Alkylacylglycerolipid domain of GPI molecules of Leishmania is responsible for inhibition of PKC-mediated c-fos expression

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Abstract Glycosylphosphatidylinositol (GPI) molecules are the most abundant molecules present in the membranes of the parasitic protozoa, Leishmania. GPIs play a significant role in manipulating normal macrophage functions, and may also be targets for early NK-T phagocyte functions, and the protein kinase C (PKC)-dependent signaling pathway. The bioactivity of Leishmania GPIs is in contrast to Trypanosoma brucei and Plasmodium falciparum GPIs, which activate the macrophage functions. To address the question of which structural domain of Leishmania GPIs is responsible for dramatic down-regulation of PKC-dependent transient c-fos expression, the chemically synthesized defined alkylacylglycerolipid domain of corresponding GPIs, and LPG and GIPLs isolated from Leishmania donovani, were evaluated for inhibition of PKC and c-fos expression in macrophages. The results presented here demonstrate that the unusual lipid domain of Leishmania GPIs is primarily responsible for inhibition of PKC-dependent transient c-fos expression. Mamta Chawla, M. and Vishwakarma. Alkylacylglycerolipid domain of GPI molecules of Leishmania is responsible for inhibition of PKC-mediated c-fos expression. J. Lipid Res. 2003. 44: 594-600.

Supplementary key words glycosylphosphatidylinositol • lipophosphoglycan • protein kinase C • c-fos

Leishmania is an intracellular parasite that colonizes the macrophage system of its vertebrate host, causing various types of leishmaniasis in humans. Upon entry into the vertebrate host, the parasite has to encounter two normal host immune mechanisms, the destruction by macrophages, and lysis by complement system. Leishmania has evolved a unique approach to deal with both these innate immune mechanisms by learning not only to avoid early destruction but also to co-opt the opsonic properties of complement to enhance its interaction with macrophages. Once inside the macrophage, the parasite subverts and attenuates normal signaling of the cells to inhibit or reduce the impact of microbicidal mechanisms. For this purpose, the parasite has devised unique molecular strategies, and one such strategy involves the structurally distinct glycosyl phosphatidylinositol (GPI) molecules, expressed abundantly on parasite cell surface.

The GPIs were first discovered as a novel mode of anchoring of a few specialized surface proteins, but subsequently a diverse family of “free” GPIs was isolated from protozoan Leishmania, Trypanosoma, and malaria parasites (2–5). Recent studies indicate that GPIs from the parasites play a significant role in manipulating normal macrophage functions, and may also be targets for early NK-T cell and humoral responses during acute infection (6). The emerging theme is that the GPIs are among the most important virulence and survival determinants for the protozoan parasites. There are mainly three types of GPIs that are expressed by Leishmania: GPI anchor for surface proteins, lipophosphoglycans (LPGs), and free GPIs called glycoinositol phospholipids (GIPLs). LPG and GIPLs are expressed in high copy numbers (more than 10^7/cell) by the parasite and are considered essential for parasite infectivity and survival (2–5). All GPIs contain a conserved structural motif, Manα1-4GlcNα1-6-PI, and differ from each other with respect to the composition of glycan head group and/or the lipid anchor (2). The lipid anchor of GIPLs is exclusively 1-O-alkyl-2-O-acyl-phosphatidylinositol (PI) or 1-O-alkyl-lysophosphatidyl-DL-serine (PS), and alkyl chains of type I and hybrid GIPLs are C_{18:0}, while type II GIPLs have longer alkyl chains, C_{24:0} and C_{26:0}. The lipid anchor of LPG is

Abbreviations: GIPL, glycoinositol phospholipid; GPI, glycosylphosphatidylinositol; HI-FBS, heat-inactivated fetal bovine serum; LPG, lipophosphoglycan; PDBu, phorbol dibutyrate; PI, phosphatidylinositol; PKC, protein kinase C; PTK, protein tyrosine kinase; TCA, trichloroacetic acid.

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1-O-alkyl-2-lys-PI with C_{24/26} saturated unbranched aliphatic chains.

For macrophages to get activated for resistance and killing of the parasite, the cells must have the capability to respond to external signals by sending information from the surface to the nucleus. Obviously, the macrophage signaling pathways are appropriate targets for the intracellular parasites, as this strategy enables them to manipulate normal gene expression of the macrophages and the normal activity of the cell without even entering the nucleus. The c-fos is among the most studied key early competence genes that are expressed immediately after the macrophage activation by infection. The c-fos expression in macrophages is controlled by two mechanisms. Protein kinase C (PKC)-mediated c-fos expression leads to a transient and rapid increase in c-fos mRNA, peaking 30 min after stimulation, whereas protein kinase A-mediated expression leads to stable c-fos transcription lasting for several hours. For these reasons, c-fos has become a useful nuclear marker to study the role of parasitic GPIs in signaling in macrophages.

