Down-regulation of MARCKS-related Protein (MRP) in Macrophages Infected with Leishmania*

(Received for publication, January 26, 1999, and in revised form, March 26, 1999)

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* This work was supported by Grant 3100-050667.97 (to J. M.) and by Grant 3100-042045.94 to G. Schwarz, Biozentrum, University of Basel from the Swiss National Fund for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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Leishmania, a protozoan parasite of macrophages, has been shown to interfere with host cell signal transduction pathways including protein kinase C (PKC)-dependent signaling. Myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP, MacMARCKS) are PKC substrates in diverse cell types. Marcks and MRP are thought to regulate the actin network and thereby participate in cellular responses involving cytoskeletal rearrangement. Because MRP is a major PKC substrate in macrophages, we examined its expression in response to infection by Leishmania. Activation of murine macrophages by cytokines increased MRP expression as determined by Western blot analysis. Infection with Leishmania promastigotes at the time of activation or up to 48 h postactivation strongly decreased MRP levels. Leishmania-dependent MRP depletion was confirmed by [3H]myristate labeling and by immunofluorescence microscopy. All species or strains of Leishmania parasites tested, including lipophosphoglycan-deficient Leishmania major L119, decreased MRP levels. MRP depletion was not obtained with other phagocytic stimuli including zymosan, latex beads, or heat-killed Streptococcus mitis, a Gram-positive bacterium. Experiments with [3H]myristate labeled proteins revealed the appearance of lower molecular weight fragments in Leishmania-infected cells suggesting that MRP depletion may be due to proteolytic degradation.

The ability of various intracellular pathogens including Leishmania to inhibit macrophage effector activities, also termed “deactivation”, is well documented (1, 2). Functional alterations in Leishmania-infected macrophages include decreases in cytokine production, oxidative burst activity, antigen presentation, and expression of major histocompatibility complex class II genes in response to interferon (IFN)-γ. One mechanism of deactivation is indirect, involving induction of autoinhibitory molecules. In addition, there is evidence for direct interference of Leishmania with macrophage signal transduction pathways including inhibition of signaling through Janus kinases and Stat1 (3), or alterations in stimulus-induced intracellular calcium gradients related to decreased production of inositol 1,4,5-trisphosphate (4). Leishmania also inhibits protein kinase C (PKC)-dependent signaling in host macrophages as evidenced by alterations in PKC translocation and activity (5) and decreased expression of the transcriptional regulatory protein c-fos (6). Some of these effects may be ascribed to the properties of lipophosphoglycan (LPG), the major surface glycoconjugate of Leishmania, which has been shown to inhibit macrophage PKC-dependent signaling (7) as well as the activity of purified PKC in vitro (8). Thus, phagocytosis of LPG-coated beads inhibited phosphorylation of both a PKC-specific substrate peptide and myristoylated alanine-rich C kinase substrate (MARCKS), an endogenous PKC substrate in murine macrophages (9). Furthermore, depletion of PKC rendered macrophages more permissive for the proliferation of intracellular Leishmania suggesting that PKC-dependent events might contribute to parasite destruction (9).

MARCKS and MARCKS-related protein (MRP), also known as MacMARCKS (Macrophage-MARCKS), are members of a highly acidic myristoylated family of PKC substrates widely distributed in diverse cell types including macrophages (10, 11). Phosphorylation of MARCKS proteins following activation of PKC has been observed in fibroblasts (12, 13), macrophages (14) and neutrophils (15). Both proteins are essential for brain development and survival as shown by mice deficient in the genes macs or mpr (16, 17).

MARCKS has been shown to cross-link actin filaments in vitro (18). In macrophages, MARCKS colocalizes with actin, vinculin, and talin at the site of attachment of the cytoskeleton to the plasma membrane (19, 20). MRP colocalizes with paxillin at membrane ruffles at the leading edge of spreading macrophages, suggesting that it also associates with the actin cytoskeleton (21). Consequently, MARCKS and MRP are thought to regulate the actin cytoskeleton and thereby participate in major cellular responses such as phagocytosis, secretion, motility, mitogenesis, and membrane trafficking.

