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Modulation des radeaux lipidiques et des propriétés de fusion des phagosomes par le lipophosphoglycane du parasite intracellulaire *Leishmania donovani*

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Modulation des radeaux lipidiques et propriétés de fusion des phagosomes par le lipophosphoglycane du parasite intracellulaire *Leishmania donovani*

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Résumé

Un élément clé de notre système immunitaire consiste en la capture rapide des microorganismes envahisseurs dans les phagosomes de macrophages et en leur destruction dans les phagolysosomes. Cependant, de nombreux pathogènes ont développé des stratégies de survie afin d'éviter leur ciblage vers l'environnement toxique des phagolysosomes soit en inhibant la biogenèse de cet organite ou en s'échappant vers le cytoplasme. Le parasite intracellulaire *Leishmania*, qui cause la leishmaniose, une maladie affectant des millions de personnes à travers le monde, utilise une molécule de surface, le lipophosphoglycane (LPG) afin de perturber certaines fonctions des macrophages incluant la biogenèse du phagolysosome.

L'objectif des travaux présentés dans cette thèse est d'étudier par quel processus le LPG modifie les propriétés fonctionnelles des phagosomes. Nos résultats montrent que les promastigotes de *Leishmania donovani* utilisent leur LPG afin de prévenir la formation des radeaux lipidiques de phagosomes ou d'en altérer la structure. Le LPG agit localement sur la membrane du phagosome et son activité dépend du polymère d'unités dissacharidiques répétitives PO4-6Galβ1-4Man présent au coeur de la molécule. Les radeaux lipidiques sont des assemblages latéraux de lipides enrichis en cholestérol et en glycosphingolipides. Ces structures, où s'assemblent certaines protéines spécifiques, délimitent des points focaux de la membrane où s'exercent des fonctions specialisées comme la transduction du signal et la fusion membranaire. Nous avons mis en évidence l'existence de radeaux lipidiques sur le phagosome en utilisant des approches morphologiques et biochimiques démontrant l'association à cet organite de la protéine flotilline-1 et du ganglioside GM1, tous deux des marqueurs reconnus comme faisant partie des radeaux lipidiques. Il existe plusieurs types de radeaux lipidiques sur le phagosome puisque la flotilline-1 et le GM1 ne colocalisent pas aux mêmes points focaux de la membrane phagosomiale. Les radeaux enrichis de flotilline-1 atteignent le phagosome par une source intracellulaire, tandis que ceux enrichies de GM1 proviennent essentiellement de la surface de la cellule. Par une étude protéomique, nous avons identifié plusieurs protéines sur les radeaux lipidiques de

phagosomes suggérant que ces structures sont impliquées dans de nombreuses fonctions, telles la transduction du signal, l'organisation du cytosquelette d'actine, l'acidification du compartiment et le choc oxydatif.

L'analyse protéomique a aussi mis en évidence la présence sur les radeaux lipidiques de plusieurs protéines impliquées dans la régulation de la fusion membranaire. En perturbant ces structures, le parasite inhiberait directement les propriétés de fusion du phagosome et la biogenèse des phagolysosomes, ce qui améliorerait sa survie. Cette hypothèse est confirmée par nos observations démontrant que le LPG inhibe effectivement la fusion phagosome-lysosome permettant aux promastigotes de *Leishmania* de résider dans un phagosome immature n'ayant pas acquis toute la machinerie nécessaire à la destruction des microorganismes. Ainsi, *Leishmania* est le premier agent pathogène dont on démontre que la stratégie de survie lors de l'établissement de l'infection dans les macrophages consiste en l 'altération des radeaux lipidiques du phagosome.

Mots-clés : *Leishmania, lipophosphoglycane, radeaux lipidiques, phagosome, fusion membranaire flotilline, protéomique, immunité.*

Abstract

A key aspect of our innate ability to fight infectious agents relies on the rapid clearance of invading pathogens by macrophages at sites of infections and their killing in phagolysosomes. Unfortunately, several pathogens have evolved strategies to avoid being cought in the toxic environment of phagolysosomes either by inhibiting phagosome maturation or by escaping to the cytoplasm. The intracellular parasite *Leishmania* which causes leishmaniasis, a disease affecting millions of people world-wide, uses a surface molecule, lipophosphoglycan (LPG) in order to disrupt some functions of macrophages including phagolysosome biogenesis.

The objective of the work presented in this thesis is to study the mecanisms through which LPG modifies phagosome functional properties. Our results demonstrate that *Leishmania donovani* promastigotes use their LPG to prevent the formation or disrupt lipid rafts on the phagosome membrane. LPG acts locally on the membrane and its action depends on the disaccharidic repeating units PO4-6Galβ1-4Man polymer located at the heart of the molecule. Lipid rafts are lateral assemblies of lipids enriched in cholesterol, glycosphingolipids. These structures, where several specific proteins assemble, represents focal points on the membrane where specialized functions like signal transduction and membrane fusion take place. We have demonstrated the existence of lipid rafts on phagosomes using morphological and biochemical approaches showing the association of the protein flotillin-1 and the ganglioside GM1 to this compartment, both well known markers of lipid rafts. There are more than one type of lipid rafts on phagosomes, since flotillin-1 and GM1 do not colocalize in the same focal points on the phagosome membrane. Flotillin-1-enriched rafts reach phagosomes from an intracellular source, whereas those enriched in GM1 originate essentially from the cell surface. In a proteomic study, we have identified several proteins resident of phagosome lipid rafts suggesting that these structures play a role in signal transduction, actin cytoskeleton organization, lumen acidification and oxydative burst.

Proteomic analysis has also revealed the presence of several proteins implicated in the regulation of membrane fusion on phagosome lipid rafts. By disturbing these structures, the parasite could directly inhibit phagosome fusion properties and phagolysosome biogenesis, improving its survival. This hypothesis is confirmed by our observations demonstrating that LPG inhibits effectively phagosome-lysosome fusion, thus allowing *Leishmania* promastigotes to reside in immature phagosomes that have not acquired all the molecular machinery essential to the destruction of microorganisms. *Leishmania* is thus the first pathogen shown to rely on disruption of phagosome lipid rafts as a survival strategy at the onset of an infection.

Keywords : *Leishmania, lipophosphoglycan, lipid rafts, membrane fusion, flotillin, proteomics, immunity.*

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Liste des abbréviations

ALF :	*amastigote-like form*
ArgBP2 :	*Arginine binding protein 2*
CAP :	*adenylyl cyclase-associated protein*
cbl:	*Casitas B-lineage lymphoma*
CD:	*cluster of differentiation*
CMH :	Complexe majeur d'histocompatibilité
CR1 :	*Complement receptor 1*
CR3 :	*Complement receptor 3*
CRP :	*C-reactive protein*
DAG :	Diacylglycerol
EEA1 :	*Early Endosomal Antigen* 1
ESA :	*Epidermal surface antigen*
FcεR :	Récepteur au Fc *epsilon*
FRET :	*Fluorescence resonance energy transfer*
GPI :	Glycosyl-phosphatidyl-inositol
GTP:	Guanosine triphosphate
HSP:	*heat shock protein*
Ig :	Immunoglobuline
IL :	Interleukine
INFγ :	Interféron gamma
iNOS:	*inducible nitric oxide synthase*
IP3 :	Inositol triphosphate
kDa :	Kilodalton
LAMP :	*Lysosome-associated membrane protein*
LDL :	*Low density lipoprotein*
LFA	*Leukocyte function-associated antigen*
LPG :	Lipophosphoglycane
LPS :	Lipopolysaccharide

M6PR :	*Mannose-6-phosphate receptor*
MAC:	*Membrane attack complex*
MAP:	*Mitogen activated protein*
MBP :	*Mannan-binding protein*
NADPH:	*Reduced Nicotinamide adenine dinucleotide phosphate*
nm	nanomètre
NSF:	*N-ethylmaleimide sensitive factor*
PAK :	*p21-activated kinase*
PDGF :	*Platelet-derived growth factor*
PE :	Phosphatidyléthanolamine
PG:	Phosphoglycane
PHB	Prohibitine
PI3K :	Phosphatidylinositol 3-kinase
PKC :	Protéine kinase C
Pyk2:	*protein tyrosine kinase 2*
SPFH	Stomatin, Prohibitin, Flotillin, Hflc
SVF :	*Semliki Forest virus*
TCR :	*T-cell receptor*
TFR:	*Transferrin receptor*
TLR:	*Toll like receptor*
T_m :	*Melting temperature* (Température de fusion)
VIH:	Virus d'immunodéficience humaine

À mon épouse Martine, pour son inconditionnel soutien.

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Chapitre I. Introduction et revue de littérature

Le terme phagocytose a été utilisé pour la première fois par Ilya Ilyich Mechnikov en 1883, et sa racine grecque signifie littéralement manger « *Phagein* » des cellules «*kytos* ». La phagocytose désigne aujourd'hui plus généralement l'internalisation de particules vivantes ou inertes de diamètre supérieur à 0,5 μm (Greenberg et Silverstein, 1993). Certaines cellules du système immunitaire, telles que les macrophages se spécialisent dans la phagocytose, ce qui leur permet d'éliminer de manière efficace des débris cellulaires, tels que les globules rouges sénescents ou des particules étrangères telles que des bactéries potentiellement pathogènes. Ces cellules exercent ainsi une fonction d'éboueur de l'organisme et servent de première ligne de défense contre les infections.

Lors de la phagocytose, les particules ingérées se retrouvent dans un compartiment intracellulaire appelé «phagosome » qui constitue un organite spécialisé du macrophage. Une fois formé, le phagosome se transforme rapidement en un compartiment lytique, le phagolysosome, par un processus complexe de maturation qui implique des échanges avec plusieurs autres organites intracellulaires. Dans le phagolysosome, les microorganismes sont rapidement tués et dégradés. Au cours de l'évolution, les pathogènes ont développé un ensemble de mécanismes de survie leur permettant d'échapper à la dégradation dans les phagolysosomes, Ces mécanismes ont été largement décrits dans la littérature (Amer et Swanson, 2002; Knodler et *al.*, 2001, Duclos et Desjardins, 2000; Méresse et *al.*, 1999; Dermine et Desjardins, article 4 en annexe). Dans cette thèse, nous traiterons surtout des stratégies de survie utilisées par le parasite intracellulaire *Leishmania donovani* sous sa forme promastigote, c'est-à-dire, la forme qui initie l'infection chez les mammifères. Ces stratégies impliquent particulièrement une molécule qui recouvre toute la surface du parasite appelée « lipophosphoglycane » (LPG).

Mécanismes de phagocytose

D'un point de vue morphologique, on distingue trois types de phagocytose qui dépendent de la nature de la particule ingérée et des récepteurs présents à la surface de la cellule phagocytaire. D'abord, il y a la phagocytose par *zipper* qui mène à la formation d'un phagosome dont la membrane est apposée de façon très serrée à la particule ingérée. La particule peut aussi s'enfoncer à l'intérieur du phagocyte à la suite d'un signal qui déclenche la déformation de la membrane plasmique créant ainsi une vague qui se referme sur elle-même (phagocytose par *trigger*). Dans certains cas, une extension de la membrane plasmique peut s'enrouler plusieurs fois autour de la particule pour former un phagosome qui a l'apparence d'une spirale au centre de laquelle se trouve la particule ingérée (phagocytose par *coiling*).

La phagocytose par *zipper*

La phagocytose par *zipper* (Silverstein et *al.*, 1977; Swanson et Baer, 1995) est le processus d'internalisation qui a été le plus étudié. Ce type de phagocytose permet l'internalisation d'agents pathogènes préalablement recouverts d'anticorps reconnaissant spécifiquement des épitopes à leur surface. On nomme aussi ce mode d'internalisation, la phagocytose médiée par les récepteurs Fc, la portion Fc étant la partie des anticorps reconnue par ces récepteurs. Cette phagocytose déclenche généralement la production de superoxides, qui peuvent tuer les microorganismes, ainsi que des cytokines proinflammatoires qui stimulent les cellules du système immunitaire. La phagocytose par *zipper* procède par l'avancement de minces extensions de membrane, les pseudopodes, qui recouvrent progressivement la particule à fur et à mesure que les portions Fc des anticorps à sa surface se lient aux récepteurs Fc à la membrane cellulaire du phagocyte (Griffin et *al.*, 1975). La formation et l'avancement des pseudopodes requiert également l'assemblage et le désassemblage du cytosquelette d'actine au site d'internalisation (Sheterline et *al.*, 1984;

Greenberg et *al.*, 1991). Cette réorganisation du cytosquelette est contrôlé par différents membres de la famille des GTPases Rho (Caron et Hall, 1998). Une fois que les pseudopodes ont complètement englobé la particule, ils se rejoignent et fusionnent pour fermer la membrane du phagosome. La fusion des pseudopodes formant le phagosome naissant se produit sous l'action de la PI3K, de certaines myosines et de la GTPase ARF6 (Araki et *al.*, 1996; Zhang et *al.*, 1998; Swanson et *al.*, 1999). Après la fusion, la particule se trouve alors comprise dans un compartiment intracytoplasmique dont la membrane est juxtaposée à sa surface. Ce mécanisme de phagocytose est induit localement et ne permet pas l'internalisation de particules adjacentes présentes à la surface du phagocyte (Griffin et Silverstein, 1974). La transduction du signal requise au cours de l'internalisation est donc limitée au site de contact entre la particule et la membrane cellulaire. Cette région de la membrane pourrait contenir des « radeaux lipidiques ». Le concept des radeaux lipidiques sera décrit plus loin (Gatfield et Pieters, 2000).

La transduction du signal requise au cours de la phagocytose par *zipper* nécessite la tyrosine kinase Syk (Matsuda et *al.*, 1996; Kiefer et *al.*, 1998). L'activation de Syk déclenche une cascade de signalisation qui comprend l'activation de la PI3K, de GTPases de la famille de Rho et de la kinase PAK, dont l'issue est la nucléation de l'actine (revue dans Takenawa et Miki, 2001). Syk active aussi la phospholipase C qui génère de l'IP3 et du DAG. La mobilisation du calcium intracellualire engendrée par l'IP3 n'est pas nécessaire à la phagocytose. Par contre, le DAG permet l'activation de plusieurs isoformes de la protéine kinase C (PKC) comme les isoformes δ et ε (Larsen et *al.*, 2000) nécessaires à la phagocytose. L e DAG active aussi la PKCα qui relie la phagocytose au processus inflammatoire c omme l a f lambée o xydative e t l a g énération d e m édiateurs l ipidiques d e l'inflammation. Dans ce contexte, le ciblage des différentes isoformes de la PKC pourrait fournir un avantage de survie important aux parasites intracellulaires. Cette stratégie est utilisée par *Leishmania* (voir plus loin).

La phagocytose sans fermeture éclair

Les particules qui ne se lient pas aux récepteurs Fc sont généralement phagocytées par un mécanisme différent du *zipper*. Dans la phagocytose du type *trigger*, très peu de pseudopodes sont formés et les particules semblent être internalisées de façon passive (Kaplan, 1977). Ce mécanisme de phagocytose débute lorsque la membrane plasmique se déforme et produit une ondulation qui recouvre la particule. Dans ces phagosomes, la membrane n'est habituellement pas accolée de manière très serrée à la particule. Ce type de phagocytose ne déclenche généralement pas la production de dérivés toxiques de l'oxygène ou de réponse inflammatoire capable de détruire efficacement les microorganismes. Les récepteurs pouvant contribuer à ce processus d'internalisation incluent le récepteur du complément, certaines intégrines, des lectines, les récepteurs de type *Toll-like* et les récepteurs de type éboueur (*scavenger*). L'activation de ces différents récepteurs dépend des molécules présentes à la surface des particules à ingérer. L'internalisation des leishmanies se produit souvent par les récepteurs du complément CR1 et CR3, une voie qui se caractérise par l'absence de réponse inflammatoire potentiellement néfaste pour le parasite (voir plus loin) (Da Silva et *al.*, 1989).

Des observations morphologiques de phagocytose de pathogènes ont permis de définir un troisième type de phagocytose, notamment la phagocytose par *coiling*. Ce type de phagocytose se caractérise par le surenroulement d'un pseudopode autour de la particule, créant ainsi un compartiment formé par une multitude de couches membranaires superposées les unes sur les autres. Parmi les microorganismes qui pénètrent les macrophages par ce processus, *Legionella* est le premier à avoir été identifié (Horwitz, 1984). Depuis, plusieurs études ont fait état de phagocytose par enroulement de membrane chez d'autres pathogènes, tels que les trypanosomatides comprenant *Leishmania*, certains spirochètes, *Burkholderia pseudomallei*, *Borrelia burgdorferi*, *Neisseria meningitidis* et *Treponema pallidum* (Rittig et *al.*, 1998a et b ; Inglis et *al.*, 2000 ; Rittig et *al.*, 1992; Kalmusova et *al.*, 2000 ; Bouis et *al.*, 2001). L'importance de ce type de phagocytose est

encore débattue puisqu'il n'est pas certain qu'il constitue un mécanisme d'internalisation distinct du *zipper* ou du *trigger*. Il pourrait plutôt s'agir d'une variante du mécanisme du *zipper* dans laquelle il y aurait absence de fusion entre les deux pseudopodes qui englobent le microorganisme (Rittig et *al.* 1998b). En absence de fusion, un des pseudopodes glisserait au-dessous de l'autre et continuerait son enroulement. Les mécanismes moléculaires qui permettent ce surenroulement et son effet sur la survie du pathogène dans les phagosomes sont encore peu connus.

Biogenèse des phagolysosomes

Lors de la phagocytose, la cellule doit fournir suffisamment de membrane pour englober complètement la particule phagocytée. Il a longtemps été supposé que la membrane plasmique du phagocyte fournissait toute la membrane nécesaire à la formation d'un phagosome. Toutefois, une étude de notre laboratoire a récemment permis de montrer que le réticulum endoplasmique fournit un apport important en membrane au phagosome au cours de sa formation, ce qui minimiserait l'utilisation de la membrane plasmique (Gagnon et *al.*, 2002). La découverte de cet apport membranaire procure une explication aux observations montrant que des macrophages peuvent internaliser des particules plus grandes qu'eux, sans affecter la fonction ni la viabilité cellulaire (Cannon et Swanson, 1992). Immédiatement après l'internalisation des particules, la membrane des phagosomes est donc formée d'un amalgame de membrane plasmique et de membrane de réticulum endoplasmique d'une composition complexe. Cependant, ces phagosomes nouvellement formés sont immatures et ne possèdent pas les propriétés nécessaires pour tuer et dégrader les microorganismes. Ils doivent donc se transformer afin d'acquérir la machinerie essentielle à ces fonctions. Cette machinerie comprend des enzymes dégradatives et d'autres composés toxiques capables d'attaquer les microorganismes. Ces substances s'accumulent au cours d'un processus de maturation des phagosomes aussi appelé biogenèse des phagolysosomes. Ces derniers forment les compartiments terminaux de la phagocytose responsables de la dégradation du contenu phagosomial. On a longtemps cru

que la formation du phagolysosome se faisait en un seul événement de fusion entre un phagosome et un lysosome, c'est-à-dire, le compartiment le plus tardif de la voie endocytaire riche en hydrolases (Rabinowitz et *al.*, 1992). Toutefois, on reconnaît aujourd'hui que ce processus implique non seulement les lysosomes, mais également de nombreuses interactions entre les phagosomes et divers compartiments intracellulaires des voies endocytaire et biosynthétique (Mayorga et *al.*, 1991 ; Desjardins et *al.*, 1994). La fusion des phagosomes avec des compartiments de la voie endocytaire nécessite le mouvement des organites le long de microtubules et du réseau d'actine, la reconnaissance des organites par des molécules d'appariement, la fusion membranaire et le recyclage des protéines (Desjardins, 1995; Blocker et *al.*, 1997; Blocker et *al.*, 1998). Les mécanismes moléculaires de tous ces processus ont été décrits dans différentes revues (Harrison et Grinstein, 2002 ; Stamnes, 2002).

