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Localization of F-actin on the Caveola-vesicle Complex of the Erythrocytes Infected with *Plasmodium vivax* and *P. cynomolgi*

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Abstract

Phalloidin-gold complexes were used to localize the distribution of F-actin in the erythrocyte infected with *Plasmodium vivax* and *P.cynomolgi.* F-actin was found in caveola-vesicle complexes (CVC), specifically in the caveola indicating that CVC have contractile activity.

Key words: *Plasmodium vivax, P. cynomolgi,* F-actin, caveola-vesicle complex (CVC)

Introduction

The membranes of erythrocytes infected with various primate malaria species undergo morphological alterations during the intracellular development of the parasite. In Giemsa-stained blood films, parasitized erythrocytes infected with the human malaria parasite, *P. vivax,* and simian vivax-type malaria species such as *P. cynomolgi,* are covered with numerous small pink to red dots known as Schuffner's stippling. Electron microscopic observations have suggested that these dots represent the membrane structures present in the infected erythrocyte membrane known as caveola-vesicle complexes (CVC) (Aikawa *et al.*, 1975; Atkinson and Aikawa, 1990). Caveolae are small flask-like invaginations of the erythrocyte membrane skeleton that measure approximately 90 nm in diameter. In vivax-type malarias, additional spherical or tube-like vesicles are associated with the base of caveolae to form the CVC. It has been suggested that the CVC could be involved in the uptake of plasma protein and/or release of specific malaria antigens (Aikawa *et al.*, 1975; Atkinson and Aikawa, 1990; Barnwell, 1990; Barnwell *et al.*, 1990; Matsumoto *et al.*, 1988; Udagama *et al.*, 1988). However, the precise role of CVC in the biology of vivax type malarial parasites remains largely enigmatic and the proposed functions are for the most part speculative. Although it has been determined that the CVC are composed of or contain several parasite encoded proteins (Barnwell, 1990; Barnwell *et al.*, 1990; Udagama *et al.*, 1988) little is known about the composition or arrangement of host erythrocyte membrane or cytoskeletal proteins in these parasite induced structures.

Characterization of these structures is needed to further our understanding of their function(s) and the interaction of the vivax-type malaria parasites with the red cell. Phalloidin, a toxin from the mushroom *Amanita phalloides,* interacts specifically with filamentous actin (F-actin) in mammalian cells (Wulf *et al.*, 1979). Here we report that phalloidin-gold particles specifically label the CVC of vivax and vivax-like malaria parasite-infected erythrocytes.

Materials and Methods

Saimiri boliviensis (squirrel monkey) erythrocytes infected with the Brazilian Belem strain of *P. vivax* or rhesus monkey erythrocytes infected with *P. cynomolgi* (Mulligan) were fixed and

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embedded as previously described (Barnwell *et al., 1990*; Matsumoto *et al.*, 1988). Phalloidin-gold complexes were prepared with purified phalloidin (Sigma Chemicals, St. Louis, MO) and colloidal gold particles having an average diameter of 8–10 nm (Frens, 1973) as previously described by Lachapelle and Aldrich (1988). Two hundred and fifty μg of phallotoxin was dissolved in 100 μl of distilled water and mixed with 10 ml of colloidal gold. The mixture was incubated for 40 minutes at 4°C, ultracentrifuged at 31,300g for 40 minutes at 4°C and the pellet was resuspended in 0.5 ml of 0.1M phosphate-buffered saline (PBS), pH 7.2, containing 0.02% polyethylene glycol (PEG: MW 20,000).

Ultrathin sections were cut and preincubated for 15 minutes with 0.1M PBS containing 1% bovine serum albumin Fraction V (BSA) and 0.01% tween 20 (PBS-BSA-Tween). Grids were transferred to the phalloidin-gold suspension of varying dilutions ranging from 1:5 to 1:100 in PBS-BSA-Tween. The specificity of the phalloidin-gold labeling was verified by the following experiment. The sections were incubated with an aqueous solution of phalloidin (1 mg/ml) for 2 hr at room temperature before incubation with the diluted phalloidin-gold complex. After 60 minutes, the grids were rinsed 6 times in 0.1M PBS containing 0.1% tween 20 for a total of 30 minutes. The grids were then stained with 2% uranyl acetate in 50% methanol and examined by a JEOL 100 CX electron microscope at 60 KV.