Expression of MARCKS and MRP is strongly up-regulated in macrophages stimulated with bacterial lipopolysaccharide (LPS) (22) or zymosan (23). LPS stimulation increases MRP steady state mRNA levels 30-fold in murine macrophages, and high levels persist for more than 8 h (22). MARCKS mRNA and protein expression can be decreased in fibroblasts through either PKC-dependent or -independent pathways by a post-transcriptional mechanism (24, 25). MARCKS concentrations may also be regulated by specific proteolytic cleavage of the unphosphorylated protein by a cysteine protease (26, 27), which has recently been identified as cathepsin B (28). To our knowledge, no reports concerning the down-regulation of MRP are avail-
able. Inasmuch as MRP is a major PKC substrate in macrophages, we have examined the expression of MRP in response to infection with Leishmania promastigotes. Our finding that Leishmania infection markedly depresses MRP levels may provide an important mechanism for regulating PKC-dependent effector function in macrophages.

**EXPERIMENTAL PROCEDURES**

*Mice—* CBA/J mice were purchased from Harlan (Horst, The Netherlands) and were used between 8 and 16 weeks of age.

*Radiolabeling of MRP—* Recombinant murine IFN-γ produced by Genentech Inc. was kindly supplied by Boehringer Ingelheim (Vienna, Austria). Recombinant human necrosis factor (TNF-α) was a gift of Dr. P. Schneider (Epalinges, Switzerland). LPS (Escherichia coli 055:B5) was purchased from Difco Laboratories, (Detroit, MI). Zymosan A, latex beads (1.07 μl), pepstatin A, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma. Heat-killed (auto-claved) *Streptococcus mitis* was a gift of Dr. D. Le Roy (Lausanne). LPG isolated from *Leishmania donovani* was kindly provided by Dr. S. Turco (University of Kentucky). Aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were purchased from Roche Molecular Biochemicals GmbH (Rotkreuz, Switzerland).

*Leishmania—* *Leishmania major* promastigotes, strains MRHO/SU/59/P and MRHO/IR/75/ER designated as LV39 and IR75, respectively, were grown at 26 °C in Dubbecco’s minimal essential medium (DME), Life Technologies, Inc., Basel, Switzerland on blood agar (29). Promastigotes of *Leishmania mexicana* strain MNKY/62/M379, *L. donovani* strain LV636 and the LPG-deficient *L. major* strain L119 (30) were propagated in 10% fetal bovine serum-supplemented HOSMEM II medium (31). For macrophage infection, stationary phase parasites were washed and resuspended in DMEM containing 10% fetal bovine serum.

**Macrophage Cultures and Activation—** Bone marrow-derived macrophages were obtained by *in vitro* differentiation of bone marrow precursors cells as described previously (32). Briefly, cells flushed from mouse tibia and femurs were grown in DMEM with 20% horse serum (Life Technologies, Inc.) and 30% L-cell conditioned medium. Day 10–11 macrophages were detached by pipetting, suspended in DMEM and 10% fetal bovine serum, and distributed in 35-mm tissue culture dishes (3 × 10⁶ macrophages/dish) or in 24-well cell culture plates (5 × 10⁵ macrophages/well), each well containing a round sterile glass coverslip. After 24 h, macrophages were washed and stimulated with IFN-γ and/or TNF-α or LPS in the presence or absence of Leishmania (5 parasites/macrophage unless indicated otherwise). To quantitate phagocytosis of Leishmania, coverslips were removed 24 h after infection, rinsed with phosphate-buffered saline (PBS), fixed and stained with Diff-Quick (Mertz and Dade, Dudingen, Switzerland) according to the manufacturer’s instructions.

**Nitrite Determination—** After 24 h of macrophage activation, 100 μl of supernatants were harvested for nitrite determination (33). Macrophage supernatants were mixed with an equal volume of Griess reagent and incubated for 10 min at room temperature. Absorbance was measured at 550 nm in a micro-enzyme-linked immunosorbent assay reader (Dynatech MR5000) using a 690-nm reference filter. NO₂⁻ concentration (μM) was determined using NaNO₂ as a standard.

**Radiolabeling of MRP—** Aliquots of [9,10-³H]myristic acid (American, Zurich Switzerland, 53 Ci/mmol) in ethanol were dried under stream of nitrogen gas, dissolved in dimethyl sulfoxide (Me₂SO) and heated to 4 °C. Microscopy was performed using a Zeiss Axioskop microscope equipped with a cooled microcamera (Photometrix, West Grove, PA). Images were captured on Kodak Traserial film (ASA 25) and scanned on the ScanJet 4c/T densitometer.