From various recent studies, it is abundantly clear that the nature of lipid domain of ‘free’ GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites. For example, the Trypanosoma brucei lipid structure of GPIs changes during parasite differentiation from epimastigotes to infective metacyclic stages, and sometimes opposite biological activities are observed. The GPIs from different parasites. For example, in the nature of lipid domain of ‘free’ GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites. For example, the Trypanosoma brucei lipid structure of GPIs changes during parasite differentiation from epimastigotes to infective metacyclic stages, and sometimes opposite biological activities are observed. The GPIs from different parasites. For example, in the nature of lipid domain of ‘free’ GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites. For example, in the nature of lipid domain of ‘free’ GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites. For example, in the nature of lipid domain of ‘free’ GPIs appears to be the most important structural feature responsible for diverse and sometimes opposite biological activities displayed by the GPIs from different parasites.
ferred from ATP to substrate per min per milligram of protein. This was followed by selective protection of primary alcohol with t-butyldimethylsilyl group (TBDMSCI, imidazole), palmitoylation at C-2 position (palmitoyl chloride, pyridine), and TBDMSCI group removal (tetrabutylammonium fluoride) to get 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycerol. The phosphorylation of this lipid with diphenyl phosphorochloridate followed by catalytic hydrogenolysis (Adams catalyst) provided the desired 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycerophosphate (alkylacylphosphatidic acid). The choice of alkyl and acyl chains was based on the structures of GPI molecules of L. donovani. The final lipid products, as well as all the intermediates, were characterized by NMR, IR, and mass spectrometry (17).

PKC assay in vitro

PKC was purified from rat brain tissue (male Wistar rats) using DE-52 and phenyl-sepharose chromatography (23), and assayed as described earlier (24) with minor modifications. The enzyme activity was measured as phosphorylation of a nonapeptide (VRKRTLRRL, 2.5 mg/ml) in a reaction mixture containing buffer (50 mM Tris-Cl, pH 7.5), 0.65 mM CaCl₂, 0.5 mM EGTA, 10 mM Mg(OAc)₂, 10 mg/ml phosphatidyserine, 2 mg/ml diacylglycerol, and 100 μM [γ-32P]ATP (10⁵ cpm/pmoland). The phospholipids dissolved in chloroform were dried under a stream of nitrogen and resuspended in buffer by sonication. Reaction was carried out at 30°C for 15 min before terminating by adding 25 μl of 20% trichloroacetic acid (TCA). A 25 μl aliquot was spotted onto a 2 x 2 cm P-81 strip, air dried, and washed with 75 mM phosphoric acid. Radioactivity incorporated in the peptide substrate was measured by scintillation counting. Nonspecific activity was measured in the absence of phospholipids and Ca²⁺. Specific activity was the difference between total activity and nonspecific activity. Enzyme activity was calculated as pmol of phosphate transferred from ATP to substrate per min per milligram of protein.

Inhibition of PKC by synthetic alkyl-acyl-glycerolipids in vitro

Different concentrations of synthetic alkylacylglycerol and alkylacylphosphatidic acid were prepared in chloroform, dried under nitrogen, and resuspended in buffer by sonication before use in in vitro PKC inhibition experiments as described above.

Culture of J774A.1 murine macrophage cell line

Murine macrophage cell line J774A.1 was cultured in DMEM (containing 2 mM glutamine, 50 mg/ml gentamicin sulfate, 2 g/l sodium bicarbonate, and 15% HI-FBS; pH 7.2). Cells incubated at 37°C in 5% CO₂ atmosphere showed adherence within 2 h, and a confluent monolayer was obtained in 4 days.

