berghei* are closely related parasites behaving very similarly in most aspects of sporozoite biology, only *P. yoelii* requires CD81 to infect hepatocytes (Silvie et al., 2003). The present study demonstrates that the two rodent parasites also display a differential requirement of host cell membrane cholesterol for invasion. By showing that cholesterol is required only in the case of CD81-dependent sporozoite infection, our results clearly indicate a functional link between CD81 and cholesterol, and strongly suggest that CD81 localization in tetraspanin microdomains is essential for sporozoite infection.

Additional studies will be required to elucidate the mechanisms involving tetraspanin microdomains during *Plasmodium* sporozoite invasion of hepatocytes. In the absence of evidence of a direct interaction between sporozoites and CD81, and given the ability of CD81 to associate with multiple proteins within the tetraspanin web, one can speculate that CD81 may actually associate with one or several hepatocyte receptors for the parasite. Previous studies have shown that association with tetraspanins up-regulates the ligand binding activity of partner proteins (Feigelson et al., 2003; Iwamoto et al., 1994; Lammerding et al., 2003; Nishiuchi et al., 2005). If CD81 associates with a receptor for *Plasmodium*, then CD81-dependent clustering of this receptor into tetraspanin microdomains could be essential to enable high avidity interactions with parasite ligands at the entry junction. Alternatively, CD81 engagement into microdomains may be essential for some signaling events required at the time of sporozoite invasion, or the host plasma membrane in tetraspanin microdomains may display specific structural properties required during the formation of the parasitophorous vacuole.

Interestingly, CD81 is a receptor for the hepatitis C virus (HCV) (Bartosch et al., 2003; Pileri et al., 1998; Zhang et al., 2004), and recent reports have pointed to a role for cholesterol during host cell infection by HCV pseudoparticles (HCVpp). In one study, MβCD treatment of Huh-7 or Hep3B human hepatocarcinoma cells specifically inhibited infection by HCVpp (Zhang et al., 2004). In another study, high-density lipoproteins (HDL) but not low-density lipoproteins (LDL) nor lipid-free HDL apolipoproteins were shown to markedly enhance HCVpp entry into Huh-7 cells, through a mechanism dependent on the scavenger receptor BI (SR-BI) (Voisset et al., 2005). In liver cells, SR-BI functions as a lipoprotein receptor responsible for the selective uptake of cholesteryl ester from HDL (Connelly and Williams, 2004). Based on these observations, it will be interesting to see whether the clustering of CD81 into cholesterol-dependent microdomains is also essential for HCV infection.

In conclusion, our study demonstrates that membrane cholesterol mediates the localization of CD81 into tetraspanin-enriched microdomains and contributes to CD81-dependent infection by *Plasmodium* sporozoites. Thus, tetraspanin microdomains constitute a novel type of lipid-rich membrane microdomain that could be used by pathogens for infection. More generally, the cell membrane cholesterol has been shown to be essential in a number of biological processes, notably through the use of MβCD-induced cholesterol depletion. The fact that MβCD affects tetraspanin microdomains raises the possibility that some of these cholesterol-dependent processes may actually involve tetraspanin microdomains.

Materials and Methods

Antibodies

Anti-human CD9 mAb TS9 and anti-human CD81 mAb TS81 (Charrin et al., 2001) were from Diaclone (Besançon, France). Anti-mouse CD9 mAb 4.1F12 was described previously (Le Naour et al., 2000). Anti-mouse α5 integrin mAb MFR5 was from BD Pharmingen (Le Pont de Claix, France). For generation of MT81 and MT81w, a rat was injected intraperitoneally twice with 10^7 L-CD9 cells and a final booster was given 3 weeks later with CD9-containing complexes collected from a Brij 97 lysate of ~10^9 L-CD9 cells. Spleen cells were fused with P3x63AG8 mouse myeloma cells to generate hybridomas according to standard techniques.