**RESULTS**

**Down-regulation of MRP Expression by Leishmania—** Murine macrophages activated with IFN-γ + TNF-α produce high levels of nitric oxide (NO) and are capable of killing intracellular *Leishmania* (37). We examined the expression of MRP in normal and activated macrophages by Western blot analysis of total cell lysates. Because of its acidic amino acid composition, MRP, whose calculated molecular mass is 20 kDa, exhibits anomalous migration on SDS gels and is recognized as a 42-kDa doublet in Western blotting. As shown in panels A and B of Fig. 1, IFN-γ + TNF-α increased the level of immunoreactive MRP protein after 4 h of culture (lane 2) though much stronger induction was observed after 24 h (lane 6). A comparison with known amounts of recombinant murine MRP (lanes 9 and 10) indicates that MRP is present at a concentration of approximately 1 ng/μg total protein in macrophages activated with IFN-γ + TNF-α. To determine whether infection by *Leishmania* modulates MRP expression, macrophages were challenged with LV39 promastigotes at the same time as stim-

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G. Vergères, unpublished data.
Leishmania Infection Down-regulates MARCKS-related Protein

FIG. 1. *Leishmania* infection decreases MRP levels in murine macrophages. Panels A and B, macrophages were cultured with IFN-γ (50 units/ml) plus TNF-α (250 ng/ml) or medium alone in the presence or absence of LV39 promastigotes (5 per macrophage). After 4 or 24 h, total cell lysates were prepared and MRP levels determined by Western blot analysis (panel B). Data from the corresponding densitometric scan is shown for comparison (panel A). Panel C, macrophages were stimulated with IFN-γ (50 units/ml, lanes 3 and 6), TNF-α (250 ng/ml), or LPS (10 ng/ml) alone or in the presence of LV39 promastigotes (5 per macrophage). MRP levels present at 24 h were determined by Western blot analysis of total cell lysates.

ulation with IFN-γ + TNF-α. Under these conditions, a strong decrease in MRP levels was consistently observed either 4 or 24 h after infection (Fig. 1, A and B, lanes 4 and 8). In many experiments, *Leishmania* also decreased the level of MRP in control unstimulated macrophages (Fig. 1B, lane 3; Fig. 4, lane 3, below and data not shown). As shown in Fig. 1C, a strong increase in MRP was also observed when macrophages were stimulated with TNF-α (lane 4) or LPS (lane 5) alone, and LV39 inhibited such induction (lanes 7 and 8). LV39 also inhibited the weak induction of MRP obtained with IFN-γ alone (lanes 3 and 6).

We then examined whether it was possible to reduce MRP levels by challenging macrophages with LV39 at various times after addition of IFN-γ + TNF-α. As shown in Fig. 2, MRP levels in lysates prepared 24 h after cytokine stimulation were strongly reduced when LV39 was added either together with the activating stimuli (lane 4) or when added 8 h after activation (lane 5). Similarly, addition of LV39 24 or 48 h after activation reduced the amount of MRP in 48-h (lane 8) or in 72-h (lane 10) lysates, respectively.

As an alternative proof that MRP levels were decreased in *Leishmania*-infected cells, the incorporation of [3H]myristic acid was examined. MRP expression was first induced for 4 h with IFN-γ + TNF-α, followed by the addition of fresh medium containing [3H]myristic acid in the presence or absence of LV39 promastigotes. After an additional 6 h, heat-stable fractions of total cell lysates were prepared and subjected to SDS-PAGE. Fluorography revealed three major proteins, a 74–78-kDa protein, most probably MARCKS, an uncharacterized protein of approximately 48–50 kDa (designated p50), and a broad 42–46-kDa doublet corresponding to MRP (Fig. 3). Western blot analyses, performed in parallel on the myristate-labeled lysates, confirmed the identity of MARCKS and MRP (data not shown). In agreement with data presented above, myristoylated MRP levels were increased upon cytokine activation and decreased in *Leishmania*-infected macrophages. Interestingly, MARCKS levels were also strongly decreased in *Leishmania*-infected cells. Although little or no induction of MARCKS expression was observed in macrophages stimulated with IFN-γ + TNF-α, it should be pointed out that constitutive levels of MARCKS are generally higher and induction of MARCKS mRNA and protein is both less pronounced and occurs with more rapid kinetics when compared with MRP (22, 39). Expression of the third heat-stable protein, p50, was increased by cytokine stimulation but, unlike MRP and MARCKS, was unaffected by *Leishmania*. In addition to the three major bands discussed above, additional lower molecular weight bands were observed for the samples from infected macrophages (lanes 3 and 4) possibly representing degradation products of MRP and/or MARCKS (see “Discussion”).