Lors de la maturation du phagosome, plusieurs de ses caractéristques biochimiques sont modifiées, plus particulièrement sa composition protéique et son pH (Mayorga et *al.*, 1991; Pitt et *al.*, 1992; Desjardins et *al.*, 1994). Des études cinétiques, où les phagosomes sont analysés à différents temps après leur formation, ont montré que certaines protéines de la membrane plasmique recrutées au cours de la phagocytose, telles que les récepteurs Fc et à mannose ainsi que des adaptines, disparaissent graduellement de la membrane phagosomiale. Ces études montrent également que peu de temps après leur formation, les phagosomes interagissent avec des organites de l'appareil endocytaire, ce qui leur permettent d'acquérir des marqueurs des endosomes précoces, tels que rab5, EEA1 et le récepteur à la transferrine. Par la suite, grâce à des interactions avec les endosomes tardifs et les lysosomes, les phagosomes perdent rapidement les marqueurs précoces au profit de marqueurs des endosomes tardifs et des lysosomes, tels que rab7, le récepteur à mannose-6-phosphate (M6PR), LAMP1 et 2, la cathepsine D et la ß-glucoronidase (Desjardins et *al.*, 1994 a et b; Scianimanico et *al.*, article 5 en annexe). La maturation du phagosome en phagolysosome s'accompagne également d'une acidification de la lumière, qui fait suite à l'acquisition de la pompe à proton v-ATPase.

Tel que mentionné plus haut, le remodellage moléculaire au cours de la biogenèse du phagolysosome nécessite de nombreuses interactions entre les phagosomes et l'appareil endocytaire. Un modèle pour expliquer ces multiples interactions, le « *Kiss and Run* », a été proposé par Michel Desjardins (1995). Selon ce modèle, les compartiments endosomiaux et phagosomiaux qui se meuvent le long de microtubules se rencontrent et fusionnent entre eux. Lors de cette rencontre, un pore de fusion transitoire se forme et permet l'échange d'une partie du contenu luminal des deux compartiments. Des protéines membranaires peuvent aussi être échangées. Ainsi, la composition biochimique de ces compartiments se modifie graduellement. Par la suite, le pore se referme par un processus de fission, ce qui permet la séparation des compartiments. Un tel processus permet aux phagosomes d'acquérir, par exemple, des hydrolases présentes dans les endosomes tardifs et les lysosomes, sans pour autant que les contenus de ces différentes vésicules se mélangent complètement. Des résultats récents de notre laboratoire ont montré que la petite GTPase Rab5 joue un rôle dans la régulation des événement s de fusion du type *Kiss and Run* (Duclos et *al.*, 2000). Le *Kiss and Run* serait aussi un mécanisme d'interaction utilisé entre les différents compartiments de la voie endocytaire (Duclos et *al.*, 2003). Des événements de fusion transitoires ont aussi été décrits au cours de l'exocytose de neurotransmetteurs dans les neurones, ce qui démontre que le *Kiss and Run* est un mécanisme important d'interactions entre différentes sortes d'organites intracellulaires (Alvarez de Toledo et *al.*, 1993; Klyachko et *al.*, 2002).

Une étude a montré que la fusion entre les phagosomes et les compartiments endocytaires requiert de l'ATP et des protéines associées aux membranes (Mayorga et *al.*, 1991). La machinerie de fusion composée des NSF (facteur sensible au N-éthyl-maléimide), SNAP (protéine soluble d'attachement au NSF) et SNAREs (récepteur du SNAP) (Rothman et Orci, 1992; Söllner et *al.*, 1993; Rothman et Warren, 1994) joue aussi un rôle dans la régulation des événements de fusion, puisque le NEM (N-éthyl-maléimide), une molécule inactivant la NSF peut inhiber la fusion entre les phagosomes et les endosomes. Certaines données suggèrent également que plusieurs autres molécules régulatrices de la fusion présentes chez les endosomes, telles que des petites GTPases

comprenant rab5 et rab7, jouent un rôle dans la fusion phagosome-endosome (Beron et *al.*, 1995). Rab5 participe aux événements de fusion précoces, tandis que rab7 participe aux événements de fusion tardifs (Chavrier et *al.* 1990; Gorvel et *al.* 1991; Méresse et *al.* 1995; Feng et *al.* 1995 ; Duclos et *al.*, 2000).

Autres fonctions du phagosome

L'analyse protéomique du phagosome a permis d'identifier près de 600 protéines associées à ce compartiment (Garin et *al.*, 2001 en annexe; Brunet et *al.*, 2003). La nature de ces protéines a permis de mettre en évidence certaines fonctions insoupçonnées du phagosome. Par exemple, plusieurs protéines du réticulum endoplasmique ont été retrouvées sur le phagosome, suggérant que ce compartiment joue un rôle actif dans la phagocytose (Gagnon et *al.*, 2002). En utilisant des approches morphologique et biochimique, il a été démontré qu'au cours de la phagocytose, le réticulum endoplasmique est recruté au niveau de la membrane plasmique sous la particule avec laquelle il fusionne. Tel que mentionné plus haut, le processus permet de former un phagosome composé en grande partie de la membrane du réticulum. Le réticulum endoplasmique pourrait servir de réservoir de membrane afin que le macrophage n'épuise pas sa membrane plasmique lors d'internalisation de grosses ou de nombreuses particules. De plus, trois groupes indépendants ont démontré que l'utilisation du réticulum endoplasmique pour former le phagosome permettrait à ce compartiment de jouer un rôle clé dans la présentation des antigènes exogènes à partir des molécules du CMH de classe I (Houde et *al.*, 2003; Guermonprez et *al.*, 2003; Ackerman et *al.*, 2003), un processus important de la réponse immunitaire contre un ensemble de pathogènes. Finalement, nous avons aussi identifié sur le phagosome plusieurs protéines reconnues comme étant présentes dans les radeaux lipidiques de la membrane plasmique, telles que la flotilline-1 et la stomatine. La présence de ces protéines nous a permis d'émettre l'hypothèse selon laquelle de telles structures sont aussi présentes sur le phagosome. Les radeaux lipidiques et la protéine flotilline-1 qui ont

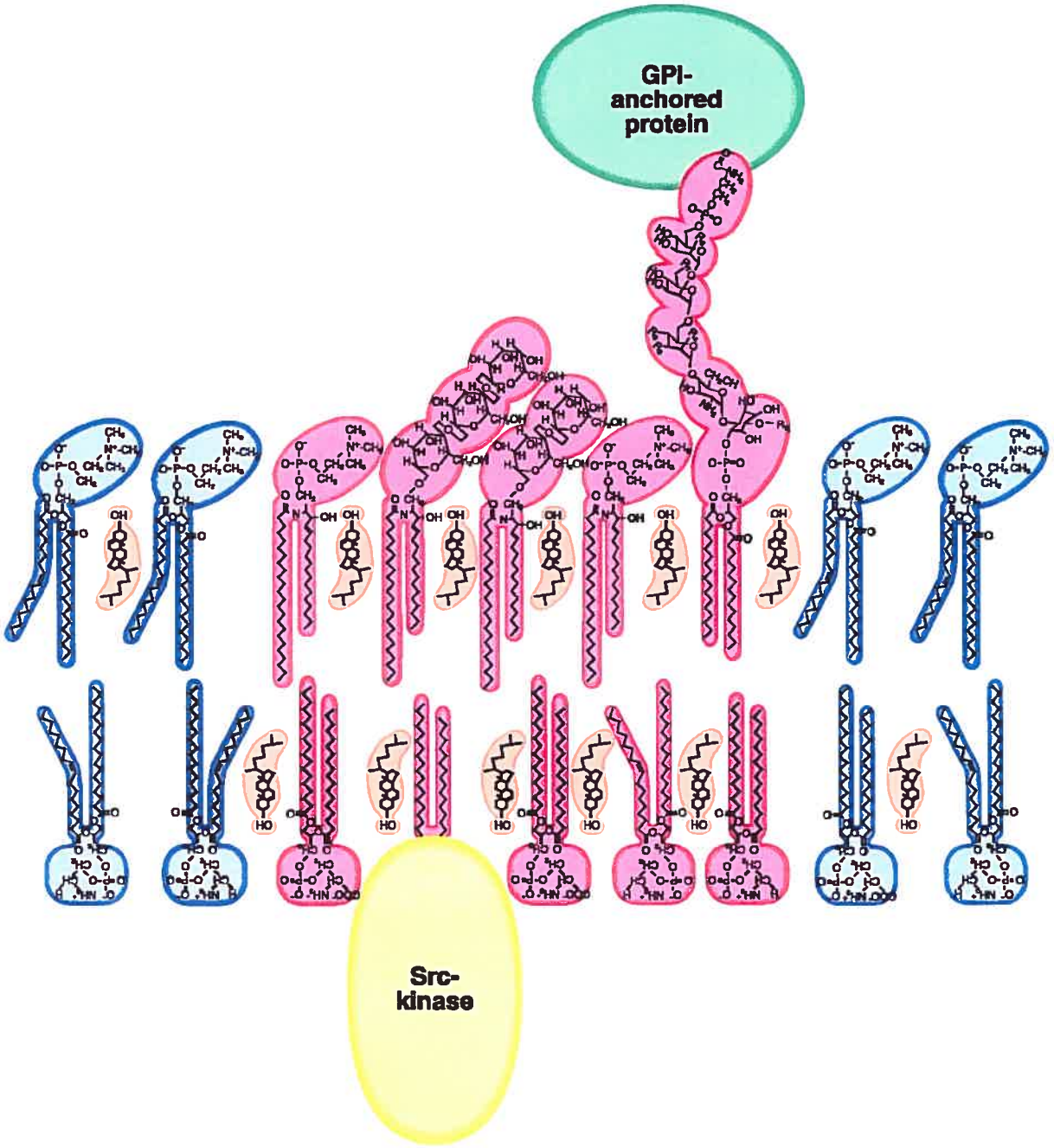
fait l'objet d'une bonne partie des travaux de cette thèse seront décrits plus en détails dans les prochaines sections.

Les radeaux lipidiques

Il y a plus de 30 ans, Singers et Nicolson (1972) proposaient le modèle de la « mosaïque fluide » selon lequel les protéines membranaires peuvent se mouvoir librement dans la bicouche lipidique formant les membranes biologiques. Grâce à de nouvelles données sur la dynamique des membranes, ce modèle a récemment été revu. En effet, certains lipides ne se meuvent pas librement, mais s'assemblent latéralement dans les membranes pour former des entités compactes ou des plates-formes que l'on a baptisées « radeaux lipidiques » par analogie à des radeaux de lipides solides qui flottent sur une mer lipidique fluide (figure 1). Les radeaux sont aussi connus sous le nom de microdomaines lipidiques. Ces plates-formes sont principalement composées de cholestérol, de sphingolipides et de glycosphingolipides et flottent du côté exoplasmique de la bicouche lipidique dans une mer de glycérophospholipides composée surtout de phosphatidylcholine (Brown et London, 1998). Bien que les radeaux soient présents du côté exoplasmique, il est possible que ces structures rassemblent des molécules de transduction du signal du côté cytoplasmique de la membrane en des domaines riches en cholestérol, ce qui ferait des radeaux des entités bilamellaires. La façon dont s'effectue l'organisation du feuillet cytoplasmique pourrait impliquer l'interdigitation des longues chaînes acyles des sphingolipides du feuillet exoplasmique avec les chaînes acyles plus courtes des glycérolipides du feuillet cytoplasmique (Simons et Ikonen, 1997; Simons et Toomre, 2000). Malgré les nombreuses études sur les radeaux lipidiques (plusieurs centaines sur PUBMED), la structure des radeaux lipidiques cytoplasmiques est encore très mal connue (Brown et London, 2000).

Figure 1 Structure fondamentale d'un radeau lipidique

Les radeaux sont enrichis en lipides à chaînes saturées et en cholestérol et incluent des protéines à ancre GPI ainsi que des protéines de transduction du signal.



D'après Simons et Ikonen. *Science* 290, 1721-1726 (2000).

Les caractéristiques physiques qui donnent aux lipides présents dans les radeaux lipidiques une forte tendance à s'associer pour former une structure compacte diffèrent de celles des glycérophospholipides présents en dehors des radeaux. D'abord, la température de fusion (T_m) des sphingolipides, c'est-à-dire, la température à laquelle un lipide passe de l'état solide à état liquide, est plus élevée que celle des glycérophospholipides. Cette différence de T_m est basée sur la longueur des chaînes acyles des acides gras et de leur degré de saturation. Par exemple, les chaînes acyles des sphingolipides sont généralement plus longues et plus saturées que les chaînes acyles des glycérophospholipides qui contiennent souvent des doubles liaisons carbone-carbone (non saturées). Ces doubles liaisons rendent la chaîne acyle moins linéaire, ce qui favorise la mobilité sur le plan de la membrane et diminue leur tendance à former des structures compactes. À la température physiologique, les longues chaînes acyles saturées des sphingolipides ont tendance à se compacter, mais sans se gélifier, formant ainsi une phase liquide ordonnée intermédiaire entre la phase gel (solide) et la phase liquide cristalline (fluide) dans laquelle se trouvent la majorité des glycérophospholipides. Dans la phase liquide ordonnée, les lipides, bien que compacts, gardent leur mobilité latérale et rotationnelle (Brown, 1998). On croit que l'action du cholestérol est essentielle à l'existence de la phase liquide ordonnée (Ahmed et *al.*, 1997 ; Silvius et *al.*, 1996). Le groupe de tête des différents lipides pourrait également influencer la tendance de certains lipides à former des entités compactes. Par exemple, le groupement de tête du phosphatidyléthanolamine (PE) est plus petit que le groupement de tête de la phosphatidylcholine, ce qui augmente sa T_m et diminue sa fluidité. Cette caractéristique pourrait être importante à la formation de radeaux dans le feuillet cytoplasmique pauvre en sphingolipide, mais riche en PE.

Les radeaux lipidiques peuvent être étudiés biochimiquement en prenant avantage de certaines caractéristiques physiques qui permettent de les isoler. En effet, ces structures résistent à la solubilisation au froid dans les détergents non ioniques, comme le Triton X-100, et forment des complexes lipidiques de faible densité qui ont la propriété de flotter lorsque soumis à une centrifugation sur gradient de densité (Simons et Ikonen, 1997). Quant aux autres lipides présents en dehors des radeaux, ils se solubilisent en micelles sous

l'action des détergents et demeurent au fond du gradient lors de la centrifugation (Brown et Rose, 1992). À cause de ces caractéristiques physiques, plusieurs noms ont été assignés aux radeaux lipidiques, tels que DRMs (*detergent-resistant membranes*), DIGs (*detergent-insoluble glycolipid-enriched membranes*), GEMs (*glycolipid-enriched membranes*) ou bien TIFF (*Triton-insoluble floating fraction*). Toutes ces appellations correspondent à la définition biochimique des radeaux. Aujourd'hui, on dénomme « radeaux lipidiques », les structures in vivo non isolées, alors que les autres appellations sont utilisées pour désigner les radeaux isolés par extraction aux détergents et flottaison sur gradient de densité.

On a longtemps douté de l'existence *in vivo* des radeaux à cause de leur petite taille et de leur dynamique de formation qui les rend difficile à observer directement par microscopie optique (Jacobson et Dietrich, 1999). Toutefois, plusieurs études récentes ont démontré leur existence *in vivo*. Par exemple, une étude a permis de visualiser ces structures après avoir induit leur coalescence à l'aide d'anticorps dirigés contre des protéines présentes dans les radeaux, formant ainsi des « superstructures » visibles en microscopie optique (Harder et *al.*, 1998). Une autre étude employant la technique du transfert d'énergie par résonance (FRET) a permis de montrer que certaines protéines à ancres GPI, reconnnues comme étant présentes dans des domaines insolubles aux détergents, ne sont pas distribuées aléatoirement dans les membranes, mais sont confinées dans des petites régions évaluées à 70 nm de diamètre (Varma et Mayor, 1998). La taille des radeaux dans les cellules vivantes demeurent un sujet de controverse. Des données indiquent que leur taille peut varier entre 70 et 100 nm (Anderson et Jacobson, 2002). Cependant, des mesures obtenues plus récemment par résonnance électronique paramagnétique (EPR) pulsé font état d'une taille d'environ 25 nm (Subczynski et Kusumi, 2003). La différence entre ces résultats peut être expliquée par l'utilisation de différentes techniques ou par la nature dynamique des radeaux lipidiques.

L'isolation biochimique des radeaux lipidiques a permis de montrer que de nombreuses protéines s'y associent préférentiellement alors que d'autres en sont exclues. Parmi celles-là, on retrouve les protéines à ancre GPI qui constituent un groupe hétérogène

de protéines ancrées à la membrane par leur groupement GPI. Ce sont les deux chaînes acyles de la molécule, généralement longues et saturées (au delà de 16 carbones) qui permettent l'ancrage et l'affinité de ces protéines aux radeaux lipidiques (Chatterjee et Mayor, 2001). Dans les radeaux, il y a aussi de nombreuses protéines de signalisation cellulaire, notamment les tyrosines kinases de la famille des Src et les protéines G trimériques qui s'associent aux radeaux par palmitoylation, myristoylation ou par prenylation (Ma et Huang, 2002). Dans les radeaux, on retrouve aussi des protéines pouvant lier le cholestérol comme la cavéoline. La cavéoline-1 est une protéine enrichie dans les cavéoles qui sont de petites invaginations de la membrane plasmique jouant un rôle dans l'endocytose (Brown et Rose, 1992; Kurzchalia et Parton, 1999). On a d'abord pensé que les radeaux lipidiques se limitaient aux cavéoles, puisqu'on y retrouvait toujours la cavéoline-1 après extraction aux détergents. Cependant, des radeaux lipidiques sans cavéoline ont été identifiés chez certains types cellulaires où il n'y a pas de cavéoles comprenant les érythrocytes, les plaquettes et les lymphocytes (Fra et *al.*, 1994). Pour cette raison, on distingue aujourd'hui deux types de radeaux lipidiques soit, ceux qui ne contiennent pas de cavéoline et forment des plates-formes, et les cavéoles, qu'on peut considérer comme des radeaux invaginés sous l'action de la cavéoline-1 (Fra et *al.*, 1995). Les radeaux lipidiques contiennent aussi des protéines de la famille des flotillines qui comporte deux membres, soit la flotilline-1 et la flotilline-2 (Bickel et *al.*, 1997). Ces protéines feront l'objet d'une discussion plus détaillée plus loin.

Rôles des radeaux lipidiques

Étant donné que les radeaux lipidiques concentrent certaines protéines, ces entités permettent à des fonctions spécialisées de se produire en des points focaux de la membrane. Parmi ces fonctions, on retrouve la transduction du signal, le transport membranaire, le ciblage des protéines, la régulation des interactions hôte-pathogène et possiblement la fusion membranaire.

Les radeaux et la transduction du signal

Un très grand nombre d'indications montrant l'implication des radeaux dans la transduction du signal proviennent d'études sur des cellules d'origine hématopoïétique. La signalisation par le récepteur FcεRI des mastocytes constitue un des modèles de signalisation médiée par les radeaux (Holowka et Baird, 2001). Normalement, les récepteurs FcεRI non liés à des anticorps sont présents dans des parties fluides solubles aux détergents de la membrane plasmique, c'est-à-dire, hors des radeaux. Cependant, lorsqu'ils s'associent à leur ligand, des anticorps du type IgE, les FcεRI s'agrègent dans les radeaux et deviennent alors insolubles aux détergents (Field et *al.*, 1997). Une fois agrégés, les récepteurs sont phosphorylés par la kinase Lyn, ce qui déclenche la transduction du signal. Seuls les récepteurs présents dans les radeaux peuvent servir de substrat à Lyn. Les récepteurs des cellules T (TCR) sont un autre bon exemple de transduction de signal médiée par les radeaux (Werlen et Palmer, 2002). En effet, il a été montré que lorsque le TCR est activé par la liaison au CMH de classe II d'une cellule présentatrice d'antigène, il se forme un complexe supramoléculaire composé de radeaux lipidiques. Il a été démontré que lors d'une telle liaison, l'intégrité de ces structures est essentielle à la transduction du signal (Xavier et *al.*, 1998). Du côté des cellules présentatrices d'antigènes, on a noté que les CMH de classe II se concentrent également dans les radeaux lipidiques afin de faciliter la présentation antigénique aux cellules T (Anderson et *al.*, 2000). Il existe d'autres voies de signalisation activées par les radeaux lipidiques. Pour n'en nommer que quelques-unes, il y a la signalisation induite par le récepteur des cellules B, la voie de signalisation médiée par l'insuline et la signalisation par certaines cytokines (revue dans Cheng et *al.*, 2001; Bickel, 2002; Manes et *al.*, 2001). À part des récepteurs membranaires, on retrouve aussi dans les radeaux lipidiques, des molécules intracytoplasmiques importantes à la transduction du signal, telles que certaines isoformes de la PKC (Khoshnan et *al.*, 2000; Becart et *al.*, 2003).