Preparation of synthetic lipid-coated RP-C₁₈ silica beads

Synthetic alkylacylglycerolipids were coated onto RP-C₁₈ derivatized silica beads according to the method first reported by Russell and Wright (25) and later applied by Descoteaux et al. (15) for presenting LPG-GIPLs to the macrophages. In a typical procedure, required aliquots of 2 mg/ml solution of synthetic alkylacylglycerolipid in CHCl₃ were mixed with 100 μl aliquots of 3 μm diameter beads (10 mg/ml) suspended in the same solvent (RP silica beads derivatized with G₁₈ alkyl groups were from Waters). The mixture was dried under N₂ gas in an Eppendorf centrifuge tube and then under vacuum overnight. The beads were resuspended in 1 ml of PBS, sonicated for 2 min, washed twice with PBS, and resuspended in 2 ml of complete medium by sonication (3 cycles, 15 s each spaced by 10 s). The final concentration was made of 2 mg beads/ml medium. Efficiency of bead-coating and their uptake by macrophages was estimated using [14C]palmitic acid as standard. For this, the 200 μl solution of labeled palmitic acid (2 mg/ml in CHCl₃, 100 μCi) was mixed with equal volume (200 μl) of beads (10 mg/ml) and suspended in CHCl₃. After mixing, the beads were dried, washed with PBS, resuspended in 2 ml of complete medium, and the labeled beads fed to confluent J774A.1 macrophages for 2 h. The monolayer was washed twice with the medium and washed further in fresh complete medium for 3 h. These were then stimulated or not stimulated with 100 nM PDBu for 15 min before PKC activity was measured in digitonin-permeabilized cells as phosphorylation of peptide substrate (16). Medium was then replaced with 60 μl of assay buffer (20 mM Hapes, pH 7.2, 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1 mg/ml glucose), supplemented with 10 mM MgCl₂, 50 g/ml digitonin, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM CaCl₂, 300 μM peptide substrate, and 100 μM [γ-32P]ATP. The reaction was carried out at 30°C for 15 min, after which it was stopped by addition of 20 μl of 20% TCA. Aliquots (40 μl) were spotted onto 2 x 2 cm P-81 strips, air dried, and washed in 75 mM phosphoric acid (5 x 100 ml). Air-dried strips were placed in cocktail-W, and incorporated activity was measured by liquid scintillation counting. Specific activity was calculated as the difference between total activity (in the presence of peptide substrate) and nonspecific activity (in absence of peptide substrate). PKC activity was calculated as pmol of phosphate transferred from ATP to peptide substrate per min per 10⁶ cells.

Analysis of c-fos expression in macrophages

Spent medium from confluent cultures was removed, and the monolayer was rinsed with 5 ml of medium. Then the synthetic lipids coated on beads and suspended in complete medium (2 ml per T-25 flask) were fed to macrophages for 2 h. Monolayers were then rinsed twice with medium and further incubated for 3 h in complete medium. These were either stimulated or not stimulated as per experimental requirement with 100 nM PDBu for 15 min. Cells were scraped, pelleted, and used for analysis of c-fos expression, as detailed below. Expression of β-actin was monitored as a housekeeping gene.

RNA extraction and Northern blot analysis

Total RNA was extracted from macrophages using Tri-reagent (MRC) or RNeasy kit (Qiagen). Samples were denatured in formamide at 65°C for 10 min, electrophoresed in 1% agarose gels (containing formaldehyde), and transferred to Hybond-N nylon membranes using 20X-SSC (175.3 g NaCl, 88.2 g sodium citrate, pH 7.0, volume made to 1 liter) as the transfer buffer. Prehybridization and hybridization were carried out at 42°C in 50% formamide with the probes previously random-labeled with [γ-32P]dCTP to 1 x 10⁸ cpm/μg DNA. Membranes were then washed once in 2X-SSPE (20X-SSPE = 175.3 g NaCl, 27.6 g NaH₄PO₄, 58.8 g Na₂HPO₄, 21.4 g EDTA/liter, pH 7.0) and twice in 0.5X-SSPE. Membranes were exposed for autoradiography.
RESULTS AND DISCUSSION

*Leishmania* GPIs (LPG and GIPLs) have been implicated in a number of functions, including the parasitie attachment and entry into macrophages, and its intracellular survival (3, 6). The most important target for *Leishmania* survival strategy is severe inhibition (nM range) of PKC, the enzyme responsible for initiation of normal oxidative mechanisms of the macrophages. In macrophages, these activation-associated events are under the control of PKC, an important enzyme and a key molecule in many signal transduction pathways. PKC comprises a family of at least 12 serine-threonine kinases, which are activated by turnover of membrane phospholipids (26). Since PKCs have a key role in the development of a protective host immune response by activation of various effector cells, it is not surprising that intracellular pathogens target this family of enzymes. This appears to be the case with *Leishmania*, which inhibits PKC directly as well as indirectly (via inhibition of PKC-mediated functions). The major cell surface molecules of *Leishmania* (LPG and GIPLs) have been shown to inhibit PKC and the downstream expression of related genes (12–16). While some studies have proposed that the majority of inhibition of LPG lied in its lipid anchors, other suggested that it was not required at all (14). The contribution of lipid anchors of GIPLs is not known. To ascertain this, one would require chemically defined molecules free from any contaminating domains. We used for the present study synthetic alkylacylglycerolipids (alkylacylglycerol and alkylacylphosphatidic acid) corresponding to *Leishmania* GIPLs (17), and studied their effect on the PKC activity and the expression of key early competence gene, c-fos, in macrophages.