Comparison of Different Species of Leishmania—Several additional species of *Leishmania* were then compared with LV39 for their effects on MRP levels. Because LPG may be responsi-
Fig. 4. Depletion of MRP by different Leishmania promastigotes. Promastigotes of LV39, L. donovani (L. dono), or L119 were added to macrophage cultures (5 parasites per macrophage) in the presence or absence of IFN-γ (50 units/ml) plus TNF-α (250 ng/ml). NO production measured as NO₂⁻ release in 24-h supernatants is shown in panel A. MRP levels at 24 or 4 h determined by Western blot analysis of total cell lysates are shown in panels B and C, respectively. Note because MRP is found at lower levels 4 h after activation compared with 24 h, the blot in panel C was exposed for a longer period as evident from the stronger rMRP signal.

Fig. 5. Failure of phagocytic stimuli to deplete MRP. Macrophages were stimulated with IFN-γ (50 units/ml) plus TNF-α (250 ng/ml) alone or together with latex beads (25 μg/ml), LV39 parasites (5 per macrophage), zymosan (zym, 500 μg/ml) or heat-killed S. mitis (10 μg/ml, approximately 7 organisms per macrophage). Data from three representative experiments is presented. Panel A, NO production determined as NO₂⁻ release in 24-h supernatants. Panel B, MRP levels at 24 h determined by Western blot analysis of total cell lysates. 

Table I

| Parasites/100 macrophages | 4 h | 24 h |
|---------------------------|-----|------|
| LV39                      | 54  | 80   |
| +IFN-γ/TNF-α              | 68  | 44   |
| L. donovani               | 68  | 5    |
| +IFN-γ/TNF-α              | 68  | 1    |
| L119                      | 84  | 8    |
| +IFN-γ/TNF-α              | 118 | 9    |

Data is presented from one of two independent experiments.

Discussion

Recently, in vitro studies have demonstrated that Leishmania is capable of interfering with host macrophage signal transduction machinery (1, 2) thereby modifying the capacity of this cell to combat infection. One well studied effect of Leishmania involves inhibition of macrophage PKC activity and consequently PKC-dependent cell function. Results presented here suggest that Leishmania might also regulate PKC-dependent cell function in a more selective fashion by decreasing levels of MRP, a major PKC substrate in macrophages. Addition of Leishmania promastigotes to macrophages strongly reduced levels of cytokine-induced MRP as early as 4 h after infection. To date, all species or strains of Leishmania promastigotes tested were capable of down-regulating MRP levels in response to IFN-γ + TNF-α. This effect did not require viable parasites as heat-killed (15 min, 56 °C) promastigotes exhibited comparable activity (data not shown). Other phagocytic stimuli including yeast cell wall zymosan, latex beads, or heat-killed S. mitis had either no effect or increased MRP levels by themselves. Interestingly, the LPG-deficient strain L119 was as efficient as LV39 suggesting that LPG is not responsible for the effect of Leishmania infection on MRP. Moreover 10 or 25 μg purified LPG from L. donovani (kind gift of S. Turco) had no...
Experimental Procedures.

Of MRP was examined by fluorescence microscopy as described under phage for 24 h. After washing and fixation, the subcellular localization of MRP in activated macrophages reflects an overall inhibition of protein synthesis or cell function for several reasons. Nitrocellulose blots stained with Ponceau red showed no significant differences in lanes containing lysates from infected versus noninfected macrophages (not shown). Secondly, the expression of an uncharacterized 50-kDa heat-stable myristoylated protein was unaffected by Leishmania infection. Third, we have previously shown that Leishmania increases bone marrow macrophage synthesis of TNF-α and prostaglandin E2 in an identical experimental system (40). Finally, as shown previously (40–42) and confirmed here, phagocytosis of Leishmania strongly up-regulates the synthesis of NO.