Results and Discussion

Phallotoxin conjugated to colloidal gold was observed to bind to the CVC structures. This labelling by gold particles was dense and highly specific over the membrane-bound CVCs in both *P. vivax*- and *P. cynomolgi*-infected erythrocytes (Fig. 1, 2). Precisely, the phalloidin-gold label appeared to be primarily associated with the caveolar membrane (insets of Fig. 1, 2) and not the membranes of vesicles attached to the caveolae or those vesicles apparently free in the erythrocyte cytosol. In addition, the membranes of the parasitophorous vacuole, the cytoplasmic clefts, and the host erythrocyte were entirely free of colloidal gold particles. The specificity of the phalloidin-gold labeling was assessed by using preincubation with the phalloidin solution. In this case, no gold particles were seen in the infected erythrocytes (data not shown).

Lachapelle and Aldrich (1988) reported that the mushroom toxin phalloidin when complexed with colloidal gold particles retained the ability to bind specifically to F-actin. We have employed this unique interaction to demonstrate the possible presence of and specific localization of F-actin in the vivax-type malaria-infected erythrocytes at the ultrastructural level.

Normally, in the erythrocyte membrane, actin occurs as short filaments of 12–19 actin monomers linked with spectrin molecules to aid in the formation of the cytoskeletal framework (Byres and Branton, 1985; Lux, 1979). Apparently, phalloidin does not interact with these short actin filaments in the normal erythrocyte cytoskeleton. Therefore, our results could denote that an accumulation of and reconstruction of F-actin in the erythrocyte membrane occurs at the site of CVC. Based on differential detergent solubility, Barnwell *et al.* (1990) speculated that one distinct parasite-encoded protein of 95 kDa might interact with cytoskeletal matrix proteins in the CVC. Thus, in addition to a possible restructuring of erythrocyte actin at the sites of CVC, an interaction between this or other parasite antigens and F-actin might also occur to induce a molecular conformation or configuration necessary for phalloidin binding. Although it is known that phalloidin specifically binds actin filaments, the exact mechanism or requirements for this interaction to occur have not been determined.

The function of CVC remains unknown but some studies have suggested they could be involved in the uptake of host plasma proteins or in the transport and release of specific malaria antigens (Aikawa *et al.*, 1975; Barnwell, 1990; Barnwell *et al.*, 1990; Matsumoto *et al.*, 1988; Udagama *et al.*, 1988). An endocytotic transport function of CVC similar to clathrin-mediated endocytosis probably does not occur. Attempts to localize clathrin on CVC in *P. vivax* and *P.*