PKC inhibition in vitro by *Leishmania* GPIs and synthetic alkylacylglycerolipids

In order to assess the contribution of lipid domains of GIPLs (LPG and GIPLs), synthetic and structurally defined alkylacylglycerolipids, 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycerol and 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycero-phosphate (alkylacylphosphatidic acid), were prepared by chemical synthesis by the following steps. First, the isopropylidene-sn-glycerol was alkylated with 1-bromooctadecane (NaH, DMF) followed by deketalation (HCl) to get 1-Octadecyl-sn-glycerol. Selective protection of primary alcohol with t-butyldimethylsilyl group (TBDMScI, imidazole), palmitoylation at C-2 position (palmitoyl chloride, pyridine), and TBDMs group removal (tetrabutylammonium fluoride) to get 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycerol. Phosphorylation of this lipid with (PhO)3POCl followed by catalytic hydrogenolysis provided desired 1-O-alkyl(18:0)-2-O-acyl(16:0)-sn-glycero-phosphate (alkylacylphosphatidic acid). For measurement of PKC activity in vitro, a highly specific arginine-rich peptide substrate (27) was used. The synthetic lipids were suspended in chloroform, dried under nitrogen, and resuspended in buffer by sonication. PKC assay was done in the presence as well as absence of diacyl-sn-glycerol, the natural physiologic activator of PKC, at various concentrations (10 to 400 μM). Interestingly, the synthetic alkylacyl-sn-glycerol showed significant PKC inhibition (Fig. 1), and more then 50% inhibition was observed at the 50 μM to 100 μM range. Increasing the concentrations further did not lead to further increase in inhibition and reached a plateau. Similarly, synthetic alkylacylphosphatidic acid showed a 50% inhibition at 50 μM. These results, in comparison with the reported PKC inhibition activity of GIPLs isolated from *Leishmania* species (10, 13) and our results on GIPLs from *L. donovani*, demonstrate that the synthetic alkylacylglycerolipids without sugar residues of intact GPIs showed almost similar inhibition (μM range). This clearly indicates that the alkylacylglycerolipid domain of the *Leishmania* GPIs contributes primarily to PKC inhibitory activity. For comparisons to PKC inhibitory activity of synthetic lipids with intact LPG, we isolated LPG from *L. donovani* promastigotes (with average 10 phosphoglycan repeats) and evaluated it for PKC assay, which showed 80% inhibition at 100 μM (data not shown).

**PKC assay in macrophages**

To assess the role of lipids or lipid-containing molecules on enzyme activity in an aqueous system, it is necessary that the molecules are available to the cells by uptake. For
this, the synthetic lipid domains were coated onto RP-18 beads by the published method (15, 25) and then fed to macrophages. The efficiency of the coating procedure was assessed by using 14C-labeled palmitic acid as control, which showed that almost 99% of the material was loaded onto the beads. The labeled beads, when fed to macrophages, were efficiently taken up. In each set of experiments, basal as well as agonist-induced (stimulation with 100 nM PDBu) PKC activity was measured. The controls were devoid of the peptide substrate for measurement of nonspecific activity, which was subtracted from total activity observed in the presence of peptide substrate, the specific activity expressed in pmol of PI transferred from [γ-32P]ATP to substrate per min per 10⁶ cells.