Les radeaux et le transport membranaire

Les radeaux lipidiques sont aussi impliqués dans le transport membranaire (Ikonen, 2001), puisque la destruction de ces plates-formes à l'aide de drogues qui déplètent le cholestérol bloque la formation de vésicules de sécrétion des voies constitutive et régulée chez certains types de neurones (Wang et *al.*, 2000). Ces drogues, telles que la cyclodextrine ou la nystatine, abolissent aussi les voies d'endocytose qui nécessitent la fonction des cavéoles et des vésicules recouvertes de clathrine (Anderson et *al.*, 1996; Rodal et *al.*, 1999). Toutefois, puisqu'il existe du cholestérol hors des radeaux, il est possible que l'inhibition de l'endocytose produite lors de la déplétion de ce lipide ne soit pas due à la destruction des radeaux lipidiques, mais à une action étendue à toute la membrane plasmique. Au-delà de la membrane plasmique, les radeaux se retrouvent à certains endroits le long de la voie endocytaire, notamment dans les endosomes précoces et de recyclage (Mayor et *al.*, 1998; Gagescu et *al.*, 2000). Par contre, ils ne sont pas abondants dans les endosomes tardifs et les lysosomes sauf dans le cas de désordres génétiques où le cholestérol n'est pas recyclé vers la membrane plasmique, mais s'accumule plutôt dans les endosomes tardifs (Lusa et *al.*, 2001). Cependant, une étude récente montre qu'il y a des radeaux lipidiques dans les endosomes tardifs chez certaines cellules épithéliales (Fivaz et *al.*, 2002). La présence des radeaux dans différents types d'endosomes portent à croire que ces structures jouent un rôle dans le recyclage du cholestérol et de certaines molécules ayant une forte affinité pour les radeaux dans la voie endocytaire. C'est peut-être le cas pour les protéines à ancre GPI (Mayor et *al.*, 1998; Chatterjee et *al.*, 2001). Par contre, le rôle des radeaux chez les endosomes tardifs reste à élucider. Les radeaux pourraient également jouer un rôle dans le trafic entre les endosomes et l'appareil de Golgi, puisque des pertubations dans la concentration du cholestérol cellulaire affectent le transport de certaines molécules des endosomes vers le Golgi (Grimmer et *al.*, 2000; Shogomori et Futerman, 2001).

Les radeaux et l'aiguillage moléculaire

Le triage moléculaire est une fonction cellulaire qui permet de maintenir des zones dans la cellule ayant une composition biochimique distincte et où se produisent des fonctions spécialisées. Par exemple, cette fonction permet aux cellules épithéliales de maintenir une polarité cellulaire qui divise la membrane plasmique en deux domaines distincts soit, le côté basolatéral et le côté apical. Étant donné que la membrane plasmique du pôle apical contient une forte densité de lipides ayant une grande affinité pour les radeaux lipidiques, contrairement au pôle basolatéral, Simons et Van Meer ont proposé un rôle pour les radeaux dans le triage moléculaire vers le côté apical. Le fait que de nombreuses protéines à ancre GPI présentes dans les radeaux soient dirigées vers le côté apical soutient cette hypothèse. En fait, les protéines à ancre GPI sont si abondantes du côté apical, que l'ancre GPI a été proposée comme motif de triage de ces protéines vers le pôle apical (Brown et Rose, 1992). Toutefois, plusieurs données expérimentales vont à l'encontre de cette hypothèse. D'abord, la sphingomyéline, un lipide des radeaux, est dirigée préférentiellement du côté basolatéral (Simons et van Meer, 1988). De plus, plusieurs protéines associées aux radeaux sont présentes dans la membrane du côté basolatéral (Melkonian et *al.*, 1995). Finalement, une étude a montré que le GPI, bien que nécessaire à l'association aux radeaux, ne suffit pas à permettre la localisation apicale des protéines à ancre GPI (Lipardi et *al.*, 2000). Le motif d'aiguillage apical consisterait plutôt en des N-glycanes (Benting et *al.*, 1999). De toute évidence, d'autres études seront nécessaires afin de déterminer le rôle exact des radeaux dans l'aiguillage apical ou basolatéral des protéines dans les cellules polarisées. En ce qui concerne les cellules d'origine hématopoïétique, dont font partie les macrophages, il n'existe pas de polarisation apicale-basolatérale comme dans les cellules épithéliales. Néanmoins, on a récemment proposé qu'un autre type de domaine polaire formé lors de la cytokinèse pourrait exister dans ce type de cellules (Rajendran et *al.*, 2003). Le rôle des radeaux dans un tel contexte reste à définir.

En plus du triage apical et basolatéral, il est possible que les radeaux jouent un rôle dans le triage des protéines résidentes de l'appareil de Golgi et de celles qui continuent leur cheminement dans la voie de sécrétion. Cette théorie s'appuie sur le fait qu'il existe un gradient de cholestérol à travers les citernes du Golgi qui augmente du côté *cis* vers le côté *trans* (Coxey et *al.*, 1993). Cette augmentation du cholestérol a pour effet d'épaissir la membrane. Or, les protéines transmembranaires de la membrane plasmique ont généralement des domaines transmembranaires plus longs que les protéines transmembranaires résidentes du Golgi. Cette propriété permettrait aux protéines destinées à la surface de se concentrer dans les domaines membranaires plus épais riches en cholestérol en vue de leur transport vers la membrane plasmique. Des résultats indiquant que l'on peut modifier la localisation d'une protéine en changeant la longueur de son domaine transmembranaire appuient cette hypothèse (Munro, 1995).

Les radeaux et la fusion membranaire

De nombreuses données prédisent que les radeaux jouent un rôle important dans la fusion membranaire. D'abord, d'un point de vue biophysique, l'eau est exclue des microdomaines en phase liquide ordonnée, puisqu'il existe peu de donneurs ou d'accepteurs de ponts hydrogène pour les molécules d'eau dans les lipides de radeaux. Cette rareté en ponts d'hydrogène disponibles provient du fait que les lipides de radeaux ont déjà construit entre eux un réseau de liaisons par ponts d'hydrogène presque saturé. L'exclusion de l'eau limitrophe aux radeaux lipidiques permettrait à deux membranes de s'accoler de façon serrée, ce qui favoriserait leur fusion (Tatulian et Tamm, 2000). Sur le plan de la composition protéique des radeaux, de nombreuses molécules impliquées dans la régulation des événements de fusion membranaire, telles que les SNARES ont été retrouvées dans ces microdomaines lipidiques (Lang et *al.*, 2001). De plus, plusieurs molécules virales, comme la protéine de fusion du SFV (Ahn et *al.*, 2002), la glycoprotéine du virus Ebola (Saez-Cirion et *al.*, 2003), l'hémagglutinine du virus de l'Influenza (Takeda et *al.*, 2003) et la gp41 du VIH (Nguyen et Hildreth, 2000), qui servent au bourgeonnement

ou à l'entrée des virus dans les cellules, deux processus nécessitant des évènements de fusion membranaire, se retrouvent enrichies dans les radeaux ou interagissent avec eux. Des sous-unités de la pompe à proton sont également présentes dans les radeaux lipidiques (article 2, Bagnat et *al.*, 2001). Or, il a récemment été proposé que le secteur V0 de la pompe à proton puisse servir de site d'appariement en trans entre deux membranes s'apprêtant à fusionner (Peters et *al.*, 2001). Finalement, un modèle du pore de fusion prédit que les pores lipidiques lors d'événements de fusion membranaire peuvent s'expandre de manière irréversible ou se refermer en fonction de changements lipidiques produits localement (Nanavati et *al.*, 1992). En ayant une composition lipidique différente du reste de la membrane, les radeaux pourraient fournir les changements membranaires nécessaires à cette modulation de l'activité du pore de fusion.

Les radeaux et les interactions hôte-pathogène

Les radeaux lipidiques sont présents dans les cellules hématopoïétiques, ce qui les rend essentiels au bon fonctionnement du système immunitaire. Pour cette raison, l'exploitation de ces structures ou la perturbation de leurs fonctions pourrait grandement favoriser la survie de microorganismes pathogènes et leur permettre d'échapper à la surveillance immunitaire. En accord avec cette idée, plusieurs études récentes démontrent un lien entre les pathogènes et les radeaux lipidiques. D'abord, les radeaux servent de portes d'entrée à plusieurs bactéries, incluant certaines souches d'*Escherichia Coli*, (Selvarangan et *al.*, 2000), *Shigella Flexneri* (Lafont et *al.*, 2002), *Brucella* (Naroeni et Porte, 2002), *Chlamydia* (Stuart et *al.*, 2003; Jutras et *al.*, 2003) et *Mycobacterium* (Gatfield et Pieters, 2000). Tel que mentionné plus haut, ils servent aussi de portes d'entrée ou de site de bourgeonnement à plusieurs virus tels le VIH (Manes et al., 2000 ; Nguyen et Hildreth, 2000), le virus ebola (Bavari et al., 2002), le virus de la rougeole (Manie et *al.*, 2000) et le virus de l'Influenza (Scheiffele et *al.*, 1999) pour n'en nommer que quelques-uns. Les radeaux servent aussi de récepteurs à différentes toxines bactériennes, notamment à la listeriolysine O de *Listeria monocytogenes* (Coconnier et *al.*, 2000), à l'aérolysine

d'Aeromonas hydrophilia (Abrami et van Der Goot, 1999) et à la toxine du choléra (Parton, 1994). Finalement, les parasites intracellulaires protozoaires *Toxoplasma Gondii* et *Plasmodium falciparum* concentrent des composants des radeaux lipidiques à la surface de la vacuole qui les abrite (Lauer et *al.*, 2000 ; Mordue et *al.*, 1999). Les raisons pour lesquelles ces parasites incluent des radeaux lipidiques de la cellule hôte dans la membrane vacuolaire demeurent obscures. Il est possible que ces parasites se servent de ce système afin d'exclure du phagosome des protéines nuisibles à leur survie ou au contraire, d'inclure des protéines inhibant la réponse immunitaire.

La caractérisation moléculaire des mécanismes par lesquels les pathogènes détournent les radeaux à leurs propres fins fournira d'importantes informations utiles dans le développement de nouvelles stratégies thérapeutiques. Déjà, une approche thérapeutique faisant appel à l'utilisation de substances qui déplètent le cholestérol suscite beaucoup d'intérêt dans la lutte contre le VIH, puisqu'on a démontré que l'ajout d'une telle substance à la thérapie conventionnelle contre ce virus réduit grandement son infectivité (Campbell et *al.*, 2002 ; Guyader et *al.*, 2002). Puisque la subversion des radeaux lipidiques est utilisée par plusieurs agents pathogènes, il est possible que cette stratégie soit un mécanisme de survie majeur utilisé par une majorité d'entre eux. Cependant d'autres études seront nécessaires afin d'en arriver à une telle conclusion.

Les flotillines

Il existe deux protéines connues dans la famille des flotillines, la flotilline-1 et la flotilline-2 connue aussi sous l'appellation de ESA (*epidermal surface antigen*). Chez le rat, ces protéines sont nommées Reggie-2 et Reggie-1 respectivement. La flotilline-1 a été découverte et clonée il y a quelques années (Bickel et *al.*, 1997). L'ESA a été clonée et mise en évidence au niveau de l'épiderme (Schroeder et *al.*, 1994). Elle a été classée dans la familles des flotillines à cause de son homologie avec la flotilline-1 (Bickel et *al.*, 1997).

La flotilline-1

La flotilline-1 a été trouvée pour la première fois dans les cavéoles de tissu pulmonaire murin (Bickel et al., 1997). Elle contient 428 acides aminés et a une masse de 47 kDa. Elle possède deux régions hydrophobes de 27 et 18 acides aminés. La fonction de ces domaines n'est pas encore connue, mais il ne s'agit pas de domaine transmembranaire, puisque la protéine s'associe plutôt aux membranes par palmitoylation (Morrow et *al.*, 2002). Cette association se produit grâce à la présence du domaine PHB, connu aussi sous le nom du domaine Stomatin, Prohibitin, Flotilline HflC (SPFH) (Tavernarakis et *al.*, 1999), qui contient un site de palmitoylation (Cys-34) (Morrow et *al.*, 2002). Cette association dépendante de la palmytoylation fait en sorte que les deux extrémités de la flotilline-1 sont cytoplasmiques. Mises à part les séquences hydrophobes et celles permettant l'ancrage aux membranes, une analyse fournissant des prédictions théoriques de domaines et de séquences consensus a permis de mettre en évidence plusieurs autres sites de la protéine pouvant lui conférer des propriétés fonctionnelles. Il y a notamment deux sites potentiels de phosphorylation par des tyrosines kinases, un site potentiel de phosphorylation par la protéine kinase A ainsi que 4 sites potentiels de phosphorylation par la protéine kinase C, ce qui suggère qu'il existe plusieurs voies de régulation de l'activité de cette protéine. L'analyse a aussi montré que la flotilline-1 ne contient pas de site de N-glycosylation. Sur le plan de la structure du gène, le promoteur possède certaines caractéristiques de gènes dits de *housekeeping*. Le gène de la flotilline-1 est donc exprimé dans tous les tissus, bien que l'expression soit plus faible dans le foie (Edgar et Polak, 2001). En plus des mammifères, un homologue fortement conservé de la flotilline-1 a été retrouvé chez la drosophile, et son expression est confinée dans le système nerveux en développement de la mouche (Galbiati et *al.*, 1998). La prépondérance de la flotilline-1 dans les tissus nerveux chez différentes espèces suggère que cette protéine pourrait y jouer un rôle, qui aurait été conservé au cours de l'évolution.

Au chapitre de la localisation subcellulaire, la flotilline-1 a été trouvée au niveau de la membrane plasmique chez différentes lignées cellulaires comprenant des fibroblastes, des adipocytes et des neurones, (Bickel et *al.*, 1997 ; Lang et *al.*, 1998), dans l'appareil de Golgi de cellules CHO (Chinese hamster ovary cell) (Gkantiragas et *al.*, 2001), dans les phagosomes de monocytes J774 (Voir article 2) et dans les endosomes tardifs (Fivaz et *al.*, 2002). Ces données suggèrent que la flotilline-1 joue des rôles différents en fonction du type cellulaire et même de sa localisation subcellulaire. De nombreuses études seront nécessaires afin de déterminer avec exactitude les rôles de cette protéine dans ces différents types cellulaires et au niveau de différentes localisations subcellulaires.

Les fonctions de la flotilline-1 demeurent encore nébuleuses. Cette protéine pourrait jouer un rôle dans le développement du cerveau à cause de son abondance dans ce tissu (Bickel et *al.*, 1997 ; Lang et *al.*, 1998). De plus, un groupe a montré que les flotillines 1 et 2 s'accumulent dans les plaques séniles caractéristiques de la maladie d'Alzheimer, ce qui suggère un rôle dans la progression de cette maladie (Kokubo et *al.*, 2000). La flotilline-1 pourrait aussi jouer un rôle dans la formation des cavéoles *in vivo* (Volonte et *al.*, 1999). En effet, le groupe de Michael Lisanti a montré que l'expression hétérologue de la flotilline-1 dans des cellules d'insecte suffit à induire la formation de vésicules similaires à des cavéoles en microscopie électronique. De plus, cette même étude a montré que la flotilline-1 et la cavéoline-1 forment des complexes hétéro-oligomériques. Cependant, ces résultats n'expliquent pas le fait qu'il n'y ait pas de cavéoles dans certaines cellules qui expriment la flotilline-1 en abondance, comme la lignée de monocytes J774 utilisée dans nos études, et dans d'autres lignées cellulaires d'origine hématopoïétique (Fra et *al.*, 1995). De plus, l'expression de l'ARN messager de cette protéine est très abondante dans le cerveau, alors qu'il n'y a pas d'expression d'aucun membre de la famille des cavéolines dans ce tissu (Scherer et *al.*, 1996 ; Tang et *al.*, 1996). Toutes ces données suggèrent que la flotilline-1 n'est pas exclusivement présente dans les cavéoles.

Dans les adipocytes, où elle est très abondante, la flotilline-1 pourrait être impliquée dans la capture régulée du glucose suite à une stimulation à l'insuline (Baumann et *al.*,

2000). Lorsque l'insuline lie son récepteur, ce dernier recrute une protéine adaptatrice appelée CAP qui recrute à son tour la protéine cbl afin que cette dernière soit phosphorylée par le récepteur de l'insuline. Après la phosphorylation de cbl, le complexe CAP-cbl se détache et se dirige vers les radeaux lipidiques en s'attachant à la flotilline-1. L'association de CAP-cbl aux radeaux lipidiques serait nécessaire à la régulation du captage du glucose dépendant de l'action de l'insuline. Ces résultats suggèrent que la flotilline-1 pourrait servir de molécule d'échafaudage au niveau de radeaux servant à la transduction du signal. La flotilline-1 pourrait également jouer un rôle dans la réorganisation du cytosquelette d'actine. En effet, on l'a retrouvé liée à un complexe de protéines comprenant la tyrosine kinase Pyk2, Cbl et ArgBP2 qui sont reconnues comme jouant un rôle dans la régulation du cytosquelette. Ce complexe colocalise avec l'actine dans les axones et les cônes de croissance dans les cellules PC12 différenciées (Haglund et *al.*, 2004). Finalement, la flotilline-1 et la flotilline-2, qui forment des plates-formes préassemblées au niveau de la membrane plasmique chez des lignées hématopoeïtiques, confèreraient une polarité à ce type de cellules (Rajendran et *al.*, 2003). Ces plates-formes ont également été proposées comme étant des sites d'échaffaudage essentiel à l'activation de la transduction du signal.

La flotilline-2

La flotilline-2 est une protéine de 379 acides aminés ayant une masse de 42 kDa (Cho et *al.*, 1995). La nature de sa structure et de sa localisation subcellulaire est moins connue que chez la flotilline-1. On a récemment découvert que la flotilline-2 s'insère dans les membranes par des ancres lipidiques palmytoyl et myristoyl (Neumann-Giesen et *al.*, 2004). Tout comme la flotilline-1, la flotilline-2 est exprimée dans de nombreux tissus, tels que l'épiderme, la rate, le thymus, le coeur, le poumon, le foie, les reins et le cerveau, ce qui suggère un rôle ubiquitaire et non spécifique à un seul type cellulaire (Volonte et *al.*, 1999). Il est possible qu'en partenariat avec la flotilline-1, cette protéine joue un rôle d'échafaudage dans la formation de cavéoles (Volonte et *al.*, 1999). Cependant, tout comme la flotilline-1, nous l'avons retrouvée dans la lignée de macrophages murins J774 dépourvus de cavéoles (article 3), ce qui met en lumière d'autres rôles possibles.

