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A Leucine-Rich Repeat Motif of Leishmania Parasite Surface Antigen 2 Binds to Macrophages through the Complement Receptor 3

Lukasz Kedzierski,* Jacqui Montgomery,* Denise Bullen,* Joan Curtis,* Elizabeth Gardiner,† Antonio Jimenez-Ruiz,‡ and Emanuela Handman*‡

Membrane glycoconjugates on the Leishmania parasites, notably leishmanolysin and lipophosphoglycan, have been implicated in attachment and invasion of host macrophages. However, the function of parasite surface Ag 2 (PSA-2) and membrane proteophosphoglycan (PPG) has not been elucidated. In this study we demonstrate that native and recombinant Leishmania infantum PSA-2, which consists predominantly of 15 leucine-rich repeats (LRR) and a recombinant LRR domain derived from L. major PPG, bind to macrophages. The interaction is restricted to macrophages and appears to be calcium independent. We have investigated the PSA-2-macrophage interaction to identify the host receptor involved in binding and we show that binding of PSA-2 to macrophages can be blocked by Abs to the complement receptor 3 (CR3, Mac-1). Data derived from mouse macrophage studies were further confirmed using cell lines expressing human CR3, and showed that PSA-2 also binds to the human receptor. This is the first demonstration of a functional role for PSA-2. Our data indicate that in addition to leishmanolysin and lipophosphoglycan, parasite attachment and invasion of macrophages involve a third ligand comprising the LRRs shared by PSA-2 and PPG and that these interactions occur via the CR3. The Journal of Immunology, 2004, 172: 4902–4906.

The Leishmania protozoan parasites shuttle between blood-feeding sandflies, colonizing the gut as flagellated promastigotes, and the mammalian hosts in which they exist as obligatory intracellular amastigotes in phagocytes. A critical point in this host-parasite interaction involves the attachment to and invasion of host macrophages, initially by the promastigotes and subsequently by amastigotes. Both promastigotes and amastigotes use receptor-mediated phagocytosis for invasion. The 63-kDa parasite surface protease (gp63, leishmanolysin) and lipophosphoglycan (LPG) have been shown to be of paramount importance in attachment, invasion, and intracellular survival of the parasite in the macrophage (1–5). On the host side, several macrophage surface receptor complexes have been implicated (6), with the complement receptor 3 (CR3, Mac-1) playing a major role in these interactions (7, 8).

LPG is a major virulence determinant in Leishmania major and L. donovani, the causative agents of cutaneous and visceral leishmaniasis, respectively. It was also shown to be a major ligand for host macrophages (5). Loss of LPG by gene targeting produced organisms severely compromised in their ability to infect and to survive in macrophages in vivo or in vitro (9, 10). The demonstration that glycans used in binding to macrophages by L. major LPG are also present in a parasite mucin proteophosphoglycan (PPG) suggested that PPG may also contribute to parasite invasion (5, 11). In support of this idea is the observation that purified PPG binds to macrophages and is internalized into the lysosomal compartment into which phagocytosed parasites are also targeted (12).

We have recently demonstrated that in addition to sharing glycans with LPG, PPG shares amino acid sequence similarity with members of the parasite surface Ag 2 (PSA-2) family of glycoproteins. This similarity is restricted to a leucine-rich repeat (LRR) motif (13). LRR motifs are involved in protein-protein interactions, signal transduction, and pathogen recognition (14, 15). Interestingly, LRRs have also been exploited for host cell invasion by bacteria. The involvement of LRR motifs in the invasion of host cells by bacteria and their function as pattern recognition domains makes their study in Leishmania particularly important.

The extracellular location of PSA-2 and PPG suggests that they are accessible for interactions with host cells. This study demonstrates the ability of the LRR domains to bind to macrophages and to mediate parasite invasion. We show that native and recombinant PSA-2 binds to murine macrophages demonstrating for the first time a function for this protein. This interaction can be blocked by mAbs directed to the CD11b subunit of CR3. We also show that PSA-2 can bind to CR3 expressed by the human monocytic line, THP-1, as well as to CR3 expressed by 293 cells transfected with a plasmid carrying the human gene.

Materials and Methods
Parasites and cell lines
The L. infantum MHOM/TN/80/IPT1 was obtained from the World Health Organization Reference Centre for Leishmaniasis (Jerusalem, Israel), and the virulent clone V121 was derived from the L. major isolate LRC-L137 (16). Promastigotes were cultured in vitro as previously described (16).