Plasmids

A human CD81 mutant lacking five cysteines representing potential palmitoylation sites (triple mutant) has been previously described (Clark et al., 2004). A sixth cysteine, located in the second transmembrane domain of CD81, was mutated using the QuickChange™ site-directed mutagenesis kit from Stratagene (Amsterdam, The Netherlands), according to the manufacturer's specifications, resulting in a CD81 mutant lacking all potential palmitoylation sites ($CD81^{plm}$).

Parasites and cells

P. yoelii (265BY strain), *P. berghei* (ANKA strain) and *P. falciparum* (NF54 strain) sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands. Mouse hepatoma cells Hepa1-6 (ATCC CRL-1830), human cervical carcinoma cells HeLa (ATCC CCL-2) and mouse fibroblastic LM cells stably expressing human CD9 (L-CD9) (Rubinstein et al., 1994) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS; Biowest, Nuaillé, France), 2 mM glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin and 100 μg/ml neomycin (Invitrogen). Human hepatocarcinoma cells HepG2 and HepG2 stably expressing CD81 (HepG2/CD81) (Bartosch et al., 2003) were a kind gift from F. L. Cosset, and were cultured in DMEM supplemented as above, in culture dishes coated with rat tail collagen I (Becton-Dickinson, Le Pont de Claix, France). Primary mouse hepatocytes were isolated as described previously (Renia et al., 1990) and cultured in William's E medium (Invitrogen) supplemented as above. Primary human hepatocytes were isolated and cultured as described previously (Silvie et al., 2004). Mouse embryonic fibroblasts were generated from E15 embryos from wild-type, CD9 (Le Naour et al., 2000) and CD81 (Maecker and Levy, 1997) knockout mice, according to a previously described procedure (Wurst and Joyner, 1993).

Sporozoite invasion assay

Hepa1-6 or HepG2 cells (15×10^4 per well), or primary mouse (9×10^4 per well) or human (18×10^4 per well) hepatocytes were seeded in 8-chamber plastic Lab-Tek

slides (Nalge Nunc International, Cergy Pontoise, France), 24-48 hours (except for human hepatocytes, 3-7 days) prior to inoculation with sporozoites (1×10^5 parasites per well, except for *P. berghei*, 3×10^4 parasites per well). After 3 hours at 37°C, cultures were washed and further incubated in fresh medium for 24-48 hours (*P. yoelii* and *P. berghei*) or 72 hours (*P. falciparum*) before quantification of infected cells (containing an EEF in their cytoplasm) in triplicate wells by immunofluorescence, as described previously (Silvie et al., 2003). Quantification of intracellular sporozoites was carried out 3 hours post-infection using a double immunostaining technique (Silvie et al., 2002). For antibody-mediated inhibition assays, mAbs (25 μg/ml, unless otherwise stated) were added to the cultures at the time of sporozoite inoculation and removed 3 hours later. Inhibition results were analyzed for statistical significance using the one-way ANOVA followed by the Tukey multiple comparison test.

Immunofluorescence labeling

For flow cytometric analysis, cells were detached using a non-enzymatic solution (Invitrogen), washed and stained with saturating concentrations (unless otherwise specified) of primary mAb. After washes in culture medium, cells were incubated with 10 μg/ml FITC-labeled secondary antibody (Beckman Coulter, Villepinte, France), washed again three times and fixed with 1% formaldehyde in PBS. All incubations were performed for 30 minutes at 4°C. Analysis of cell-surface staining was performed using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). For fluorescence microscopy, cells were fixed in paraformaldehyde and incubated with anti-CD9 mAb 4.1F12 coupled to Alexa Fluor 488 and biotin-conjugated MT81 or MT81w followed by incubation with streptavidin-Alexa Fluor 594, before examination with a Leica DMR fluorescence microscope equipped with an appropriate set of filters.