Récemment, la flotilline-2 a été retrouvée au niveau des centrosomes dans les lymphocytes B (Solomon et *al.*, 2002), ce qui suggère un rôle dans l'organisation du cytosquelette formé par les microtubules. Cette fonction pourrait s'exercer dans les cellules hématopoïetiques et permettrait la formation de plates-formes riches en flotilline-1 et 2 lors de la cytokinèse (Rajendran et *al.*, 2003, voir plus haut). Finalement, on a découvert que la surexpression de la flotilline-2 induit la formation de filopodes chez différentes lignées cellulaires, ce qui sous-tend cette fois-ci un rôle de cette protéine dans l'organisation du cytosquelette d'actine (Neumann-Giesen et *al.*, 2004).

Le parasite intracellulaire *Leishmania donovani*

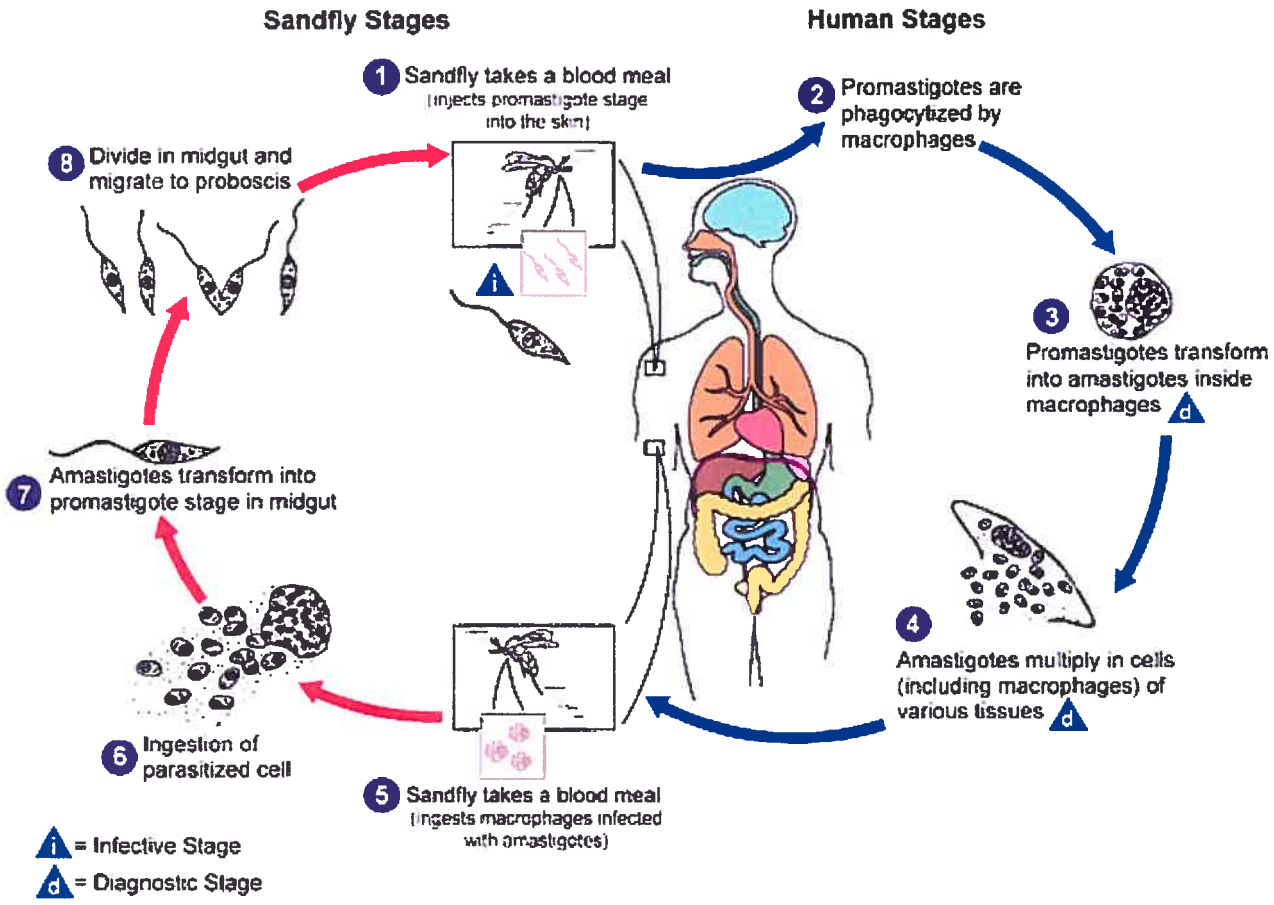
Les protozoaires du genre *Leishmania* sont les agents étiologiques des leishmanioses. Il en existe trois types soit : le type cutané causé principalement par *L. major*, *L. tropica*, et *L. mexicana*; le type mucocutané causé par *L. braziliensis*; et le type viscéral causé par *L. donovani* (Herwalt, 1999). Le type le plus dangereux est de loin la maladie viscérale, mieux connue sous le nom de Kala-azar. Elle atteint le foie et la rate causant souvent la mort. Jusqu'à présent, il existe peu de traitements efficaces contre la leishmaniose viscérale. De plus, ces traitements, qui utilisent des agents à base d'antimoine pentavalent et l'amphotericine B sont toxiques et provoquent de graves effets secondaires (Berman, 2003).

Cycle de vie

Le cycle de vie des leishmanies comprend deux stades. Elles existent sous une forme flagellée, appelée promastigote, que l'on retrouve chez les mouches phlébotomes, et sous une forme aflagellée, appelée amastigote, que l'on retrouve chez les mammifères à l'intérieur des phagolysosomes de macrophages (Alexander et Russell, 1992). Lorsque la mouche prend un repas sanguin chez un mammifère infecté, elle ingère des macrophages

parasités. Les amastigotes contenus dans les macrophages sont par la suite libérés dans le système digestif de la mouche où ils se différencient en promastigotes réplicatifs, appelés procycliques, capables de s'attacher à l'épithélium intestinal (Sacks et *al.*, 1995). À ce stade, les promastigotes ne sont pas infectieux. Ces derniers subissent ensuite un processus de maturation, appelée métacyclogenèse, au cours duquel ils se différencient en une forme métacyclique non réplicative, mais très virulente (Sacks, 1989). Lors d'un repas sanguin subséquent, les promastigotes infectieux sont relachés dans le derme des mammifères, après quoi ils sont rapidement phagocytés par des macrophages dans lesquels ils se redifférencient en amastigotes.

Figure 2 Cycle de vie du parasite *Leishmania*



Source: Centre for disease control

Survie dans la circulation

Après une morsure de la mouche, les promastigotes injectés sous le derme peuvent rejoindre la circulation sanguine ou lymphatique où ils subissent un assaut par le système du complément. Ce système a pour fonction de lyser les microorganismes envahisseurs. Toutefois, les promastigotes métacycliques sont bien équipés pour résister à ce genre d'attaque et utilisent même des protéines faisant partie du système du complément afin de favoriser leur entrée dans les macrophages. Par exemple, chez *Leishmania major* le composant C3b du complément se lie aux parasites sans générer le complexe lytique C5b-9 (MAC), puisque les leishmanies possèdent une molécule de surface, le lipophosphoglycane (LPG), qui empêche l'insertion de ce complexe lytique (Puentes et *al.*, 1990). Dans le cas de *Leishmania donovani*, une protéase de surface, la gp63, coupe le C3b en un fragment inactif, le C3bi qui recouvre le parasite et facilite ainsi la capture par les macrophages. Cette capture se fait en empruntant les récepteurs du complément sans toutefois activer la cascade lytique du complément (Brittingham et *al.*, 1995; Mosser et Brittingham, 1997). Deux autres protéines peuvent faciliter la capture des leishmanies par les macrophages. D'abord, une protéine du sérum, la *Mannan-binding protein* (MBP), peut lier les mannoses terminaux du LPG et, par cette liaison, enclencher la formation du C3b qui opsonise le parasite (Green et *al.*, 1994). Il y a aussi la *C-reactive protein* (CRP), qui lie le LPG et interagit par la suite avec des récepteurs spécifiques à la surface des macrophages (Culley et *al.*, 1996). L'entrée de *Leishmania* par les récepteurs de la CRP ne déclenche par l'activation des macrophages et par conséquent, favorise la survie du parasite (Bodman-Smith et *al.*, 2002).

Entrée dans les macrophages

Les leishmanies pénètrent dans les macrophages par phagocytose médiée par des récepteurs. Tel que mentionnée plus haut, les promastigotes métacycliques sont opsonisés par les composants C3b et C3bi du complément qui lient les récepteurs des compléments CR1 et CR3 à la surface des macrophages et activent la phagocytose. L'entrée par les CR1 et CR3 procure un avantage aux leishmanies, puisque la phagocytose médiée par ces récepteurs ne déclenche pas de flambée oxydative (Wright et Silverstein, 1983), contrairement à la phagocytose médiée par les récepteurs Fc. De plus, le CR3 supprime la production de l'interleukine 12, une cytokine essentielle à l'immunité cellulaire (Marth et Kelsall, 1997). Les autres récepteurs identifiés comme étant responsables de la capture des promastigotes par les macrophages incluent le récepteur à mannose-fucose (Wilson et Pearson, 1986), le récepteur de la fibronectine (Rizvi et al., 1988), le récepteur pour les AGEs (*advanced glycosylation end-products*) (Mosser et *al.*, 1987) et le récepteur pour la CRP (Culley et *al.*, 1996). À l'instar des récepteurs à CRP, les récepteurs à mannose-fucose ne déclenchent pas les fonctions bactéricides des macrophages (Wilson et Pearson, 1986; Astarie-Dequeker et *al.*, 1999). La capacité des leishmanies d'utiliser différents récepteurs facilite grandement leur entrée dans les macrophages et favorise leur survie en évitant d'être exposés directement aux autres défenses de notre système immunitaire.

Survie à l'intérieur des macrophages

Après la liaison d'un promastigote à la surface des macrophages, la phagocytose se déclenche et enferme le parasite à l'intérieur d'un phagosome dans le cytoplasme de la cellule. Une fois dans le phagosome, la leishmanie doit s'adapter afin de survivre dans ce nouvel environnement fort différent de celui présent dans l'appareil buccal et les intestins de la mouche. L'adaptation du parasite dépend de sa différenciation de la forme promastigote en sa forme amastigote. L'augmentation de la température et la baisse du pH

à l'intérieur des phagosomes sont les facteurs déterminants qui provoquent cette différenciation. Les amastigotes sont plus résistants que les promastigotes à l'environnement des phagolysosomes (Zilberstein et Shapira, 1994).

Plusieurs études ont montré que les amastigotes résident dans des phagosomes possédant plusieurs caractéristiques des phagolysosomes. Par exemple, les phagosomes contenant *L. mexicana* et *L. amazonensis* maintiennent un pH acide (Antoine et *al.*, 1990), ont accès à des traceurs chargés dans les endosomes et possèdent une activité enzymatique d'acide phosphatase présente surtout chez les lysosomes (Alexander et Vickerman 1975; Chang et Dwyer 1976; Shepherd et *al.*, 1983; Rabinovitch et *al.*, 1985). De plus, des études plus récentes ont montré que les phagosomes contenant *L. donovani* possèdent des marqueurs d'endosomes tardifs et de lysosomes, tels que les cathepsines, les peptidases, le CMH de classe II et LAMP1 (Prina et *al.*, 1990; Russell et *al.*, 1992; Lang et *al.*, 1994). Toutes ces données montrent que les amastigotes survivent à l'intérieur de phagolysosomes. La machinerie moléculaire qui intervient dans la survie des amastigotes dans l'environnement hostile de ces compartiments est encore peu connue, mais il est certain que ces parasites résistent à l'action des hydrolases. Cette résistance n'est pas due à la présence d'une enveloppe protectrice puisque, contrairement aux promastigotes, les amastigotes ne produisent pas de glycocalyx riche en LPG capable de les protéger des hydrolases (Pimenta et *al.*, 1991). Elle serait plutôt due à des protéases, comme la gp63 (Chaudhuri et *al.*, 1989; Seay et *al.*, 1996) synthétisées par les amastigotes et impliquées dans la dégradation de molécules de l'hôte comme les hydrolases lysosomiales (Pupkis et *al.*, 1986). De plus, les amastigotes possèdent plusieurs activités enzymatiques, telles que la glutathione peroxydase, la catalase et la superoxyde dismutase, capables de neutraliser les composés oxydatifs toxiques produits dans les phagolysosomes des macrophages lors de la flambée oxydative (Channon et Blackwell, 1985).

Une autre façon pour les promastigotes de survivre à l'intérieur des macrophages consiste à inhiber les voies de signalisation de la cellule hôte en vue de perturber certaines fonctions cellulaires. Chez les cellules infectées par *Leishmania*, on a observé une

diminution de la réponse de la cellule au LPS, à l'IFNγ et à certains activateurs de la protéine kinase C (Descoteaux et *al.*, 1991; Ray et *al.*, 2000). L'inhibition de la PKC est causée par le LPG de surface des promastigotes. La molécule du LPG sera discutée en détail plus loin. Quant à eux, les amastigotes expriment des phospholipides glycosylinositol qui, à l'instar du LPG, inhibe la PKC. Cependant, le mécanisme d'action de ces molécules seraient différent de celui du LPG (McNeely et *al.*, 1989). La modification de la transduction du signal peut aussi survenir par la perturbation de certaines kinases ou phosphatases de la cellule hôte. Par exemple, *L. donovani* module la phosphorylation et l'activation de certains enzymes en réponse à l'IFNγ, un effet attribuable à l'activation de la tyrosine phosphatase SHP-1, ce qui contribue également à la survie du parasite. (Olivier et *al.*, 1998; Forget et *al.*, 2001).

En plus de perturber la transduction du signal, les leishmanies affectent également la production de cytokine par les macrophages. Par exemple, les parasites réduisent la production de l'IL-12, une cytokine nécessaire à la réponse antiparasitaire (Carrera et *al.*, 1996). De plus, la phagocytose des amastigotes via les récepteurs Fc induit la production de IL-10 associée à une baisse de l'activité antimicrobienne des macrophages (Sutterwala et *al.*, 1998).

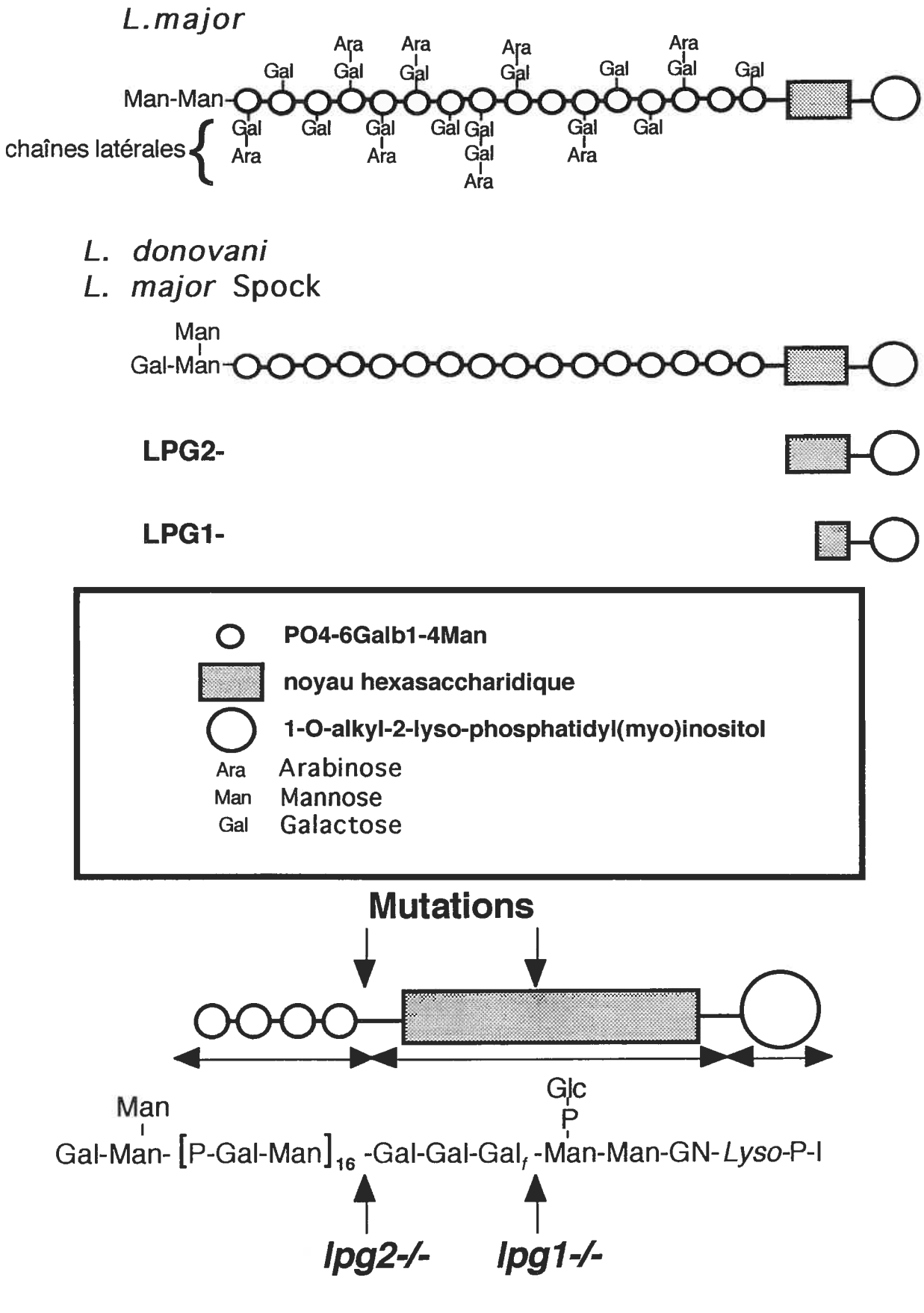
Contrairement aux amastigotes, les promastigotes de *Leishmania* résident à l'intérieur de compartiments qui ne possèdent pas les caractéristiques des phagolysosomes en début d'infection (Scianimanico et *al.*, 1999, Annexe 1, article 5). Des études de notre laboratoire ont montré que l'inhibition de la fusion phagosome-endosome, un processus essentiel à la formation des phagolysosomes, contribue à la survie des promastigotes de *Leishmania* dans les phagosomes. L'environnement favorable de ces compartiments immatures permet aux promastigotes d'initier leur différenciation en amastigotes à l'abri de l'attaque des enzymes lytiques présentes dans les phagolysosomes (Desjardins et Descoteaux, 1997). Il apparaît donc évident que la stratégie de survie des promastigotes est liée à leur capacité à moduler les propriétés de fusion des phagosomes. Il a été démontré

que l'inhibition de la fusion est due à la présence du LPG à la surface des promastigotes (voir plus bas, Desjardins et Descoteaux, 1997; voir Article 1).

Le lipophosphoglycane

Le LPG est le glycoconjugué majeur des différentes espèces de *Leishmania*. Chez *Leishmania donovani*, il est constitué d'une longue chaîne d'unités disaccharidiques phosphorylées répétitives (PO_4-6Galβ1-4Man) dont la longueur dépend du stade de développement du parasite (en moyenne jusqu'à 16 unités). Ces unités répétitives sont liées entre elles par des liens phosphodiester. Cette chaîne se termine au bout par une structure composée du trisaccharide Gal(β1,4)-Man(α1,2)-Manα1 (Thomas et *al.*, 1992). Ce polymère d'unités disaccharidiques est attaché par l'autre extrémité à un noyau hexasaccharidique composé d'une unité glucosamine non acétylée, de deux résidus mannose, d'un résidu galactose 6-phosphate, d'un résidu galactopyranose et d'un résidu galactofuranose (Turco et *al.*, 1989). Ce noyau est lui-même fixé à une ancre lipidique, 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol, dont la chaîne aliphatique contient un hydrocarbone saturé sans embranchement de 24 ou 26 carbones, insérée dans la membrane plasmique du parasite (Orlandi et Turco, 1987; Turco et *al.*, 1987, 1989). Cette structure peut varier entre les espèces de *Leishmania*. Les différences entre les espèces se retrouvent principalement au niveau des chaînes saccharidiques latérales. Ainsi, *L. major* contient des chaînes oligosaccharidiques latérales formées de résidus galactose et arabinose liées à la position 3 du résidu galactose des unités répétitives du LPG (McConville et *al.*, 1990). Il existe aussi des différences à la hauteur des sucres terminaux. Il y a donc un polymorphisme structural du LPG entre les différentes espèces. La figure 3 décrit de façon schématique la structure du LPG chez *L. donovani* et *L. major* ainsi que chez les mutants utilisés pour effectuer les études décrites dans cette thèse.