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The murine macrophage cell line J774 was from the American Type Culture Collection (ATCC, Manassas, VA). The NIH 3T3 fibroblasts and the myeloid FD cell lines were provided by K. Shortman and D. Metcalf, respectively (Walter and Eliza Hall Institute, Melbourne, Australia). The human kidney 293 cells expressing human Mac-1 (from L. Zhang, American Red Cross, Rockville, MD) (17) and control 293 cells were cultured in DMEM (Life Technologies, Rockville, MD) containing 10% (v/v) FBS at 37°C in 5% CO₂. The monocyte THP-1 cell line was provided by J. Hamilton (University of Melbourne, Melbourne, Australia) and was maintained in RPMI 1640 supplemented with 10% (v/v) FBS. Differentiation of THP-1 cells was induced by treatment with 1 ng/ml TGF-β and 50 nM 1,25-(OH)₂ vitamin D₃ (18). The peritoneal thioglycollate-elicited macrophages were collected from C57BL/6 mice as previously described (12).

**Purification of water soluble PSA-2**

PSA-2 was purified from a promastigote lysate by affinity chromatography on immobilized mAb 142.17 (13). Cells were solubilized in 40 ml of 0.5% (v/v) Triton X-114 in ice-cold PBS pH 7.3 in the presence of a mixture of protease inhibitors. After detergent phase separation, the water phase, which included all the water-soluble cytoplasmic proteins, was loaded onto the column and PSA-2 was eluted with 0.1 M glycine HCl pH 2.6 as previously described (19). The pH of the eluate was neutralized and eluted protein was quantitated using the BCA Protein Assay (Pierce, Rockford, IL) according to the manufacturer’s instructions and its purity was assessed by SDS-PAGE and silver staining.

**Recombinant proteins**

Vector pMAL-c2T was used for the production of maltose-binding protein (MBP) fusion proteins in E. coli. This vector was modified from pMAL-c2X (New England Biolabs, Beverly, MA) by J. Goding (Monash University, Melbourne, Australia) by replacing the Factor Xa cleavage site with a thrombin cleavage site. The construction of MBP-LRR derived from PPG was previously described (13). MBP-PSA-2 was constructed by cloning the MBP-PSA-2 fusion into pMAL-c2T (20). The recombinant protein was produced in Escherichia coli strain BL21(DEL3) (Stratagene, La Jolla, CA) and purified by affinity chromatography on amylose resin as recommended by the manufacturer. Briefly, the bacterial lysate in a buffer containing 20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA was loaded onto the amylose column under gravity. After thorough washing, the bound protein was eluted in the same buffer containing 10 mM maltose. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce), and purity was determined by SDS-PAGE and Coomassie blue staining.

**Macrophage infection**

J774 macrophages (5 x 10⁴) in DMEM containing 10% (v/v) FBS were plated on glass coverslips in Nunclone 4-well multidishes (Nalge Nunc International, Rochester, NY) and grown overnight to a density of ~1 x 10⁵/well. A total of 20 µg of mAb in medium was added and incubated for 30 min at 37°C, 5% CO₂ before infection. V121 promastigotes or amastigotes were added to each well and allowed to invade at 37°C for 2 h, free parasites were removed, and after 25 h, the cells were washed twice in medium, fixed in 100% methanol and stained in 5% (v/v) Giemsa (Sigma-Aldrich, St. Louis, MO) in Sorensen’s buffer pH 7.2. Infection was assessed by counting five areas of 100 cells each from duplicate coverslips, and results were plotted as a percentage of infected cells.

**Flow cytometry analysis**

J774 and peritoneal macrophages, 3T3, FD, 293, and THP-1 cells, were resuspended in PBS pH 7.2 containing 10 µg/ml of the anti-FcγR rat mAb 2.4G2 (Fc block solution; ATCC). The cells (5 x 10⁵) were incubated on ice with 20 µg of MBP, MBP-LRR, MBP-PSA-2, or 4 µg native PSA-2 for 60 min, as were untreated controls. The cells were pelleted through an FBS cushion and resuspended in Fc block solution containing rabbit anti-MBP (1/1000) or rabbit anti-PSA-2 serum (1/500). After a further incubation of 60 min on ice and a rapid centrifugation through a FBS cushion, the cells were incubated with FITC-conjugated sheep anti-rabbit IgG (1/150; Chemicon International, Temecula, CA) for 45 min, washed and resuspended in 200 µl Fc block solution containing 2 µg/ml propidium iodide for analysis by flow cytometry (FACScan; BD Biosciences, San Jose, CA). In blocking experiments, a total of 100–200 µg of various Abs was used per 5 x 10⁵ cells, which were then incubated for 1 h with rotation at 4°C before the addition of 4 µg of L. infantum native PSA-2 followed by the steps previously described.