We considered the possibility that down-regulation of MRP resulted from an effect of Leishmania on TNF-α receptor expression. However, similar results were obtained with other stimuli capable of up-regulating MRP levels including LPS and zymosan. Moreover, other markers of macrophage activation such as NO production or TNF-α synthesis (40) are enhanced under the same conditions. MRP levels in activated macrophages were also dramatically decreased when parasites were added 24 or 48 h after stimulation.

As mentioned above, examination of myristic acid incorporation revealed the presence of a 48–50-kDa protein (designated as p50 in our studies) in macrophages stimulated by IFN-γ + TNF-α. The identity of this protein remains unknown though at least two groups (39, 43) have previously described myristoylated macrophage proteins of comparable size. Although p50 levels were similar in normal and infected macrophages, the same studies suggested a profound effect of Leishmania on the levels of MARCKS, a PKC substrate closely related to MRP. Further studies are now in progress to examine Leishmania-dependent modulation of MARCKS expression.

Although MRP has been shown to be induced at the transcriptional level by LPS (22), there are no reports concerning factors capable of down-regulating its expression. Down-regulation of MARCKS in fibroblasts can occur through a post-transcriptional decrease in MARCKS mRNA upon incubation with bombesin or platelet-derived growth factor (24). Down-regulation could be mimicked by short term treatment with phorbol esters and was inhibited by PKC depletion. Somewhat paradoxically, Spizz and Blackshear (28) showed that PKC-dependent phosphorylation of MARCKS protects the protein from another down-regulatory pathway involving proteolysis by lysosomal cathepsin B. They speculated that targeting of MARCKS to the lysosomal membrane via a putative LAMP1-specific sequence might permit the interaction of cytosolic MARCKS and the lysosomal enzyme. That similar mechanisms might be involved in the regulation of MRP levels is suggested by our observations that the disappearance of radiolabeled MARCKS proteins in Leishmania-infected macrophages correlates with the appearance of lower molecular weight species. Moreover, we recently demonstrated that rMRP is rapidly cleaved by LV39 lysates or by purified Leishmania surface metalloprotease, leishmanolysin, in a cell-free in vitro assay. It remains to be determined if this proteolytic event occurs within the macrophage and, if so, how a Leishmania enzyme, which is presumably restricted to the phagosomal/phagolysosomal compartment might interact with a cytosolic protein such as MRP. In this regard, a recent report by Rittig et al. (44) provided intriguing evidence that some intracellular promastigotes of L. major are localized in the cytosol of infected macrophages.

The implications of MRP down-regulation during Leishmania infection are purely speculative for the time being. It has been proposed that down-regulation of PKC might favor parasite survival (9). Decreasing the expression of a given PKC

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3 S. Corradin, G. Corradin, J. Mauel, A. Ransijn, M. Rogerro, P. Schneider and G. Vergéres, submitted for publication.
substrate could represent an important mechanism for inhibiting specific PKC-dependent effector functions in the macrophage. Evidence of functional alterations in fetal cells from animals lacking MARCKS family proteins or from cell lines expressing incomplete or dominant-negative mutants of MRP or MARCKS is somewhat contradictory (45). In a recent investigation, Underhill et al. (46) reported that MRP is not essential for phagocytosis by macrophages. However, the authors speculated that due to their high effector domain homology, MARCKS and MRP might play overlapping roles explaining the normal phagocytic phenotype of MRP-deficient cells. It is, thus, particularly interesting that Leishmania infection appears to decrease levels of both MARCKS proteins in macrophages. Our data, taken together with the previously documented inhibitory effect of LPG on PKC activity, further establish the ability of Leishmania parasites to circumvent normal PKC-dependent function in macrophages.

Finally, we recently showed that peptides corresponding to the effector domain of MARCKS and MRP induce polymerization of monomeric actin and bundling of filamentous actin in contrast to comparatively moderate effects found with the intact MARCKS and MRP proteins (18). We postulated that in vivo proteolysis might facilitate the interaction between MARCKS proteins and actin by exposing their effector domain. Thus it is interesting to speculate that Leishmania-dependent degradation of MRP might in some way modulate the structure and function of the actin cytoskeleton in infected macrophages.

Acknowledgments—We are grateful to Dr. Sam Turco for providing purified LPG and to Dr. Giampietro Corradin for advice in preparing the MRP peptide construct. We thank Jeanette Holenstein for excellent technical assistance.

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