Figure 3 Structure du LPG chez le type sauvage et certains mutants



Fonctions du LPG

Le polymorphisme structural du LPG permet aux espèces de *Leishmania* d'utiliser des vecteurs différents, ce qui favorise leur dissémination sans que les parasites ne compétitionnent entre eux (Pimenta et *al.*, 1994). Par exemple, les leishmanies qui expriment un LPG contenant des unités répétitives non liées à des chaînes oligosaccharidiques latérales, comme c'est le cas chez *L. donovani*, sont transmis surtout par les mouches de l'espèce *Phlebotomus argentipes* tandis que *L. major*, qui contient de telles chaînes latérales, est transmis par *Phlebotomus papatasi* (Revue dans Sacks, 2001).

La structure du LPG est modifiée au cours du développement du parasite. Les modifications les plus importantes se produisent lors de la métacyclogenèse du parasite à l'intérieur du système digestif de la mouche. Tel que mentionné plus haut, ce processus constitue un passage d'une forme non infectieuse, appelée procyclique, où les parasites se multiplient activement, vers une forme infectieuse, où les promastigotes ne se divisent plus et migrent vers la bouche de l'insecte (Turco, 1990). La métacyclogenèse s'accompagne d'une élongation marquée (environ le double) de la molécule du LPG associée à une augmentation du nombre d'unités disaccharidiques répétitives, qui peut atteindre 30 dans le cas de *Leishmania donovani* (McConville et Blackwell, 1991). L'élongation du LPG masque certains sucres terminaux de la molécule et, ainsi, permet aux parasites métacycliques de se détacher de la paroi intestinale pour se diriger vers la bouche de l'insecte afin d'être subséquemment injectés dans un mammifère (Sacks et Perkins, 1984, 1985; Pimenta et *al.*, 1992, Sacks et *al.*, 1995).

Plusieurs rôles du LPG ont été proposés dans la survie des parasites (McNeely et Turco, 1990). Chez la mouche, le LPG forme un glycocalyx dense apte à protéger les promastigotes contre la dégradation par les enzymes digestives (Schlein et *al.*, 1990). Chez le mammifère, le LPG accomplit plusieurs fonctions avant l'internalisation des parasites dans les macrophages. Une des fonctions extracellulaires du LPG consiste à protéger le

parasite contre les attaques du complément, puisque les promastigotes métacycliques infectieux, dont le LPG est très long, sont beaucoup plus résistants à la lyse que les promastigotes procycliques non infectieux, dont le LPG est beaucoup plus court (Franke et *al.*, 1985; Puentes et *al.*, 1988). Une autre fonction extracellulaire du LPG est de favoriser la fixation des *Leishmania* aux macrophages (Handman et *al.*, 1985), en facilitant le captage de la protéine C3b du complément (Puentes et *al.*, 1988), ce qui favorise par la suite les interactions avec les récepteurs à complément CR1 et CR3 situés à la surface des macrophages (Da Silva et *al.*, 1989; Mosser et Edelson, 1985; Talamàs-Rohana et *al.*, 1990; Wilson et Pearson; 1988).

Tel que mentionné précédemment, à l'intérieur des phagosomes, les parasites sont exposés à un environnement riche en enzymes hydrolytiques. Pour survivre, les leishmanies doivent donc s'adapter, soit en neutralisant l'action de ces enzymes dégradatives, soit en y résistant (Alexander et Vickerman, 1975; Chang et Dwyer, 1976). Une étude suggère que le LPG pourrait jouer un rôle dans l'inactivation des enzymes dégradatives contenues dans les lysosomes (El-On et *al.*, 1980). Grâce à ses charges négatives, le LPG servirait aussi de chélateur de calcium et d'autres cations divalents, ce qui préviendrait la production de radicaux hydroxyles toxiques lors de l'activation des macrophages (Eilam et *al.*, 1985; Homans et *al.*, 1992). Le LPG influence aussi la transduction des signaux chez les macrophages en inhibant la PKC (Descoteaux et *al.*, 1992). Cette kinase joue un rôle clé dans la signalisation transmembranaire qui contrôle plusieurs fonctions cellulaires (revu dans Farago et Nishizuka, 1990; Houslay, 1991) comme le déclenchement de la flambée oxydative chez les macrophages (revu dans Baggiolini et Wyman, 1990) qui mène à la production de radicaux oxygénés toxiques pour les leishmanies (Reiner et Kazura, 1982). De plus, il a été montré récemment que l'isoforme α de la PKC inhibibée par le LPG joue un rôle dans la maturation du phagosome en phagolysosome (Ng Yan Hing et *al.*, 2004). Le mécanisme précis de l'inhibition de la PKC par le LPG est encore méconnu. Toutefois, on sait que le LPG n'empêche pas la translocation de cette kinase du cytosol vers la membrane plasmique (Descoteaux et *al.*, 1992). Le LPG agirait plutôt sur la PKC en empêchant le changement de sa conformation

nécessaire à son activation (Giorgione et *al.*, 1996). Le LPG peut inhiber la PKC, même si les deux molécules se retrouvent de part et d'autre de la bicouche lipidique, ce qui suggère que le LPG n'interagit pas directement avec cette protéine. Les autres fonctions possibles du LPG incluent: le captage de radicaux toxiques avant qu'ils ne causent des dommages (Chan et *al.*, 1989), l'inhibition de la production de IL-1 (Frankenburg et *al.*, 1990), une cytokine importante pour l'activation des lymphocytes T (Unanue et Allen, 1987), l'inhibition de la chimiotaxie (Frankenburg et *al.*, 1990) et la réduction de la capacité des macrophages à lier le TNF, une cytokine qui joue un rôle dans l'activation des macrophages (revu dans Rosenblum et Donato, 1989).

Finalement, des données expérimentales ont démontré que le LPG inhibe la fusion phagosome-endosome (Desjardins et Descoteaux, 1997). En effet, les promastigotes mutants de *Leishmania donovani lpg2-/-*, qui expriment un LPG tronqué sans unité disaccharidique répétitive (Descoteaux et *al.*, 1995), induisent la formation de phagosomes très fusogéniques comparativement aux phagosomes formés lors de l'internalisation de promastigotes de type sauvage. L'étude de Desjardins et Descoteaux (1997) a aussi montré que l'opsonisation du mutant *lpg2-/-* avec du LPG purifié permet à ce parasite d'induire la formation de phagosomes peu fusogéniques semblables à ceux contenant la forme sauvage. Le nombre d'unités disaccharidiques répétitives du LPG serait la clef d'une inhibition efficace de la fusion, puisque le mutant RT5 de *L. donovani*, qui exprime seulement de 3 à 5 unités répétitives au lieu de 16 (McNeely et *al.*, 1990), se retrouve dans des phagosomes dont le taux de fusion est intermédiaire entre ceux contenant la forme sauvage et le mutant *lpg2-/-*.

Biosynthèse du LPG

La polymérisation des unités disaccharidiques répétitives (PO_4-6Galβ1-4Man) se produit dans l'appareil de Golgi (Carver et Turco, 1991). Ce processus implique l'addition séquentielle et en alternance de résidus mannose et galactose liés à des précurseurs nucléotidiques directement à la chaîne naissante du LPG. Le gène *lpg2* code pour un transporteur, la mannosyl transferase qui permet la translocation de résidus mannose du cytoplasme vers l'appareil de Golgi (Ma et *al.*, 1997). Chez le mutant lpg2-/-, où le gène *lpg2* n'est plus fonctionnel, l'absence de résidu mannose dans le Golgi ne permet pas la synthèse des unités (PO_4-6Galβ1-4Man) répétitives et par conséquent, l'ajout de telles unités à un ensemble de molécules incluant, en plus du LPG, la phosphatase alcaline et les phosphoglycanes sécrétés n'est plus possible (Ilg et *al.*, 1994). L'utilisation du mutant lpg2-/- ne permet donc pas de déterminer directement laquelle de ces molécules est responsable de l'altération des propriétés de fusion des phagosomes. La figure 3 indique l'endroit où la synthèse du LPG est affectée par la mutation au gène *lpg2*.

Un autre gène, le *lpg1*, contribue à la biosynthèse du LPG (Huang et Turco, 1993). Ce gène codant pour une galactofuranosyl transférase joue un rôle dans la synthèse du noyau hexasaccharidique présent exclusivement sur la molécule du LPG (voir figure 3). Étant données les modifications du noyau hexasaccharidique, chez le mutant lpg1-/- l'ajout des unités répétitives disaccharidiques à la molécules n'est plus possible. Cependant, contrairement au gène *lpg2*, la mutation du gène *lpg1* n'a aucun effet sur la synthèse des autres molécules qui possèdent des sous-unités disaccharidiques. Par conséquent, le mutant *lpg1-/-* permettrait de déterminer si d'autres molécules possédant des unités répétitives ou seulement le LPG sont impliquées dans l'inhibition de la fusion des phagosomes avec les endosomes (Voir article 1).

Dans les travaux de cette thèse, j'ai d'abord cherché à mieux caractériser l'inhibition de la fusion phagosome-endosome causée par le LPG (article 1). Par

l'utilisation du mutant *lpg1-/-*, où seul le LPG fait défaut, nous avons montré que le LPG est la seule molécule contenant des unités disaccharidiques répétitives responsable de l'inhibition phagosome-endosome. Nous avons aussi montré que l'inhibition de la fusion phagosome-endosome est plus efficace envers les endosomes tardifs qu'envers les endosomes précoces, ce qui favoriserait la survie du parasite. Dans un deuxième temps, j'ai consacré une partie de mes efforts à la caractérisation de la protéine flotilline-1 que nous avons retrouvée associée au phagosome par une approche protéomique (article 2). Grâce à cette protéine, nous avons mis en évidence pour la première fois la présence de radeaux lipidiques sur le phagosome. Une étude protéomique préliminaire nous a ensuite permis de proposer plusieurs rôles à ces structures sur le phagosome, tels que la transduction du signal, la nucléation de l'actine et l'acidification du compartiment (article 2). Finalement, en constatant que les phagosomes contenant des mutants non virulents de *Leishmania* dépourvus de LPG acquièrent beaucoup de flotilline-1 contrairement aux phagosomes contenant le parasite du type sauvage, nous avons émis l'hypothèse d'une action du LPG sur les radeaux lipidiques afin de favoriser la survie du parasite dans les macrophages (article 3). Les résultats montrent que le LPG peut perturber l'acquisition et la distribution des radeaux lipidiques sur le phagosome. À notre connaissance, il s'agit de la première démonstration qu'une molécule microbienne puisse perturber directement les radeaux lipidiques de phagosomes. Le parasite intracellulaire *Leishmania donovani* rejoint donc un groupe de pathogènes qui interagissent activement avec les radeaux lipidiques de leur cellule hôte afin de favoriser l'infection.

Chapitre II. Résultats. Article I

Dermine J.-F., Scianimanico S., Privé C., Descoteaux A., Desjardins M. (2000) *Leishmania* promastigote require lipophosphoglcyan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. *Cell Microbiol* **2**(2), 115-126.

Leishmania promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis

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Running title: LPG alters phagosome fusion

Key words: lipophosphoglycan; _Leishmania_; phagocytosis, phagosome fusion; macrophages

Summary

The lipophosphoglycan (LPG) of *Leishmania* promastigotes plays key roles in parasite survival both in the insect and mammalian hosts. Evidence suggest that LPG decreases phagosome fusion properties at the onset of infection in macrophages. The mechanisms of action of this molecule are, however, poorly understood. In the present study, we used a panoply of *Leishmania* mutants displaying modified LPG structures to determine more precisely how LPG modulates phagosome-endosome fusion. Using an *in vivo* fusion assay measuring, at the electron microscope, the transfer of solute materials from endosomes to phagosomes, we provided further evidence that the repeating (PO_4-6Galβ1-4Man) units of LPG are responsible for the alteration of phagosome fusion. The inhibitory effect of LPG on phagosome fusion was shown to be more potent towards late endocytic organelles and lysosomes than early endosomes, explaining how *Leishmania* promastigotes can avoid degradation in hydrolase-enriched compartments. The involvement of other repeating unit-containing molecules, including the secreted acid phosphatase, in the inhibition process was ruled out since a LPG-defective mutant ($lpg1^-$) which secretes repeating unit-containing glycoconjugates was present in highly fusogenic phagosomes. In *L. major*, oligosaccharide side chains of LPG did not contribute to the inhibition process, as Spock, a *L. major* mutant lacking LPG side chains, blocked fusion to the same extent as wild type parasites. Finally, dead parasites internalized from the culture medium were not as efficient as live parasites to alter phagosome-endosome fusion despite the presence of LPG. However, the killing of parasites with vital dyes after their sequestration in phagosomes, had no effect on the fusion properties of this organelle. Collectively, these results suggest that living promastigotes displaying full-length cell surface LPG can actively influence macrophages at an early stage of phagocytosis to generate phagosomes with poor fusogenic properties.

Introduction

Leishmania are intracellular parasites causing a spectrum of diseases called leishmaniases which afflict millions of people worldwide. Parasites are transmitted to human through the bite of infected sand flies. At the onset of infection, promastigotes of *Leishmania* are internalized by macrophages into phagosomes, also referred to as parasitophorous vacuoles (for a review see Desjardins and Descoteaux, 1999; Antoine et al., 1998; Alexander and Russell, 1992). Whereas most microbes are engulfed in phagosomes that engage in a maturation process to generate potent microbicidal compartments (see Aderem and Underhill, 1999; Dermine and Desjardins, 1999; Finlay and Falkow, 1997), *Leishmania* promastigotes have evolved strategies to retard their delivery inside phagolysosomes. We have shown recently that *L. donovani* promastigotes modulate the fusion properties of the phagosomes in which they reside by excluding key molecules such as the late endosome-associated GTPase rab7 (Scianimanico et al., 1999; Desjardins and Descoteaux, 1997). Absence of this host molecule impairs the maturation of phagosomes, allowing promastigotes to reside in intracellular compartments not displaying the lytic environment of phagolysosomes. Using a combination of genetic and biochemical approaches, we provided evidence that the repeating unit moiety of LPG, the major promastigote surface glycoconjugate (Turco and Descoteaux, 1992), is essential for the transient inhibition of phagosome maturation (Desjardins and Descoteaux, 1997). The backbone of this molecule consists of a polymer of the repeating (PO_4-6Galβ1-4Man) unit, attached via a glycan core to a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol anchor (Turco et al., 1989, 1987; Orlandi and Turco, 1987). The non-reducing end of LPG is terminated with one of several small neutral oligosaccharides containing galactose and mannose residues. While the lipid anchor and the glycan core are conserved in all *Leishmania* species examined, sugar composition and sequence of the repeating units and the cap structure are species specific (McConville et al., 1995). Complex oligosaccharide side chains are also present in certain species (Turco and Descoteaux, 1992).

The contribution of repeating units in the alteration of phagosome-endosome fusion was strongly suggested by the observation that mutants of *L. donovani* (*lpg2*-) defective in repeating units biosynthesis, induced the formation of phagosomes that fuse extensively with endocytic organelles and mature normally into phagolysosomes (Scianimanico et al., 1999; Desjardins and Descoteaux, 1997). In addition, opsonization of a LPG-defective mutant with purified LPG conferred the ability to alter phagosome fusion. Thus, while there were solid evidence that the presence of LPG repeating units at the surface of promastigotes is required for slowing down phagosome maturation, the contribution in this process of other repeating unit-containing molecules, also altered in the *lpg2*- mutant, could not be ruled out. Of the molecules containing repeating units, LPG was shown to be a major virulence factor of *Leishmania*. Beside inhibiting phagosome-endosome fusion, LPG confers resistance to the lytic action of complement (Puentes et al., 1990, 1988) and acts as a potent inhibitor of the protein kinase C (Giorgione et al., 1996; Descoteaux et al., 1992) which plays a central role in signal transduction and in the regulation of macrophage microbicidal activities. These findings emphasized the role of LPG in *Leishmania* pathogenesis.

In the present study, we used *L. donovani* and *L. major* mutants displaying altered forms of LPG to further determine how this molecule alters phagosome-endosome fusion. The results obtained indicate that repeating units present on LPG, but not on secreted molecules, are responsible for the transient inhibition of phagosome fusion with endocytic organelles. Fusion inhibition is more potent towards late endocytic organelles and lysosomes than early endosomes, suggesting that *Leishmania* promastigotes use LPG to avoid contact with hydrolase-enriched compartments.

Results

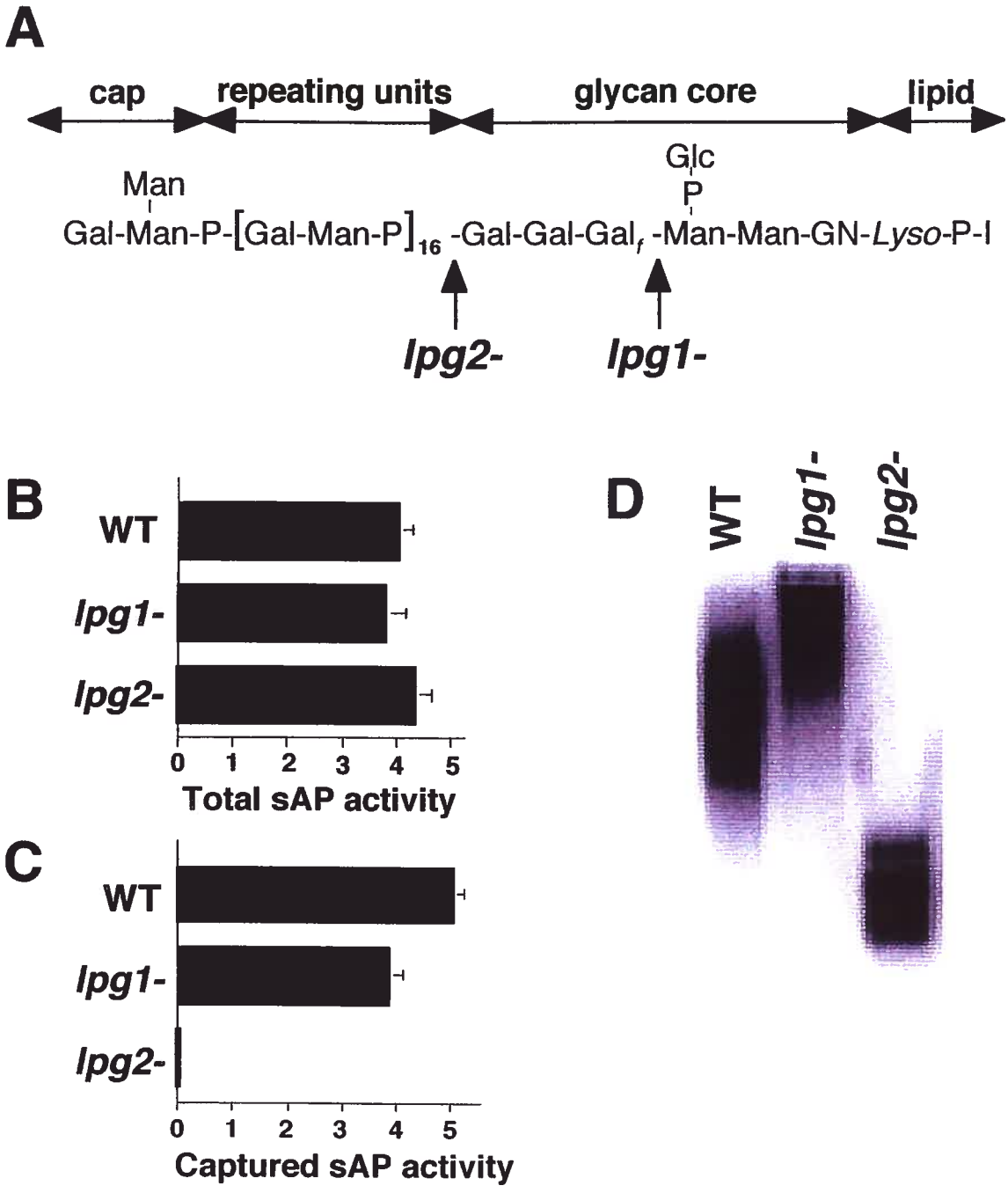
In the present study, we have used a combination of various *Leishmania* species and LPG-defective mutants to further study the role and mechanism(s) of action of LPG and its components, as well as other repeating unit-containing molecules, in the alteration of phagosome-endosome fusion.