**Results**

The LRR domain binds specifically to J774 macrophages

We have previously shown that the filamentous form of PPG binds to the murine macrophage cell line J774 and to murine resident peritoneal macrophages (12). At that time, we had assumed that this interaction was mediated by PPG glycan side chains because PPG contains oligosaccharides that in LPG bind to macrophages (5, 11). To investigate whether the LRRs contribute to this interaction and can in their own right mediate adherence to macrophages, J774 cells were incubated with recombinant L. infantum MBP-PSA-2 or L. major MBP-LRR. The L. infantum PSA-2 has been chosen because it contains 15 LRRs spanning almost the entire length of the molecule (20). Binding was detected using Abs to the MBP tag and analyzed by flow cytometry. Fig. 1A illustrates that both the MBP-PSA-2 and MBP-LRR proteins, but not MBP protein alone, bound strongly to macrophages as demonstrated by a substantial shift in fluorescence. The addition of 0.1 M maltose had no effect on binding of MBP-PSA-2, MBP-LRR, or MBP (data not shown). It was important to assess whether this interaction is specific for macrophages, which are the host cell for Leishmania. L. major promastigotes and amastigotes are readily phagocytosed by the J774 cells in which they survive and replicate. In contrast, both parasite forms are rarely phagocytosed by the murine fibroblast line NIH 3T3, and the few intracellular parasites detected do not appear to survive (our unpublished observations). There was
negligible binding of either the MBP-PSA-2 or the MBP-LRR recombinant proteins to the fibroblast cell line (Fig. 1B), strongly suggesting specificity of the observed interaction.

Because the recombinant PSA-2 was able to bind to macrophages, it was of interest to examine the binding of the native PSA-2 to macrophages and other cell types. In these experiments we used a mAb, which recognizes several members of the PSA-2 family in L. major, L. donovani, and L. infantum to purify the native form of PSA-2. Fig. 1C shows that native L. infantum PSA-2 binds specifically to the J774 cells, but not to the control DF myeloid cell line (Fig. 1D). The native soluble PSA-2 also bound to thiglycollate-induced peritoneal macrophages (Fig. 1E) and bone marrow derived macrophages (data not shown). To assess whether the binding of PSA-2 to J774 cells was Ca\(^{2+}\)-dependent, experiments were performed in the presence of the chelating agent EDTA. Intact cells were incubated with the native L. infantum PSA-2 with or without the addition of 10 mM EDTA and the binding was analyzed using flow cytometry (Fig. 1F). The addition of EDTA had no effect on binding of PSA-2 to J774 macrophages, suggesting that the interaction was Ca\(^{2+}\)-independent.

The LRR region of PSA-2 and PPG can adhere to murine macrophages and potentially this binding may induce internalization of parasites by receptor-mediated phagocytosis. To investigate this possibility we have assessed the ability of mAb 142.17 to block invasion of J774 macrophages by L. major promastigotes and amastigotes. This mAb recognizes the LRR domain on both PSA-2 and PPG. The 142.17 mAb had a profound inhibitory effect on the invasion of macrophages (data not shown). Using flow cytometry, we examined the ability of Abs against CR1 and CR3 to block binding of PSA-2 to macrophages. The anti-CR1 (clone 8C12) blocking Ab had no effect on the interaction of the native PSA-2 with J774 macrophages (Fig. 3A), but mAb M1/70 and 5C6, both directed against the CD11b subunit of CR3, were able to block binding (Fig. 3, B and C, respectively). Independent blocking experiments with either 5C6 or M1/70 mAb resulted in ~70% inhibition of PSA-2 binding to J774 macrophages (data not shown). Interestingly, the blocking with M1/70 appeared to be dose dependent, whereas this was not the case for 5C6 (data not shown). The inhibitory effect of M1/70 was stronger than that caused by 5C6. However, 5C6 was able to block significantly the attachment of amastigotes to macrophages, suggesting that the functional concentration of Ab required for the parasite invasion is lower than that required for binding of the protein used in these experiments. Purified rat IgG did not have any inhibitory effect (Fig. 3D) neither did I21-7-7 mAb directed against the CR3-related integrin, LFA-1 described receptors involved in host-parasite interactions, we used a candidate approach to identify the host receptor for PSA-2. Use of Abs against the scavenger receptor type I and type II and fibronectin ruled out the involvement of these molecules in interactions with PSA-2 (data not shown). Using flow cytometry, we examined the ability of Abs against CR1 and CR3 to block binding of PSA-2 to macrophages. The anti-CR1 (clone 8C12) blocking Ab had no effect on the interaction of the native PSA-2 with J774 macrophages (Fig. 3A), but mAb M1/70 and 5C6, both directed against the CD11b subunit of CR3, were able to block binding (Fig. 3, B and C, respectively). Independent blocking experiments with either 5C6 or M1/70 mAb resulted in ~70% inhibition of PSA-2 binding to J774 macrophages (data not shown). Interestingly, the blocking with M1/70 appeared to be dose dependent, whereas this was not the case for 5C6 (data not shown). The inhibitory effect of M1/70 was stronger than that caused by 5C6. However, 5C6 was able to block significantly the attachment of amastigotes to macrophages, suggesting that the functional concentration of Ab required for the parasite invasion is lower than that required for binding of the protein used in these experiments. Purified rat IgG did not have any inhibitory effect (Fig. 3D) neither did I21-7-7 mAb directed against the CR3-related integrin, LFA-1