Small interfering RNA and plasmid transfection

We used small double stranded RNA oligonucleotides targeting mouse CD81 (5′-CGT GTC ACC TTC AAC TGT A-3′), mouse CD9 (5′-GAG CAT CTT CGA GCA AGA GAA-3′), human CD81 (5′-CAC GTC GCC TTC AAC TGT A-3′) or human CD9 (5′-GAG CAT CTT CGA GCA AGA A-3′). A siRNA oligonucleotide targeting human CD53 (5′-CAA CTT CGG AGT GCT CTT C-3′) was used as a control siRNA throughout the study. Hepa1-6 cells ($5\text{-}10\times10^6$ cells in 400 μl RPMI) were transfected with 200 pmol of siRNA by electroporation (Weil et al., 2002) using the Gene Pulser apparatus (Bio-Rad, Ivry, France). Human hepatocytes were transfected with siRNA using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's recommendations. Following siRNA transfection, cells were cultured for 48 hours before flow cytometry analysis or sporozoite infection. Plasmid transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's specifications.

Immunoprecipitation

Surface labeling of cells with EZ-link-Sulpho-NHS-LC-biotin (Pierce, Rockford, IL, USA) was performed as described previously (Charrin et al., 2001). Biotin-labeled and non-labeled cells were lysed at 4°C for 30 minutes in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN_3, protease inhibitors and either 1% Brij 97 (Sigma), 1% digitonin (high purity; Calbiochem, San Diego, CA, USA), 1% CHAPS (Roche Applied Science, Meylan, France) or 1% Triton X-100 (Roche Applied Science). Immunoprecipitations were then performed using protein G-Sepharose beads (Amersham Biosciences, Rainham, Essex, UK), as described previously (Berditchevski et al., 1996; Charrin et al., 2001). For precipitation of cholesterol with digitonin, a 1/10th volume of 10% digitonin in methanol, or methanol as a control, was added to the Brij 97 supernatants. After 30 minutes at 4°C, the insoluble material was removed by centrifugation at 12,000 ***g***, and the supernatant was used for immunoprecipitation as above. The immunoprecipitates were separated by SDS-PAGE (5–15% gel) under non-reducing conditions and transferred to a PVDF membrane (Amersham Biosciences). Biotin-labeled surface proteins were revealed using streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences) revealed by enhanced chemiluminescence (Perkin Elmer Life Sciences, Zaventem, Belgium). Western blotting on immunoprecipitates was performed using biotinylated mAbs, followed by streptavidin-biotinylated horseradish peroxidase complex and visualization by enhanced chemiluminescence.

Cholesterol depletion

Cholesterol depletion was carried out by incubating cell monolayers with 10 mM methyl-β-cyclodextrin (MβCD; Sigma) in serum-free medium at 37°C for 15 minutes, as described previously (Charrin et al., 2003c). Treatment with MβCD removed ~40% of the cholesterol content of Hepa1-6 cells, as determined using the Amplex Red cholesterol kit (Invitrogen-Molecular Probes) according to the manufacturer's instructions. Cholesterol replenishment of cholesterol-depleted cells was achieved by incubating monolayers with 1:10 (mol/mol) complex of cholesterol (Sigma) and MβCD, using a 1 mM final cholesterol concentration, in serum-free medium at 37°C for 15 minutes as described previously (Charrin et al., 2003c).

We thank K. Farhati and T. Houpert for technical assistance. The CD81knockout mice were provided by S. Levy (Stanford University Medical Center, Stanford, California, USA) and the HepG2 and HepG2/CD81 cells by F.L. Cosset (Ecole Normale Supérieure, Lyon, France). O.S. was supported by a fellowship from Inserm. S.C. was supported by fellowships from the Association pour la Recherche sur le Cancer and the Fondation pour la Recherche Médicale. This work was supported in part by grants from Association Nouvelle Recherche Biomédicale, Association pour la Recherche sur le Cancer, by the European Union FP5 contract number QLK2-CT-2002-00774 and by the NIH grant AI55052 to Kansas State University.

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