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Cellular localization of *Babesia bovis* rhoptry-associated protein 1 in the merozoite stage with immuno-electron microscopy

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ABSTRACT

We examined the detailed cellular localization of the rhoptry-associated protein-1 (RAP-1) of *Babesia bovis* merozoites by the post-embedding method of immuno-electron microscopy (IEM) using the anti-RAP-1 monoclonal antibody (mAb) 1C1 in order to substantiate the result in our previous report by an indirect immunofluorescent antibody test and the pre-embedding method of IEM. RAP-1 slightly distributed only in cytoplasm of merozoites, especially around the nucleus, but not in the rhoptry organelle at an early stage after invasion into a red blood cell. Then, the RAP-1 was accumulated in the rhoptry organelle and was also found in the iRBC cytoplasm, which suggests that synthesis and release of RAP-1 may occur in the iRBC. Finally, the RAP-1 was found within and around merozoites after the breakdown of the iRBC. The results of the present study suggest that the RAP-1 of *B. bovis* merozoites functions not only in the invasion into RBC but also in the escape from iRBC.

INTRODUCTION

Babesia bovis is a hemoprotozoan parasite that causes great economic losses to the cattle industry worldwide. *B. bovis* is transmitted by tick vectors and has an asexual intraerythrocytic cycle in the infected cattle (De Waal, 2000; Homer et al., 2000). However, the molecular mechanism of the asexual intracellular development, particularly the process of merozoite invasion into and escape from infected red blood cells (iRBC) is still not fully understood.

Apicomplexans utilize several rhoptry proteins in their invasion into and development within RBC (Sam-Yellowe, 1996; Preiser et al., 2000). Extracellular merozoites attach to the RBC and reorient to bring the apical organelles close to the attachment interface, and the rhoptry products are released at the point of membrane invagination through the interaction of protozoan ligands with several surface receptors (Sam-Yellowe, 1996; Preiser et al., 2000). *B. bovis* RAP-1 is contained within the rhoptries but has not been detected in iRBC (Suarez et al., 1993), and spherical body proteins are released into the RBC after invasion, localize to the host cell plasma membrane and/or cytoskeleton, and apparently contribute to intracellular habitation (Dowling et al., 1996). Additionally, dense granule proteins of other apicomplexan parasites are discharged form merozoite post-host cell invasion, while rhoptry and microneme proteins appear to be involved in host membrane binding and penetration (Schwartzman, 1986; Sam-Yellowe et al., 1988; Adams et al., 1990; Entzeros et al., 1992; Perkins and Ziefer, 1994). Although RAP-1 is considered to be an important factor at invasion into RBC and during the intracellular development of *B. bovis* merozoites, we

currently lack any conclusive evidences of participation of the rhoptry products in their escape from the iRBC. On the other hand, proteins derived from another organelle, the dense granule, take part in their escape from the host cells as the nucleotide triphosphatase in *Toxoplasma gondii* (Carruthers, 1999). The understanding of the process of merozoite invasion and escape is important for the development of effective drugs and vaccines.

The rhoptry-associated protein 1 (RAP-1) of *B. bovis* merozoites, which is a protozoan secretion from the rhoptry, bears substantial sequence homology with the RAP-1 of other *Babesia* parasites (Dalrymple, 1993; Dalrymple et al., 1993). The purified recombinant RAP-1 has proved effective in inducing protective immunity in the vaccinated cattle (Wright et al., 1992). Recently, we demonstrated that the RAP-1 showed erythrocyte binding activity and that the anti-RAP-1 monoclonal antibody (mAb) 1C1 presented the interaction of the RAP-1 with RBC and inhibited the development of *B. bovis* in an *in vitro* culture (Yokoyama et al., 2002). In an indirect immunofluorescent antibody test (IFAT), RAP-1 was detectable in all developmental stages of merozoites and in extracellular merozoites and was also found in the cytoplasm and membrane of iRBC by immuno-electron microscopy (IEM) using the pre-embedding method. These results suggested the possibility of the participation of RAP-1 in not only the invasion into RBC but also the escape from iRBC (Yokoyama et al., 2002). However, the pre-embedding method was preliminarily used for IEM in a previous study, which made it difficult to ascertain the localization of RAP-1. In the present study, we examined the detailed cellular localization and movement of RAP-1 by IEM using the post-embedding method in order to substantiate our previous results with IFAT.