L. infantum PSA-2 binding to macrophages can be blocked by anti-CR3 Abs

The interaction of the LRR motif with macrophages prompted the search for the identity of the host receptor. Based on previously

FIGURE 2. Monoclonal Ab inhibition of L. major invasion of murine macrophages. Promastigotes (A) and amastigotes (B) were treated with 20 μg/ml mAb as indicated on the graph and incubated with J774 macrophages for 2 h. Ab 2G11 is a negative control anti-T. gondii mouse mAb. WIC-79.3 and LT6 are mouse anti-LPG mAbs directed to the (Galβ1-3Gal) repeats and (Galβ1-4Man) backbone repeats, respectively (11, 25). Mouse mAb 142.17 recognizes PSA-2 and PPG (13). Rat mAb 5C6 is a mAb (ATCC) directed to CR3 (30, 21). The mean value with SE bars represents experiments performed in triplicate. *, p < 0.05; **, p < 0.005.

FIGURE 3. Flow cytometry analysis of blocking of L. infantum PSA-2 interactions with J774 macrophages. The Abs used for blocking are shown in the left top corner of each histogram and are as follows: rat anti-mouse CR1 (8C12) (A), rat anti-mouse CR3 (M1/70) (B), rat anti-mouse CR5 (5C6) (C), and rat anti-mouse LFA-1 (I21-7-7) (E). D, Purified rat IgG was used as a negative control. All Abs were used at 100–200 μg total Abs per reaction.
(Fig. 3E). Taken together, the previous observations strongly suggest that the LRR domain present in PSA-2 and PPG binds to the CR3 and contributes to parasite attachment and invasion.

PSA-2 binds to human CR3

We have further investigated binding of PSA-2 to CR3 using the human monocytic cell line THP-1 as well as the 293 cell line expressing the human CR3. THP-1 cells were treated with TGF-1β/vitamin D₃ to induce differentiation, which leads to a 2-fold up-regulation of CR3 expression (22). As shown in Fig. 4A, native PSA-2 bound to non-differentiated as well as differentiated cells as demonstrated by a shift in the fluorescence peak. The analysis of the mean FITC fluorescence revealed that there is an ~2-fold increase in binding of PSA-2 to TGF-1β/vitamin D₃-treated cells compared with non-treated cells consistent with the increased expression of CR3 (Fig. 4B). Similar experiments were performed using the 293 cells transfected with an expression vector pCIS2M harboring human CR3 (17). Native PSA-2 bound to both untransfected and transfected cells as demonstrated by the shift in fluorescence peaks (Fig. 4C) with an ~2-fold increase in binding to CR3 present on transfected cells (Fig. 4D).

It has been reported that the CR3-specific mAb, ICRF444 (Imperial Cancer Research Fund, London, U.K.), reduced the adhesion of neutrophils to fibronectin and serum-coated slides (23). However, the ICRF444 Ab had no effect on binding of PSA-2 to either THP-1 cells or CR3-expressing 293 cells in our blocking experiments at the reported saturating concentration of 10 μg/ml per 2 × 10⁷ cells (23) (data not shown). Because the adhesion blocking effect described for neutrophils was partial, it is possible that the amount of Abs used was not sufficient to block the interaction of PSA-2 with CR3. In the case of mouse macrophages, 100–200 μg of Ab was necessary to block protein binding.

Discussion

*Leishmania* have been shown to use multiple host receptors for attachment and invasion of macrophages. These include CR1 and CR3, the mannose-fucose receptor, the fibronectin receptor and the macrophage receptor for advanced glycosylation end products (6). There is mounting evidence that the parasite also uses multiple ligands for its interaction with the host. Among these, gp63 and LPG have been well characterized. However, it is now clear that some of the reagents used in the studies on LPG could not have distinguished between LPG and PPG because of similar glycans present on both molecules (11, 21, 24, 25).