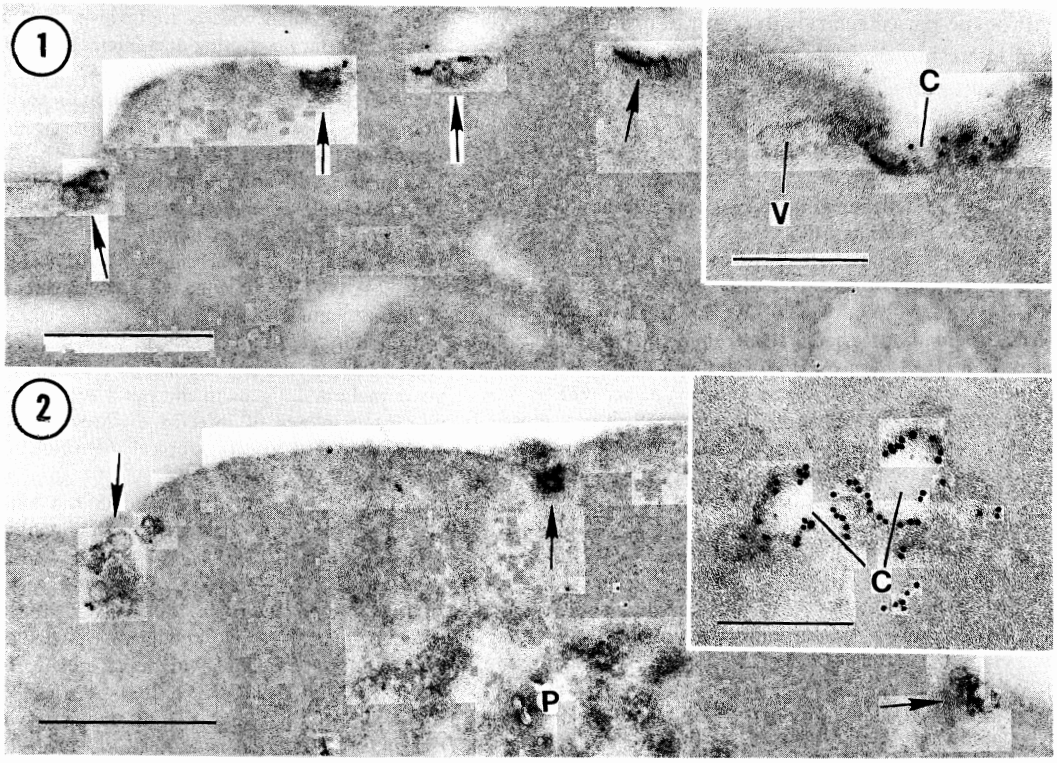

Fig. 1 LR white section of an erythrocyte infected with *Plasmodium vivax,* labeled with phalloidin-gold complexes. Caveolae of CV complexes (arrows) are labeled with colloidal gold. C: caveola, V: vesicle. Bar = 1 μm. Inset: Phalloidin-gold label concentrated on the caveolar membrane. Bar = 0.1 μm.

Fig. 2 LR white section of an erythrocyte infected with *Plasmodium cynomolgi,* labeled with phalloidin-gold complexes. Caveolae of CVC (arrows) are labeled with colloidal gold. P: parasite. Bar = 1 μm. Inset: Phalloidin-gold label is concentrated on the caveolar membrane. C: caveola. Bar = 0.1 μm.

brasilianum-infected erythrocytes using a variety of different antibodies have not been successful (Atkinson and Aikawa, 1990). An endocytotic mechanism involving nonclathrin-coated vesicles has also been proposed (Atkinson and Aikawa, 1990; Barnwell, 1990) as it had been described in other kinds of cells (Brown *et al.*, 1987; Rothberg *et al.*, 1990a, b). Payne and Schekman (1985) demonstrated a secretory pathway in *Saccharomyces cerevisiae* that uses vesicles without clathrin coating. The CVC might also be involved in exocytosis of products of the intra-erythrocytic malaria parasite. One soluble 70 kD parasite protein associated exclusively with cytoplasmic vesicles and the CVC (Barnwell *et al.*, 1990) is released into the extracellular environment in the absence of host cell rupture (J. Barnwell, unpublished data). Whatever the CVC function may be, the results have demonstrate the possible restructuring of host cell actin in *P. vivax* and *P. cynomolgi* infected erythrocytes exclusively at the caveolar membrane of the CVC. This could be, in conjunction with parasite proteins and other cytoskeletal elements, the basis for the formation of these classic flask-shaped membrane indentations. Such reorganization of the membrane and skeletal matrix could provide the signal(s) for vesicle "docking" or, alternatively, the formation of the associated sac-like vesicles through actin polymerization and depolymerization. As the CVC in vivax-type malarias are most likely involved in an important process for intra-

erythrocytic parasite survival, continuing studies are of interest to elucidate the molecular structure and function of CVC in vivax infected red cells.

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