MATERIALS AND METHODS

Parasite

The Texas T2B strain of *B. bovis* (Suarez et al., 2000) was maintained in bovine RBC using the microaerophilus stationary-phase culture system (Levi and Ristic, 1980). Cell cultures that had between 5-10% parasitemia were washed three times with cold phosphate-buffered saline (PBS) and immediately used in immuno-electron microscopy.

Immuno-electron microscopy (IEM)

Infected iRBC were fixed in 4% paraformaldehyde with 0.1 % glutaraldehyde in PBS overnight at 4 ˚C, washed thoroughly in PBS, and embedded in 2% agarose. After dehydration with an ethanol series, the samples were embedded in LR Gold resin (Polysciences Inc., PA). Thin sections (about 80nm thick) were cut on a Leica UCT ultramicrotome using a diamond knife and put on nickel grids. Sections were exposed to 5 % skim milk PBS as a blocking agent at room temperature (RT) for 30 minutes and then incubated with the mAb 1C1 (Yokoyama et al., 2002) or normal mouse IgG (= negative control) overnight at 4 ˚C and subsequently with 10 nm gold-labeled goat anti-mouse IgG + IgM antibody (Amersham Biosciences, UK) at RT for two hours. Finally, these sections were counter-stained with uranyl acetate and lead citrate and examined with a H-7500 transmission electron microscope (HITACHI, Japan).

RESULTS AND DISCUSSION

RAP-1 slightly distributed only in the cytoplasm, especially around the nucleus, but not in the rhoptry organelle of merozoites within iRBC (Fig. 1a). Then, the RAP-1 was mainly found in the rhoptry organelle of divided merozoites and slightly in the iRBC cytoplasm (Fig. 1b) and was then observed mainly in the

cytoplasm of iRBC but not in the rhoptry organelle at a later stage (Fig. 1c). Finally, RAP-1 was found in and around the merozoites following the breakdown of iRBC (Fig. 1d).

Earlier studies have identified and characterized a 60kDa RAP-1 that has an apical location on the surface of *B. bovis* merozoites (Goff et al., 1988; Reduker et al., 1989; Palmer et al., 1991; Dalrymple, 1993) and was detectable as a rhoptry component by IEM (Suarez et al., 1993). Previously, we reported the localization of RAP-1 in the different developmental stages of intracellular merozoites (the ring and subsequent pear-shaped forms) and extracellular merozoites by IFAT using confocal laser scanning microscope and in the cytoplasm and confirmed the accumulation of RAP-1 in the cytoplasm of iRBC near the membrane by IEM the using the pre-embedding method. However, details of the localization of RAP-1 within the merozoite cytoplasm had never been achieved with IFAT, and the pre-embedding method was not suitable in conjugation with IEM for a more precise examination of the localization of RAP-1.

For the first time, the present study shows, using IEM with the post-embedding method, distribution pattern of RAP-1 in intracellular merozoites of *B. bovis* within iRBC at various stages. RAP-1 was observed in the cytoplasm of merozoites in iRBC, especially around the nucleus as shown in Fig. 1a. The rough endoplasmic reticulum (RER) and the Golgi apparatus are usually found around nucleus, so the result may suggest that the RAP-1 of *B. bovis* is synthesized in the RER and the Golgi apparatus after invasion into RBC. Since RAP-1 has a signal sequence (Dalrymple, 1993), it may be assumed that RAP-1 had been secreted from merozoites. Then distribution of RAP-1 in the rhoptry organelle of merozoites after division of parasites and movement of RAP-1 (Fig. 1b to c) may indicate that RAP-1 was accumulated in the rhoptry organelle secreted into the iRBC cytoplasm for the breakdown of iRBC and release of merozoites from iRBC. This hypothesis can be confirmed by the fact that RAP-1 was found in and around a merozoite and near the membrane of destroyed iRBC (Fig. 1d).

The results of the present study confirmed previous results by IFAT and strongly support the hypothesis that the RAP-1 of *B. bovis* merozoites functions not only in the invasion into RBC but also in the escape from iRBC. In addition, the RAP-1 family of proteins does not possess any significant homologies with any known proteases (Dalrymple, 1993). However, the proteolytic activity of RAP-1 that is non-covalently associated with protease of a similar size and functions as a co-factor has been proposed earlier (Commins et al., 1985). In view of these earlier reports, the biochemical characterization of the recombinant *B. bovis* RAP-1 gene product should help clarify the protease and/or protease-associated protein function of RAP-1.

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