LPG, PPG, and PSA-2 are expressed extracellularly, in secreted and membrane-anchored forms, so they are ideally placed to interact with receptors on host macrophages and in the midgut of the insect vector. We have previously shown that secreted PPG can bind to macrophages and modulate their biology (12). We assumed that this interaction was mediated by glycans shared by PPG and LPG. However, when the gene encoding a membrane-bound PPG was cloned the sequence analysis revealed the presence of an LRR motif with remarkable similarity to the LRR sequence previously described in members of the PSA-2 family of glycoproteins (13, 20, 26). The shared sequence includes not only the typical leucine residues, but almost invariant glycine, proline, and tryptophane residues at conserved positions. A three-dimensional model of the LRRs based on the known structure of several LRRs shows that the conserved amino acids cluster on a compact region on the surface of a typical horseshoe structure (13). This clustering suggests a role in binding, and the similarity between the LRRs in PPG and PSA-2 suggests that they may interact with the same ligand.

LRRs are common motifs involved in protein-protein interactions and in pathogen recognition. Pathogen pattern recognition by the LRRs present in Toll-like receptors and the identification of cytoplasmic LRR-containing proteins (NOD1 and NOD2) as intracellular sensors of microbial products indicate an important role for LRRs in host-pathogen interactions (14, 27). LRR domains have been exploited by *Listeria monocytogenes*, in which the internalin LRRs are both necessary and sufficient for invasion of a range of host cells (28). Like PSA-2 and PPG, the internalins are encoded by a large multigene family and are expressed as cell surface and secreted forms.

The role of CR3 in the macrophage-*Leishmania* interaction has been demonstrated for promastigotes and amastigotes as well as LPG itself (1, 6, 8, 29). CR3 appears to be one of the major receptors for *Leishmania*, but there may be a hierarchy of receptor-ligand interactions depending on parasite ligand accessibility. These may occur sequentially or simultaneously thus strengthening the signal for phagocytosis. The leishmanial LRRs appear to bind to macrophages via CR3 as shown by the PSA-2 binding experiments. Because the similarity between PSA-2 and PPG is restricted to the LRRs, these results suggest that PSA-2 and PPG may interact with a common ligand. The recombinant MBP-PPG consists of a protein fragment containing LRRs only, and it is very likely that this protein binds to the same receptor as native and recombinant PSA-2, which are almost entirely composed of LRRs. A similar type of interaction between LRR and CR3 has been described for the platelet glycoprotein Ibα, which binds to leucocyte CR3 (18). Binding has been mapped to the glycoprotein Ibα LRR domain and I domain (inserted) present on the CD11b subunit of CR3. The I domain has been shown to be a major ligand binding domain of CR3 (30). It is likely that the same type of interactions is true for the LRR motif present in both leishmanial proteins. This observation is strengthened by the fact that both mAbs used in our blocking experiments are directed to CD11b. Nevertheless, the I domain may not be the only binding site for leishmanial LRRs because CR3 offers several binding sites. The involvement of β-chain (CD18) of CR3 in ligand binding to integrins is yet to be established, but is seems unlikely that it plays a...
significant role in the interaction between LRR and CR3. The possibility that other receptors are also involved in the LRR-macrophage interaction cannot be ruled out at this stage. In the case of gp63, although CR3 is also a major receptor, the molecule has been reported to bind to β2 integrins, fibrinogen receptor, and mannos-fucose receptor (2, 4, 24, 31). The fact that we were not able to fully block PSA-2 binding may suggest that other macrophage receptors may be involved in LRR-cell surface interaction. In contrast, the ability of anti-CR3 Abs to block almost 70% of PSA-2 binding to J774 clearly points to CR3 as a major ligand for the leishmanial LRR motif. The role of CR3 as a host ligand for LRR binding was further demonstrated by the ability of PSA-2 to bind to human CR3. Whereas the native protein bound to undifferentiated and differentiated THP-1 cells, there was a 2-fold increase in binding to differentiated cells, consistent with the fact that cytokine stimulation results in up to 2-fold up-regulation in Mac-1 expression. Similarly, there was a 2-fold increase in binding of PSA-2 to 293 cells transfected with Mac-1 compared with untransfected cells. The latter was most likely due to the inherent “stickiness” of this particular cell line. Bodary and McLean (32) investigated the presence of integrin receptors on 293 cells and demonstrated that this particular cell line. Bodary and McLean (32) investigated the presence of integrin receptors on 293 cells and demonstrated that this particular cell line.

References