LPG, and not other repeating unit-containing molecules, is responsible for the impairment of phagosome-endosome fusion

In a previous study (Desjardins and Descoteaux, 1997), we provided genetic and biochemical evidence that LPG repeating units are required for the inhibition of phagosome-endosome fusion. However, the possibility that repeating unit-containing molecules other than LPG could also participate in the alteration of phagosomal maturation remained to be addressed. To this end, we used the *lpg1-* mutant (Privé and Descoteaux, submitted), which lacks a glycosyltransferase required for completion of the LPG glycan core, thereby preventing the addition of repeating units to the LPG molecule (Huang and Turco, 1993; Ryan et al., 1993). In contrast to the *lpg2-* mutant, the *lpg1-* mutant adds repeating units to secreted molecules, including the secreted acid phosphatase (Fig. 1). In this regard, the *lpg1-* mutant is phenotypically identical to the R2D2 mutant which has been well characterized with respect to the synthesis of phosphoglycan-containing molecules (Huang and Turco, 1993; Ryan et al., 1993). Therefore, the *lpg1-* mutant represents an unique tool to address the possible role of secreted repeating unit-containing molecules in the alteration of phagosome-endosome fusion.

Figure 1 Characterization of the secreted acid phosphatase from *lpg1-* and *lpg2-* mutants.

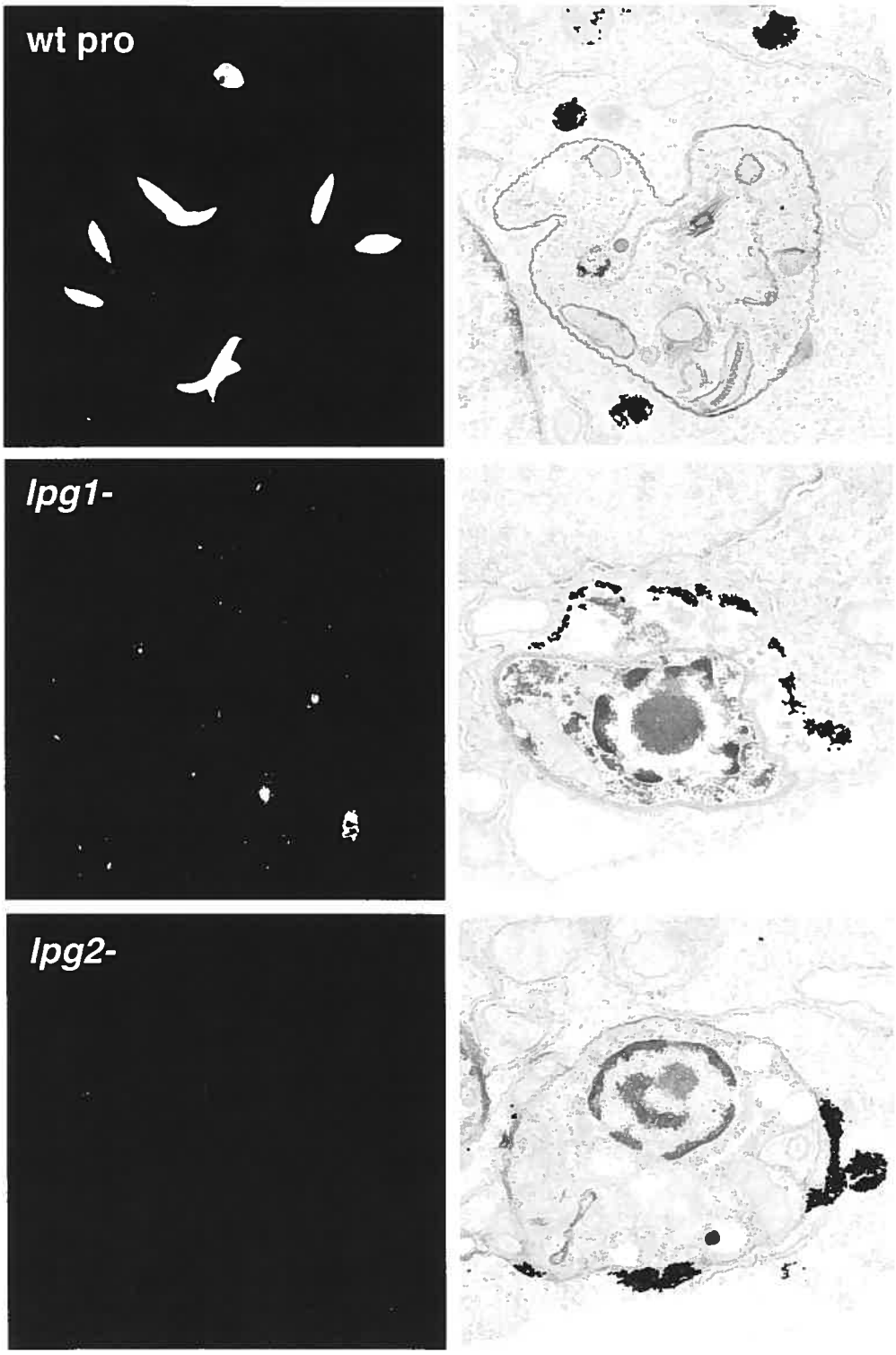
(A) Structure of LPG from *L. donovani* and the truncated LPG accumulating in *lpg1-* and *lpg2-* mutants. *L. donovani* LPG comprises four domains: (i) an oligosaccharide cap, (ii) a polymer of repeating disaccharide-phosphate units (n=16 to 30 units per molecule), (iii) a glycan core, and (iv) a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor (PI). The *LPG1* gene is required for the addition of the galactosylfuranose (Gal*f*) within the glycan core (Ryan et al, 1993). The *lpg1-* mutant expresses the truncated LPG [Glc(α1-P)]Man(α1,3)Man(α1,4)GN(α1,6)-PI, and retains the ability to secrete the various repeating units-containing glycoconjugates (Huang and Turco, 1993). The *LPG2* gene encodes for a transporter required for the transport of GDP-mannose into the Golgi lumen where repeating units are assembled on LPG and the various repeating unit-containing glycoconjugates (Descoteaux et al, 1995). The *lpg2-* mutant expresses the truncated LPG Gal(α1,6)Gal(α1,3)Gal*f*(α1,3)[Glc(α1-P)]Man(α1,3)Man(α1,4)GN(α1,6)-PI, and does not synthesize repeating units. Gal, galactose; Glc, glucose; GN, glucosamine; Man, mannose; P, phosphate. (B) Wild type promastigotes, the *lpg1-* and the *lpg2-* mutants secrete similar levels of acid phosphatase activity. (C) and (D) The sAP from the *lpg1-* mutant was captured by the anti-repeating unit monoclonal antibody CA7AE, indicating that similar to wild type, the *lpg1-* mutant secretes a repeating unit-containing AP. Of interest, the *lpg1-* sAP displayed a slower electrophoretic mobility than the wild type sAP, which may reflect an increase in the number of repeating units per molecule. As previously shown, the sAP from the *lpg2-* mutant lacks repeating units, as it failed to react with the CA7AE antibody (C) and displayed a rapid electrophoretic mobility with respect to wild type sAP (D) (Descoteaux et al, 1995).


A
cap
repeating units
glycan core
lipid
Man
Glc
P
Gal-Man-P-[Gal-Man-P]16 -Gal-Gal-Gal$_f$ -Man-Man-GN-*Lyso*-P-I
lpg2-
lpg1-
B
WT
lpg1-
lpg2-
0 1 2 3 4 5
Total sAP activity
C
WT
lpg1-
lpg2-
0 1 2 3 4 5
Captured sAP activity
D
WT
lpg1-
lpg2-

Immunofluorescence with antibodies directed against repeating units showed an uniform distribution of LPG on the surface of wild type promastigotes (Fig. 2). Whereas no labeling was detected at the surface of the *lpg*2- mutant, a punctate surface staining was observed with the *lpg*1- mutant, which may represent secreted phosphoglycans and proteophosphoglycans. These results confirmed that the *lpg1*- and *lpg2*- mutants are well suited to address the role of secreted repeating unit-containing molecules in the alteration of phagosome-endosome fusion. The *in vivo* fusion assay performed with these mutants revealed that phagosomes containing either *lpg1*- or *lpg2*- mutants displayed high levels of fusion with endocytic organelles when compared to phagosomes containing wild type promastigotes (Fig. 2). The quantitative analysis of fusion indicates that a 50 to 60% increase in fusion is observed for phagosomes containing mutant parasites (Fig. 3A). In this case, the average level of fusion measured for phagosomes containing wild type promastigotes was around 40%, a value similar to the one published previously (Scianimanico et al., 1999). These results clearly demonstrate that LPG is the main molecule responsible for the alteration of the fusion properties of phagosomes. The other repeating unit-containing molecules, secreted by the *lpg1*- mutant, do not seem to contribute significantly to the fusion inhibition process.

Figure 2 Immunofluorescence analysis of LPG expression on *L. donovani* promastigotes

Promastigotes were processed for immunofluorescence analysis with the CA7AE monoclonal antibody directed against the repeating units of LPG. Top panel: results indicate that wild type promastigotes display high levels of LPG at their surface, but the antibody fails to stain the *lpg2-* mutants (bottom panel). Middle panel: the punctuated staining on the *lpg1-* mutants may represent secreted repeating unit-containing molecules. Right panels: Fusion of *L. donovani* promastigote-containing phagosomes with endosomes. J774 macrophages were infected either with stationary phase *L. donovani* wild type, *lpg2-* mutants or *lpg1-* mutants promastigotes 60 min and further incubated for 60 min in culture medium. Cells were then fed 16 nm BSA-gold particles for 30 min, followed by a chase of 30 min and processed for electron microscopy. Top panel: phagosomes containing *L. donovani* display low fusogenic properties with endocytic organelles. Phagosomes containing the *lpg1-* (middle panel) and *lpg2-* (bottom panel) mutants fuse extensively with endocytic organelles, as shown by the presence of numerous gold particles within the phagosomes.

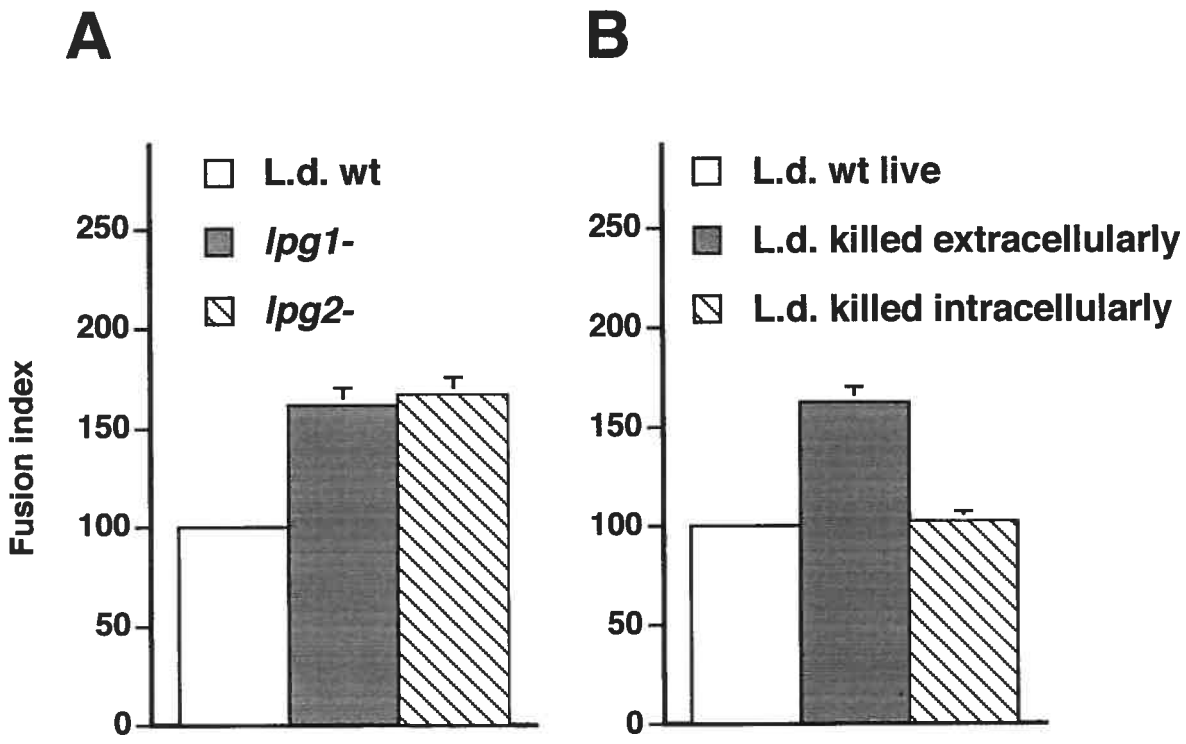

wt pro
lpg1-
lpg2-

Leishmania promastigotes actively modulate phagosome fusion properties early during phagocytosis

Several pathogens, whose survival depends on their ability to inhibit the fusion between phagosomes and endocytic organelles, are rapidly routed to phagolysosomal compartments when internalized as killed particles (see Sinai and Joiner, 1997). These observations suggest that microbes play an active role in the generation of phagosomes with altered fusion properties. To determine whether the impairment of phagosome-endosome fusion by *Leishmania* promastigotes is an active process, we compared the fusion properties of phagosomes formed by the internalization of either live or killed promastigotes (heat-killed or paraformaldehyde-fixed). The results obtained showed that phagosomes formed by the internalization of heat-killed parasites, which still display LPG, fused about 60% more with endosomes than phagosomes containing live parasites (Fig. 3B). Immunofluorescence analysis showed that dead parasites still display LPG at their surface (not shown), ruling out the possibility that alleviation of the fusion inhibition was caused by the possible loss of LPG during heat treatment. Furthermore, similar results were obtained with paraformaldehyde-fixed parasites or parasites killed with crystal violet prior to their internalization (not shown). We next determined whether killing *Leishmania* after their internalization in poorly fusing phagosomes would have any effect on the fusion properties of this organelle. Our data showed that killing the internalized parasites with crystal violet, an electron carrier efficient at killing *Leishmania* intracellularly without harming host cells (Mauel et al., 1984 and data not shown), had no significant effect on the level of fusigenicity of this compartment (Fig. 3B). This observation suggested that the active involvement of the parasite in modulating the fusion properties of *Leishmania*-containing phagosomes takes place at the time of or shortly after phagocytosis.

Figure 3 Quantitative analysis of the fusion between *Leishmania*-containing phagosomes and endosomes.

(**A**) J774 macrophages were infected with either stationary phase *L. donovani* wild type promastigotes, *lpg2-* or *lpg1-* mutants and processed as described in Fig. 2. Fusion between phagosomes and endocytic organelles was measured as described in Materials and Methods. Data are expressed as a fusion index (ratio of the percentage of fusion of the various *Leishmania*-containing phagosomes over that of *L. donovani* promastigotes which was given a value of 100). Results show that both *lpg1-* and *lpg2-* mutant–containing phagosomes are 60% more fusogenic than wild type-containing phagosomes, indicating that repeating units present on LPG but not on other secreted molecules are involved in phagosome fusion impairment. (**B**) In these experiments, macrophages were first fed 16 nm gold particles for 30 min followed by a 120 min chase. Wild type parasites were then internalized for 60 min followed by a 60 min incubation in culture medium. At this point, cells were treated for 10 min with 10^{-5} M crystal violet diluted in culture medium to kill the parasites inside the phagosomes. Cells were then incubated for 2 h in culture medium to allow interactions between phagosomes and endosomes and processed for electron microscopy. The results indicate that killing parasites after their internalization has no effect on the phagosome fusion properties, while internalization of heat-killed parasites leads to the formation of fusogenic phagosomes.

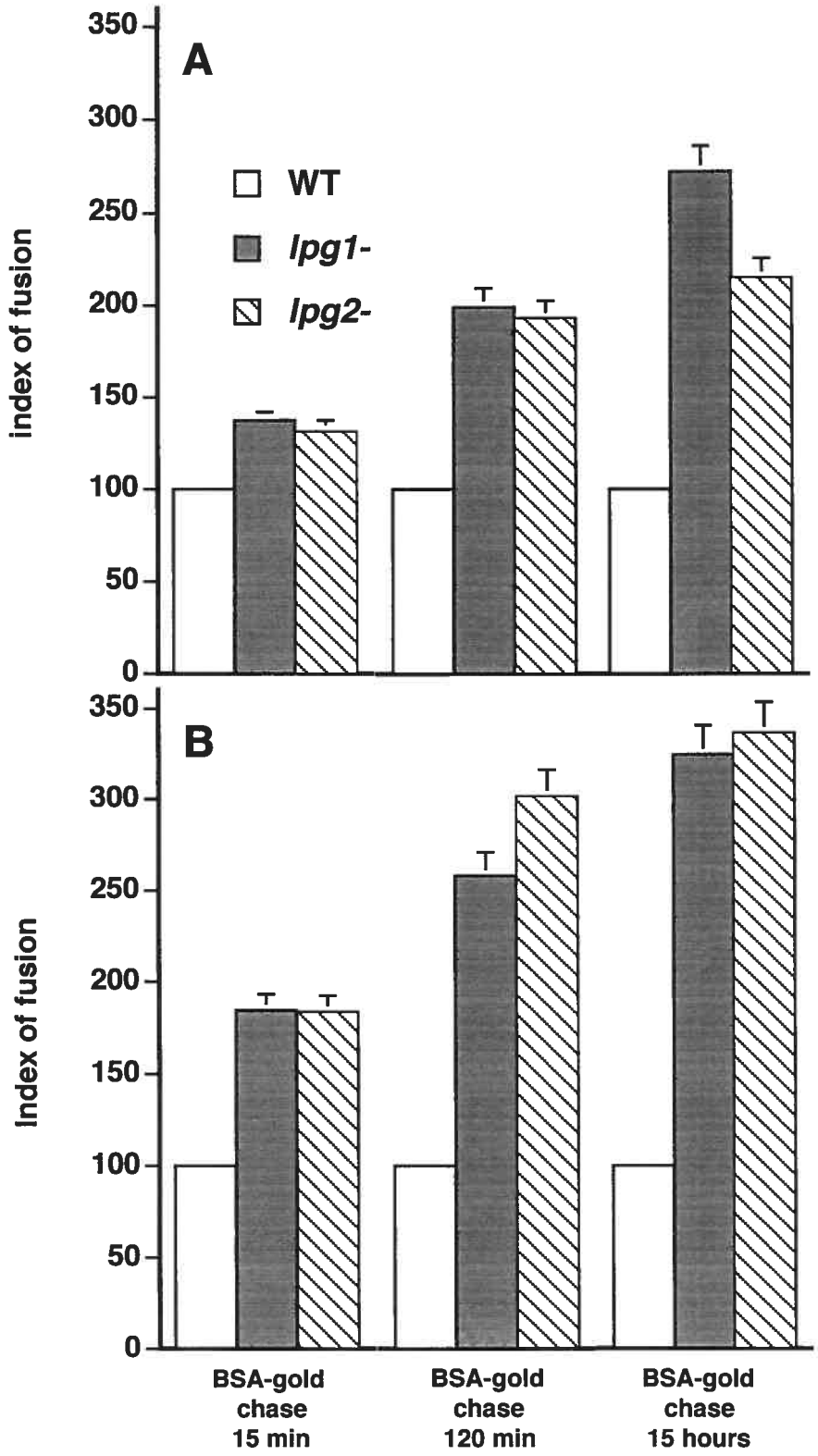

A
B
L.d. wt
lpg1-
lpg2-
L.d. wt live
L.d. killed extracellularly
L.d. killed intracellularly
Fusion index
250
200
150
100
50
0

LPG preferentially impairs fusion between phagosome and late endocytic organelles

If the survival strategy of *Leishmania* promastigotes is to avoid ending up in phagolysosomes, one could assume that inhibition of fusion will be more efficient towards late endocytic organelles and lysosomes, which contain most of the molecules involved in parasite degradation, than towards early endosomes not particularly enriched with hydrolases. To verify this hypothesis, we measured the levels of fusion occurring between phagosomes containing the various *Leishmania* strains and endocytic organelles of increasing ages. Macrophages were allowed to internalize BSA-gold particles, which were then chased for increasing periods of time in order to preferentially label either early endocytic organelles, late endocytic organelles or lysosomes. Macrophages were then infected with either wild type promastigotes, the *lpg1*- mutant or the *lpg2*- mutant for 60 min followed by chase periods of either 60 min or 4 h. Measurements of the transfer of gold particles from the various endocytic organelles to phagosomes (60 min chase) revealed that phagosomes containing either mutants fused about 30 to 40% more with early endosomes than phagosomes containing wild type parasites (Fig. 4A) (the wild type value is 62.4%). This difference was more important when phagosomes interacted with late endosomes and terminal lysosomes. Indeed, mutant-containing phagosomes fused about 100% more with 2 h-old endosomes and 200% more with lysosomes than wild type-containing phagosomes (the wild type values were of 35.2% and 17.3% respectively). Differences in the fusion properties of mutant-containing phagosomes and wild type-containing phagosomes were also important when parasites were chased for 4 h (Fig. 4B). These results clearly support our hypothesis that one of the strategy used by *L. donovani* promastigotes to establish themselves within macrophages consists in limiting fusion of phagosomes with late, hydrolase-enriched endocytic organelles.