Evaluation of PKC activity and c-fos expression in macrophages

For measurement of both the PKC activity as well as the expression of c-fos in macrophages, synthetic lipids were coated onto RP-18 beads and fed to macrophage (J774A.1) cells. Efficiency of coating of beads and their uptake by macrophages was determined using [14C]palmitic acid as a standard that showed that coating of the beads was 99% and lipid-coated beads were efficiently taken up by macrophages. The reported concentrations of synthetic lipids are the concentrations of the lipid solutions used for coating beads. The controls were incubated with the same amount of uncoated beads that were processed the same way as the experimental materials. The viability of macrophages after treatment with lipid-coated beads was routinely checked microscopically. After incubation for 2 h, the lipid-coated beads were removed and macrophage monolayers washed and further incubated in complete medium for 3 h. Macrophages were then either stimulated with 100 nM PDBu, or not stimulated as for the control, before replacing the medium with assay buffer for PKC assay or cells were harvested for RNA extraction. The synthetic lipids showed a similar pattern of PKC inhibition in macrophages, as shown in the in vitro experiments (data not shown), and an increase in inhibition as the concentration was increased up to 100 μM, beyond which a plateau was observed. Northern blot and densitometric analysis showed that the synthetic lipid 1-O-alkyl-2-O-acetyl-sn-glycerol dramatically inhibited c-fos gene expression by 60% at 100 μM, which remained static at higher concentrations (Fig. 2). A similar inhibition level was observed with alkylacylphosphatidic acid (data not shown). Significantly, the intact LPG, isolated from L. donovani, showed significant inhibition (90%) of PKC activity in macrophages at 100 μM, and almost complete inhibition of c-fos expression at the same concentration. On comparison with intact LPG and GPIPs of L. donovani, the synthetic alkylacylglycerol lipid domains showed significant PKC inhibition (both in vitro as well as in macrophages) and transient c-fos expression.

The question as to how parasitic cell-surface GPIs (LPG and GPIPs) bring about the extremely high levels of inhibition of PKC-dependent c-fos gene expression inside macrophages without even entering the cell, and which structural domain is responsible for this dramatic inhibition, has been a subject of speculation and debate (3, 6). A recent biophysical study (28) proposed that LPG brings about conformational changes in the lipid bilayer of macrophage plasma membrane, and these conformational changes directly or indirectly affect the binding of PKC to the membrane and/or prevent it from folding correctly in an active confor-

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**Fig. 2.** Effect of varying concentrations of alkylacylglycerol on c-fos expression in macrophages: total RNA extracted from macrophages was subjected to c-fos analysis as detailed in Experimental Procedures. Samples in even-numbered lanes are from cells stimulated with 100 nM phorbol dibutyrate. A: β-actin m-RNA, (B) c-fos m-RNA, and (C) densitometric analysis of c-fos signals.
tion. In either case, the outcome would be inhibition of PKC-dependent downstream signaling cascade. Since it is not known as yet whether LPG-GIPLs can flip across the lipid bilayer, another possible mode of action could be through their association with GPI-sphingolipid-cholesterol-rich lipid “rafts” and inhibition of normal signal transduction. An alternative hypothesis is that the GPIs are first hydrolyzed by secretory GPI-specific phospholipase (PI-PLC or PI-PLD types) generating alkylacylglycerolipids, which then can easily flip across the plasma membrane. Due to their structural similarity with diacyl-sn-glycerol (natural physiologic activator), the released alkylacylglycerolipids can compete for the binding pocket of PKC and act as competitive DAG analog inhibitors. The first pointer toward such a possibility is a recent study (29) showing that several proteases of Leishmania play an important role in the secretion of gp63, a major GPI-anchored surface metalloprotease believed to promote parasite attachment and entry into host macrophages, and proteolytic cleavage of gp63 by membrane GPI-PLC contributes to disease pathogenesis. It is likely that these GPI-PLC enzymes also promote the release of lipid anchors of LPG-GIPLs, and such mechanisms would promote a parasite’s entry into and survival thereupon in macrophages. Our results presented in this paper show that alkylacylglycerolipid domain alone is sufficient for observed inhibition of PKC-dependent c-fos gene transcription by Leishmania GPIs. It is also intriguing that minor differences in lipid structure of the GPIs of various parasites manifest in opposite biological activities. For example, GPIs of T. brucei and P. falciparum containing diacyl-glycerolipid structural domain activate (10, 11, 30) macrophage functions (induction of proinflammatory cytokines, and activation of PKC and PTK), whereas Leishmania GPIs containing alkylacyl- or alkyllysoglycerolipid domain severely inhibit (12–16) macrophage functions (PKC and downstream signaling). Another factor complicating the issue further is the microheterogeneity present in the lipid domains, and most of the GPIs isolated from the parasites are heterogeneous, and it is not clear as to what really contributes to the opposite biological activities observed within the same class of molecules from different parasites. We have addressed this by using synthetic and chemically defined glycerolipids corresponding to Leishmania GPIs, which unambiguously demonstrate that signaling inhibitory activities of GPIs reside in their unusual lipid domains. Our ongoing efforts focus on the chemical synthesis of full-length alkylacyl- and diacyl-GPIs of Leishmania and P. falciparum, respectively, to address the issue of the opposite biological activities.

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