Figure 4 Quantitative analysis of the fusion between *L. donovani* -containing phagosomes and different endosome populations.

(**A**) In this assay, macrophages were fed with 16 nm BSA-gold particles for 30 min and chased for 15 min, 2 h and 15 h to load early endocytic organelles, late endocytic organelles and lysosomes respectively. Cells were then infected with either wild type *L. donovani* promastigotes, the *lpg1-* mutant or the *lpg2-* mutant for 60 min and further incubated for 60 min in culture medium. Results show that wild type parasites (in comparison with mutant parasites) alter preferentially the fusion of phagosomes with late endocytic organelles and lysosomes rather than with early endocytic organelles. (**B**) Cells were treated as in (A) except that *Leishmania* were chased for 4 h instead of 60 min.

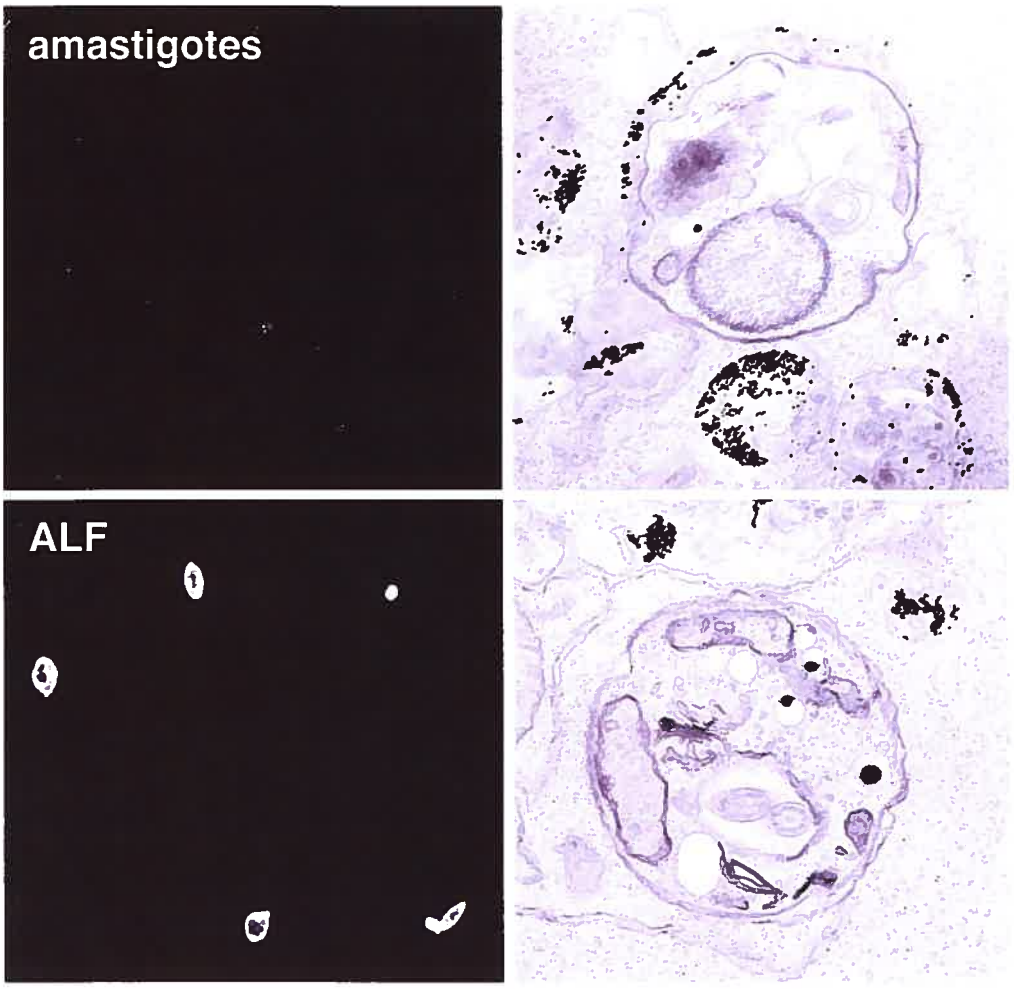

A
B
WT
lpg1-
lpg2-
index of fusion
index of fusion
350
300
250
200
150
100
50
0
BSA-gold chase 15 min
BSA-gold chase 120 min
BSA-gold chase 15 hours

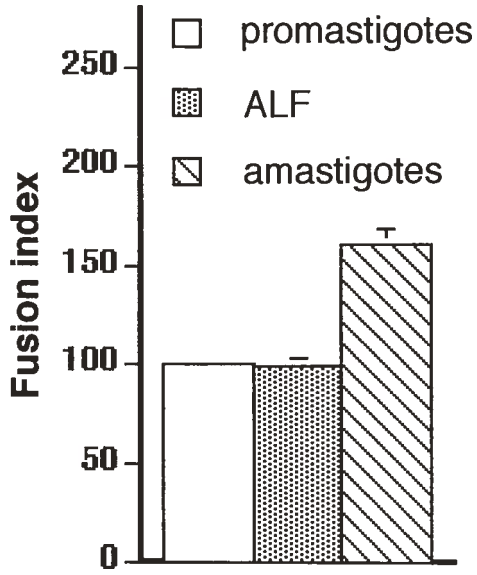
Amastigotes, lacking LPG, are internalized in fusogenic phagosomes

Several laboratories have shown that *Leishmania* amastigotes reside in fusogenic compartments displaying phagolysosomal characteristics (Lang et al., 1994; Russell et al., 1992; Antoine et al., 1990; Prina et al., 1990). In contrast, results reported in the present study and in previous work indicate that promastigotes are first internalized in phagosomes displaying poor fusogenic properties (Scianimanico et al., 1999; Desjardins and Descoteaux, 1997). Collectively, these results suggest that the modifications occurring during the differentiation of promastigotes into amastigotes are responsible for the loss of phagosome fusion inhibition. To investigate whether these changes could influence phagosome fusion properties, we performed the *in vivo* fusion assay with lesion amastigotes purified from the spleen of infected hamsters and with *in vitro* amastigotes. Immunofluorescence studies showed that lesion amastigotes do not display detectable level of LPG at their surface (Fig. 5). On the other hand, despite presenting a rounded amastigote-like morphology, ALFs are covered with LPG. As expected, amastigotes, which lack LPG, were present in phagosomes displaying high fusogenic properties compared with phagosomes housing promastigotes (Fig. 5). ALF, which lost their flagellum and express LPG, altered phagosome-endosome fusion to the same extent than promastigotes (Fig. 5). These results further support a role for LPG in the inhibition of phagosome-endosome fusion. They also support the previous suggestion that the loss of LPG during promastigote-to-amastigote transformation is responsible for the restoration of fusion between phagosomes and endosomes at later stages of infection (Desjardins and Descoteaux, 1997; Russell et al., 1992).

Figure 5 Immunofluorescence analysis of LPG expression on *L. donovani* amastigotes

Left panels: Lesion amastigotes and *in vitro* generated amastigotes (ALF) were processed for immunofluoresence as described in Fig. 2. Results indicate that lesion amastigotes (upper left panel) do not display LPG molecules to a detectable level at their surface. However, ALFs display a high amount of LPG at their surface (lower left panel). Right panels: Fusion of *L. donovani* amastigote-containing phagosomes with endosomes. Macrophages were processed as described in Fig. 2. Upper right panel: phagosomes containing *L. donovani* amastigotes freshly harvested from an infected hamster fuse extensively with endocytic organelles. Lower right panel: phagosomes containing *in vitro* generated amastigotes (ALFs) fuse poorly with endosomes.


amastigotes
ALF


promastigotes
ALF
amastigotes
Fusion index
250
200
150
100
50
0

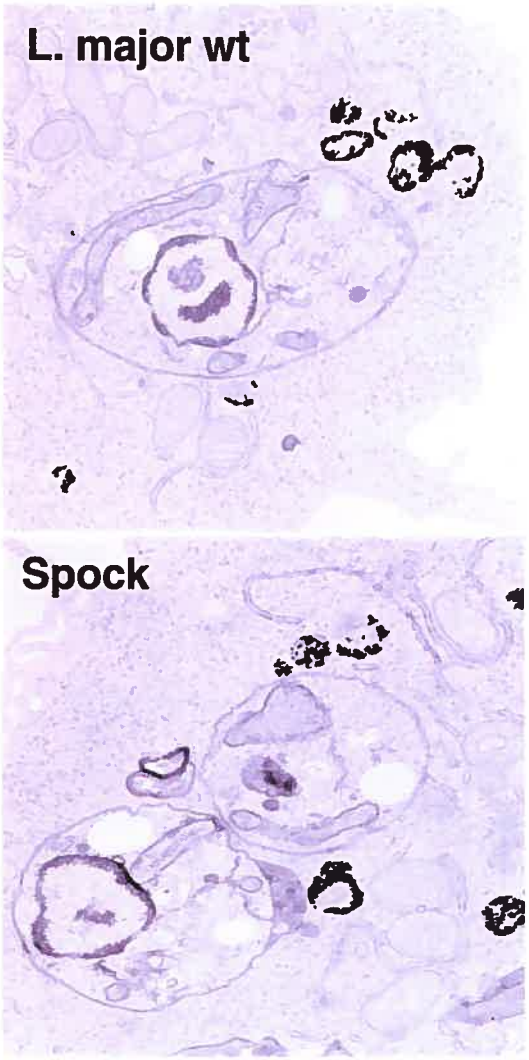
Various species of *Leishmania* can alter phagosome-endosome fusion

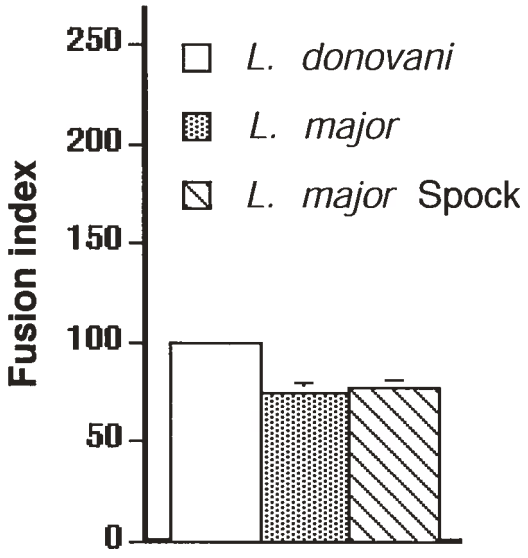
We then verified if *Leishmania major* was also able to alter the fusion properties of phagosomes. Since *L. major* promastigotes express a LPG displaying additional oligosaccharide side chains linked at the position 3 of the galactose of the repeating units, it was of interest to determine whether the increased complexity of this molecule would lead to a more potent inhibition of phagosome-endosome fusion. The results obtained show that, similar to *L. donovani* (see Fig. 2), *L. major* promastigotes (Fig. 6) reside in phagosomes in which few gold particles were transferred. Quantitative analysis confirmed that parasites from both species reside in phagosomes that display similar low fusogenic properties (Fig. 6). The level of fusion of these phagosomes was considerably lower than generally observed for latex bead-containing phagosomes (not shown) or phagosomes containing LPG repeating units mutants (see Fig. 2). These results indicated that species of *Leishmania* displaying LPG of different structures are equally able to impair phagosome endosome fusion.

Next, we determined whether the oligosaccharide side chains of *L. major* LPG could contribute to the impairment of phagosome fusion properties. To this end, we compared the fusion properties of phagosomes containing either wild type *L. major* promastigotes or Spock, a *L. major* mutant defective in LPG oligosaccharide side chain biosynthesis. Our results showed that Spock impaired phagosome-endosome fusion to the same extent as wild type *L. major* promastigotes, indicating that LPG oligosaccharide side chains are unlikely to play a major role in the alteration of phagosome-endosome fusion (Fig. 6).

Figure 6 Fusion of *L. major*-containing phagosomes with endosomes

J774 macrophages were infected with stationary phase *L. major* promastigotes or the Spock mutant and processed as described in Fig. 2. Top panel: phagosomes containing wild type *L. major* have low fusogenic properties with gold-filled endosomes, as shown by the absence of gold particles in the phagosome. Bottom panel: similar results are observed with Spock, a side chain-defective mutant, which induces phagosomes with low fusogenic properties. Quantitative analysis: Fusion events between phagosomes and endosomes were measured and are represented as described in Fig. 2. Results indicate that different species of *Leishmania* are able to alter phagosome fusion. Moreover, the oligosaccharide side chains of *L. major* (Spock) are not involved in this process.


L. major wt
Spock


Fusion index
250
200
150
100
50
0
L. donovani
L. major
L. major Spock

Discussion

Leishmania, the etiological agent of leishmaniasis, has evolved strategies to survive and replicate in the unfriendly environment of both the insect and mammalian hosts. In mammals, parasites are rapidly internalized in macrophages by phagocytosis and reside in phagosomes where promastigotes transform, within few days, into amastigotes, the replicative form responsible for the spread of infection. This transformation is triggered by the rise in temperature and decrease in pH encountered in phagosomes (Zilberstein and Shapira, 1994). Several studies have shown that *Leishmania* amastigotes replicate within phagolysosomes (Alexander and Vickerman, 1975; Chang and Dwyer, 1976; Shepherd et al., 1983; Rabinovitch et al., 1985; Prina et al., 1990; Antoine et al., 1990; Russell et al., 1992; Lang et al., 1994), which are hydrolase-enriched organelles arising from phagosome maturation (Desjardins, 1995; Haas, 1998). This clearly indicates that amastigotes are well adapted for survival within the harsh environment of phagolysosomes. In the present study, we provided evidence that *Leishmania* promastigotes, the form responsible for the initiation of infection, rely on a different strategy to adapt and survive in the new environment encountered in macrophages.

Using an *in vivo* fusion assay, we have previously shown that *L. donovani* promastigotes are internalized in phagosomes displaying low levels of fusion with endocytic compartments (Desjardins and Descoteaux, 1997). As a result, these phagosomes fail to mature properly (Scianimanico et al., 1999). Based on a genetic approach, we also demonstrated that molecules displaying repeating unit moieties of PO_4-6Galβ1-4Man were responsible for the inhibition of phagosome-endosome fusion (Desjardins and Descoteaux, 1997). In addition to LPG, several *Leishmania* molecules contain these repeating units (Ilg et al., 1994), and might therefore play a role in the alteration of phagosome fusion properties induced by *L. donovani* promastigotes. Our inability to demonstrate the possible role of repeating unit-containing molecules other than LPG in the fusion inhibition process was related to the fact that the *lpg2-* mutant used in our previous studies completely lacks repeating units (Descoteaux et al., 1995). In the present study, we addressed this issue

using *lpg1-*, an LPG-defective mutant which retains the ability to add repeating units to secreted molecules (Privé and Descoteaux, submitted, Fig. 1). The use of the *lpg1-* and *lpg2-* mutants clearly demonstrated that LPG is the only repeating unit-containing molecule that possesses the ability to impair phagosome-endosome fusion and that secreted molecules containing repeating units had no effect in this process. Presumably, the GPI-anchor allows insertion of the repeating units into the phagosome membrane bylayer, a phenomenon required for membrane stabilization and hence, modification of its fusogenic properties (Miao et al., 1995). *L. major* promastigotes were slightly more efficient at inhibiting phagosome-endosome fusion than *L. donovani* promastigotes. The increased complexity of *L. major* LPG, characterized by a high proportion of repeating units with oligosaccharide side chains could have explained this difference in fusion level. However, the finding that Spock, a mutant lacking the oligosaccharide side chains (Butcher et al., 1996), impaired fusion to the same extent than wild type *L. major* promastigotes ruled out any major role for side chains in the alteration of phagosome-endosome fusion. The ability of the Spock mutant to alter phagosome-endosome fusion is not surprising, considering that this *L. major* mutant produces a LPG similar to that of *L. donovani*. Further similarities in the functional properties of these LPG molecules have been reported. Both the Spock mutant and *L. donovani* are unable to infect the sand fly *P. papatasi*, the natural *L. major* vector (Butcher et al., 1996). This is in contrast with the apparent superior ability of *L. major* LPG to inhibit HIV-induced syncytia formation (Easterbrook et al., 1995), which led the authors to propose that the ability of LPG to inhibit membrane fusion events may reside in the size of its carbohydrate moiety. More likely, the increased number of repeating units might account for the improved performance of this parasite at inhibiting phagosome fusion. A relation between the number of repeating units on LPG and its ability to inhibit membrane fusion in various biological systems has been reported (Miao et al., 1995; Giorgione et al., 1996; Desjardins and Descoteaux, 1997).

The precise way by which LPG alters phagosome fusion properties is not completely understood. Evidence suggest that LPG can alter the physical properties of membranes. Studies have shown that LPG is able to inhibit virus-induced membrane

fusion in a dose-dependent way by raising the hexagonal phase transition temperature of lipid bilayers (Miao et al., 1995; Martin et al., 1998; Rasmusson et al., 1998). LPG was shown to inhibit fusion from both side of the membrane lipid bilayer (Martin et al., 1998). These results support the model that LPG alters membrane fusion from the luminal side of phagosomes (Scianimanico et al., 1999). We have shown that phagosomes containing *L. donovani* promastigotes fail to acquire normal levels of the small GTPase rab7 (Scianimanico et al., 1999), a molecule involved in the regulation of fusion between phagosomes and late endocytic organelles (Feng et al., 1995; Méresse et al., 1995; Vitelli et al., 1997). It is still not clear, however, whether the absence of rab7 is responsible for the low fusion properties of phagosomes or whether phagosomes fail to acquire this molecule because they do not fuse with late endocytic organelles, a possible source of rab7. In the later case, modifications to a molecule or sets of molecules, upstream of rab7, would be responsible for the alteration of phagosome fusion properties. In this context, results from the present study indicate that *Leishmania* promastigotes actively influence the fusion properties of phagosomes at an early step of phagocytosis. This is based on the finding that the internalization of heat-killed *L. donovani* promastigotes results in the formation of phagosomes that fuse extensively with endocytic organelles despite the presence of LPG. This indicates that an active process over the sole presence of LPG is exerted on host cells by parasites. Second, killing of parasites after their internalization in phagosomes failed to restore the fusion properties of this organelle, suggesting that phagosome fusion properties are determined early after host-pathogen interaction rather than by a persistent active action of the parasite on the phagosome. It is also possible that once phagosomes are formed, the presence of LPG is sufficient to maintain the transient inhibition of fusion. This is consistent with the finding that phagosomes become more fusogenic as LPG is down-regulated during the promastigote to amastigote transformation (Russell et al., 1992), even if in this case, the fusion properties were evaluated at much longer time points after infection. Similar observations were made with a variety of pathogens, including *Mycobacteria*, *Chlamydia*, and *Legionella* which all reside in phagosomes with restricted fusion properties unless killed parasites are internalized (for a review see Sinai and Joiner, 1997).

Differences in the fusion properties observed for phagosomes containing live or dead parasites could also be related to observations showing that several species of *Leishmania*, including *L. donovani*, tightly bind to parasitophorous vacuoles through their posterior pole (Benchimol and de Souza, 1981; Lang et al., 1994; Antoine et al., 1998), a process that might require LPG and live parasites.

Data accumulated so far strongly suggests that the onset of infection by *Leishmania* promastigotes is characterized by their internalization into phagosomes unable to mature properly into phagolysosomes (Scianimanico et al., 1999; Desjardins and Descoteaux, 1997). Phagolysosome biogenesis is a complex process allowing the transformation of newly formed phagosomes into phagolysosomes mainly by a sequential series of regulated fusions with early endosomes, late endosomes and lysosomes (Desjardins et al., 1997; Desjardins et al., 1994). The sequential interaction of phagosomes with endocytic organelles and lysosomes enables the acquisition of the machinery and molecules needed to kill and degrade microorganisms and to process antigens for presentation by the major histocompatibility class II system (Prina et al., 1996). We have shown that phagosomes containing either wild type *Leishmania* promastigotes or LPG-defective mutants initiate their maturation in a similar way by recycling plasma membrane receptors and markers associated to early endosomes (Scianimanico et al., 1999). However, while phagosomes containing LPG-defective mutants further mature and acquire the late endocytic and lysosomal markers rab7 and Lamp1, phagosomes containing wild type promastigotes acquire much less of these molecules, or acquire them in a delayed fashion. In these conditions, a recent study showed that LPG mutants were killed more rapidly in macrophages than wild type parasites (St-Denis et al., 1999). These results suggest that to provide a survival advantage, *L. donovani* promastigotes must prevent phagosome fusion with late endosomes and lysosomes. This hypothesis was tested in the present study and the results obtained indicated that the ability of *Leishmania* promastigotes to inhibit the fusion of phagosomes with early endosomes is, indeed, lower than their ability to inhibit phagosome fusion with late endosomes and lysosomes. Intriguing questions are why and

how LPG prevents fusion with late endocytic compartments but not with early endosomes? Although there are no obvious answers at the moment, similar observations were made with other microorganisms. *Brucella abortus*-containing phagosomes were shown to be able to fuse with early endocytic organelles but not with lysosomes (Pizarro-Cerda et al., 1998, 1999,). *Salmonella*-containing phagosomes interact with early endocytic organelles (Steele-Mortimer et al., 1999), while conflictual data are reported regarding their possible interaction with lysosomes (see Finlay and Falkow, 1997). *Mycobacteria* prevent phagolysosome biogenesis by stopping phagosome maturation between stages regulated by the rab5 and rab7 molecules (Via et al., 1997). Indeed, whereas *Mycobacteria*-containing phagosomes do not fuse with late endosomes and lysosomes (Armstrong and Hart 1971; de Chastellier et al., 1993; Xu et al., 1994; Clemens and Horwitz 1995; Barker et al., 1997), they fuse significantly with early endocytic organelles (de Chastellier et al., 1995). Recently, a coat protein of phagosomes termed TACO was shown to restrict the delivery of *Mycobacteria* to phagolysosomes (Ferrari et al., 1999). However, the possible involvement of TACO in the inhibition of fusion of *Mycobacteria*-containing phagosomes was not studied. It would be of interest to verify if this protein is also associated to *Leishmania*-containing phagosomes. Systematic comparisons of the composition of phagosomes containing wild type *Leishmania* promastigotes and their LPG-defective counterparts should provide insights into the molecules and mechanisms altered by LPG and responsible for the respective fusion properties of these organelles.

Experimental procedures

Cell culture

The murine macrophage-like cell line J774 was cultured in Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were grown to approximately 80 % confluence in Petri dishes prior to each experiment, as described previously (Desjardins et al., 1994), and were not kept in culture more than 15 passages after thawing.

The wild type *L. donovani* promastigotes (strain 1S) and the LPG-defective mutants *lpg1*- (Privé and Descoteaux, submitted) and *lpg2*- (Scianimanico et al., 1999) were grown at 26°C in modified M199 medium (Life technologies, Grand Island, NY) as described (Descoteaux et al 1994). *L. major* promastigotes were cultured in the same medium supplemented with 10 % heat-inactivated FBS (Hyclone, Logan, UT) buffered with 10 mM Hepes at pH 7, 3. Spock, a *L. major* mutant lacking the oligosaccharide side chains of LPG was kindly provided by David Sacks (Butcher et al., 1996).

Analysis of secreted acid phosphatase (sAP) activity

Total acid phosphatase activity present in the supernatants of promastigote cultures was determined by adding 50 µl of promastigote culture supernatants to 50 □l of 100 mM Na Acetate pH 5.0 containing 1.6 mg/ml p-nitrophenyl phosphate. After 30 min incubation at 26°C, 10 □l 2N NaOH were added and O.D. at 405 nm was determined. A previously described antigen capture assay performed with the CA7AE monoclonal antibody was used to determine the presence of repeating PO_4-6Galβ1-4Man units on the sAP (Descoteaux et al., 1995). To determine the electrophoretic pattern of sAP, concentrated (5X) promastigote culture supernatants were electrophoresed in non-denaturing conditions in 7%

acrylamide gels in the absence of SDS. Gels were stained for acid phosphatase activity as described (Katakura and Kobayashi, 1988).

Leishmania amastigotes and amastigote-like-form (ALF)

L. donovani amastigotes were propagated in female Syrian hamsters and were recovered from the spleen of infected animals as described (Descoteaux and Matlashewski, 1989). Amastigotes were used immediately after recovery to infect macrophages as described below. ALFs were induced by transferring end-log phase/stationary phase promastigotes to 37^{0}C in a 5% CO_2 atmosphere, for at least 36 hours to simulate conditions prevailing in mammals. After that period of time, parasites presented a rounded morphology, lacked flagella but still displayed LPG at their surface as shown by immunofluorescence (see below).

Immunofluorescence analysis

In vivo, the transformation of promastigotes into amastigotes is accompanied, among other molecular modifications, by the loss of the flagellum and the down-modulation of LPG from the parasite surface. We have used these two criteria to assess the state of transformation of the various parasites used in the present study. For immunofluorescence, 10 □l of *Leishmania* cultures were placed on coverslips and fixed in 20% methanol/80% acetone for 20 min at –20°C. Fixed *Leishmania* were washed and rehydrated in PBS 2 times 5 min and 2 times 10 min and blocked 10 min with PBS 2% BSA (fraction V, Sigma, St-Louis, MO), 0, 2% gelatin. Coverslips were then incubated with the mouse monoclonal antibody CA7AE (1:1000) directed against the repeating units of LPG (Tolson et al., 1989) for 1 h at room temperature. After three washes in PBS 1% BSA, a goat anti-mouse IgM antibody coupled to Texas Red was added for 30 min at room temperature. Coverslips were then washed 3 times in PBS 1% BSA and once in PBS, mounted on slides with polyvinyl alcohol (Gelvatol, Air Products & Chemicals, Allentown, PA) and observed at

the epifluorescence microscope. Controls included *Leishmania* incubated only with the secondary antibody.

In vivo fusion assay at the electron microscope

To evaluate the levels of fusion between *Leishmania*-containing phagosomes and endosomes, an in vivo fusion assay at the electron microscope level allowing precise quantitative analysis was used (Desjardins et al., 1997; Desjardins and Descoteaux, 1997). This assay monitors the transfer of BSA-gold particles from endocytic organelles to *Leishmania*-containing phagosomes in infected macrophages. J774 cells were plated 2 days prior to the experiments onto 35 mm Petri dishes (Falcon) and grown to 80% confluence. To form phagosomes, the culture medium was removed and replaced with 1 ml of fresh medium containing *Leishmania* at a parasite to host-cell ratio of 10:1 except for the *lpg1*-and *lpg2*- mutants for which a ratio of 5:1 was sufficient to yield the same number of phagosomes per cell. Macrophages were allowed to internalize parasites for 60 min at 37°C and were then thoroughly washed with cold PBS to eliminate free parasites. Cells were subsequently incubated for another 60 min in culture medium at 37°C to allow complete internalization of bound parasites.

To load endosomes, infected cells were incubated in culture medium containing 16 nm BSA-gold particles for 30 min. Cells were then washed 3 times 5 min in cold PBS and further incubated for 30 min at 37°C in fresh medium to allow extensive interaction between phagosomes and endosomes. At the end of each experiment, cells were fixed in the dishes with 2% glutaraldehyde, post-fixed in 1% OsO_4 and flat-embedded in Epon 812 as described previously (Desjardins et al., 1997). Thin sections of the flat cells were then prepared and observed at the electron microscope. The levels of fusion between endosomes and phagosomes were scored based on the presence of gold particles inside phagosomes (Desjardins and Descoteaux, 1997). The presence of one particle was sufficient to score a fusion event. At least 50 phagosomes per section were analyzed for each sample. All

experiments were performed at least 3 times in triplicates. Fusion levels were measured as percentage of phagosome profiles that contain BSA-gold on the electron microscope sections. To compare each experiment, the fusion levels were expressed as ratios over the *L. donovani* wild type levels that were assigned a value of 100. Note that wild type parasites were used in every experiments performed in this study to ensure direct comparisons.

The ability of phagosomes containing wild type promastigotes or LPG-defective mutants to fuse with late endocytic organelles and lysosomes was also investigated. To perform these experiments, various endocytic organelles were loaded first by internalizing BSA-gold particles for 30 min followed by incubation in culture medium for 15 min, 2 h or 15 h at 37°C. Parasites were then internalized for 60 min and further incubated in normal culture medium for 60 min or 4 h at 37°C. The samples were then processed as described above.

In some experiments, killed *L. donovani* promastigotes were used to determine whether viable parasites are required to alter phagosome-endosome fusion. Promastigotes were killed before internalization by an incubation at 45°C in culture medium for 20 min. Since killed parasites are degraded very rapidly inside phagosomes, which makes their identification difficult, the fusion assay was slightly modified so that killed parasites reside in phagosomes for the shortest time possible. Thus, endocytic organelles were loaded first with BSA-gold particles for 30 min at 37°C followed by short washes with cold PBS and a 2h incubation in culture medium at 37°C to get rid of non-internalized particles. Cells were then allowed to internalize live or dead promastigotes for 60 min at 37°C and processed for electron microscopy as above.

As our results indicated that killed wild type promastigotes were present in fusogenic phagosomes, in contrast to live parasites present in low-fusogenic phagosomes, we tested whether the internalization of live parasites followed by their killing in phagosomes would restore the fusion properties of these organelles. In this case, the assay was performed as

follows. BSA-gold was internalized for 30 min and chased 120 min at 37°C. Parasites expressing luciferase were then internalized for 60 min and chased for 60 min at 37°C. At this point, cells were treated with 10^{-5}M crystal violet diluted in culture medium for 10 min to kill parasites intracellularly as described (Mauel et al., 1984). After 3 x 2 min washes in cold PBS, cells were incubated 2 h in medium to allow interactions between phagosomes and endosomes. As a control, all the above steps were repeated with latex beads instead of *Leishmania* to verify that crystal violet does not modify the fusion properties of phagosomes. To ensure that crystal violet was efficient at killing internalized *Leishmania*, the viability of parasites after treatment was evaluated by measuring the luciferase activity generated by living parasites.

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Chapitre III. Résultats. Article 2

Dermine J.-F., Duclos S., Garin J., St-Louis F., Rea S., Parton R.G., Desjardins M. (2001) Flotillin-1-enriched domains accumulate on maturing phagosomes. *J. Biol. Chem.* **276,** 18507-18512.

Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes

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Running title: Lipid rafts on phagosomes

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Abstract

Flotillin-1 was recently shown to be enriched on detergent resistant domains of the plasma membrane called lipid rafts. These rafts, enriched in sphingolipids and cholesterol, sequester certain proteins while excluding others. Lipid rafts have been implicated in numerous cellular processes including signal transduction, membrane trafficking and molecular sorting. In this study, we demonstrate both morphologically and biochemically that lipid rafts are present on phagosomes. These structures are enriched in flotillin-1 and devoid of the main phagosome membrane protein LAMP1. The flotillin-1 present on phagosomes does not originate from the plasma membrane during phagocytosis but accumulates gradually on maturing phagosomes. Treatment with bafilomycin A1, a compound that inhibits the proton pump ATPase and prevents the fusion of phagosomes with late endocytic organelles, prevents the acquisition of flotillin-1 by phagosomes, indicating that this protein might be recruited on phagosomes from endosomal organelles. A proteomic characterization of the lipid rafts of phagosomes indicates that actin, the α and β subunits of heterotrimeric G proteins, as well as subunits of the proton pump V-ATPase are amongst the constituents of these domains. Remarkably, the intracellular parasite *Leishmania donovani* can actively inhibit the acquisition of flotillin-1-enriched lipid rafts by phagosomes, and the maturation of these organelles. These results indicate that specialized functions required for phagolysosome biogenesis may occur at focal points on the phagosome membrane, and therefore represent a potential target of intracellular pathogens.

Introduction

Lateral assemblies of lipids, termed lipid rafts, have been postulated to represent a general feature of the plasma membrane of eukaryotic cells (Simons and Ikonen, 1997; Harder and Simons, 1997). Rafts apparently form due to the biophysical properties of sphingolipids and cholesterol which pack tightly into liquid-ordered (l_o) domains that partition away from the more disorganized glycerophospholipids in the bulk of the membrane (Brown and London, 1998). Lipid-modified proteins and some transmembrane proteins are concentrated in the rafts whereas other proteins are excluded. Lipid rafts have been implicated in many important cellular processes, such as polarized sorting of apical membrane proteins in epithelial cells and signal transduction (Kurzchalia and Parton, 1999). Recent evidence further indicates that a raft-based mechanism might be involved in the sorting of SNAREs to the plasma membrane and in their function in apical membrane docking and fusion events (Lafont et al., 1999). As this is in no way an exhaustive list of the potential function of lipid rafts, it appears that membrane subdomains represent important sites conferring specialized properties to foci within biological membranes.

In the present study, we provide evidence showing that lipid rafts are present on phagosomes. These specialized regions, devoid of the major phagosomal protein LAMP 1, are enriched in flotillin-1. The phagosomal lipid rafts are unlikely to be simply transferred from the plasma membrane to phagosomes during phagocytosis since early phagosomes display low amounts of flotillin-1. Instead, flotillin-1 is recruited to phagosomes during phagosome maturation, possibly through fusion with late endocytic organelles. The identification of lipid rafts on phagosomes suggests that specific functions occur at focal points on the phagosome membrane. Further proteomic characterization allowed us to identify sets of proteins indicating that phagosome lipid rafts might be involved in signal transduction, interaction with actin, and phagosome acidification.

Experimental procedures

Cell culture and phagosomes formation and isolation

The murine macrophage-like cell line J774 was cultured in Dulbecco's modified Eagle medium high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were grown to approximately 80 % confluency in Petri dishes prior to each experiment, as described previously (Desjardins et al., 1994).

To form phagosomes, J774 macrophages were fed with 0.8 µm blue-dyed latex beads (Sigma) diluted 1:50 in culture medium. Depending on the experiment, cells were allowed to internalize beads for 30 to 90 min at 37°C. Cells were then washed 3 times 5 min with ice-cold PBS to remove non internalized beads and further incubated for increasing periods of time to obtain early and late phagosomes. Phagosomes were then isolated on sucrose step gradients as described previously (Desjardins et al., 1994). Purified phagosomes were resuspended in Laemmli or rehydration/lysis buffers for Western blotting and 2-D gel electrophoresis respectively.

Sensitivity to bafilomycin A1

To determine if phagosome maturation is required for the acquisition of flotillin-1, we internalized latex beads in J774 macrophages for 30 min. Cells were then incubated for 3 h in the presence of bafilomycin A1 (0,5 µM), a drug that inhibits the vacuolar H+ ATPase and prevents lysosome biogenesis (Clague et al., 1994), or without drug (DMSO vehicle only). To further determine if the association of flotillin-1 present on phagosomes is modulated by lumenal pH, we internalized latex beads for 60 min followed by a 3 h chase, a time point at which flotillin-1 is present in high amounts on phagosomes (see

below). Cells were then incubated for 60 min with bafilomycin as above. Phagosomes were then isolated and prepared for Western blotting.

Sensitivity to pronase

In order to determine if flotillin-1 is exposed on the cytoplasmic side of the phagosome membrane, phagosomes (1 h pulse/3 h chase) isolated as described above were treated with pronase, a mixture of proteases, as described previously (Garin et al., 2001). As demonstrated, in our previous study, pronase treatment did not affect proteins present in the lumen of phagosomes, such as cathepsins and other hydrolases, indicating that phagosomes in our preparations were intact.

Triton X-114 extraction

The phase separation of membrane proteins using Triton X-114 was performed according to the procedure previously described (Bordier, 1981) using isolated phagosomes as starting material. Proteins from the separated phases (aqueous and detergent) were solubilized in Laemmli buffer for Western blot analysis.

Lipid raft isolation

To prepare phagosome rafts, phagosomes were formed by internalizing latex beads for 60 min followed by incubation in culture medium without beads for 3 hours. For each experiment, 42 X 10 cm Petri dish were used and phagosomes isolated as described above. The purified phagosome pellet was resuspended in 1,5 ml of TNE-Triton buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail (Boehringer Mannheim), pH 7.4 and 1% Triton X-100), transferred to an Eppendorf tube and shaken 30 min at 4°C to

solubilize phagosomal membranes. Latex beads were then removed by centrifugation and the supernatant containing solubilized and insoluble phagosome components was added to 1,5 ml sucrose 90% to obtain a final concentration of 45% sucrose which was then poured at the bottom of an Ultraclear centrifuge tube (Beckman). Finally, 4 ml of 35% sucrose and 4 ml of 5% sucrose (with protease inhibitors) were layered. After a 17-20 h centrifugation at 38 000 rpm (SW41 rotor) to float the insoluble rafts, 1 ml at the 5%/35% interface containing the rafts was collected. The proteins in this fraction were then precipitated with methanol/chloroform according to Wessel and Flugge (Wessel and Flugge, 1984) and resuspended in the appropriate buffers for western blotting or 2-D gel electrophoresis. In some cases, in order to determine the distribution of flotillin-1 and LAMP1 in the gradient after the flotation step, 1 ml fractions from the top of the gradient were collected , the proteins precipitated by methanol/chloroform and resuspended in Laemmli buffer for western blot analysis.

Western blotting

For Western blot analysis, each sample in a given experiment contained the same number of phagosomes determined by evaluating the number of latex bead by FACS analysis as done previously (Desjardins et al., 1994). Western blotting was performed according to standard procedures. In the kinetic study, the membrane was cut in half between the molecular weight markers 52 and 80. The upper part was probed with the 1D4B rat monoclonal antibody (Developmental Studies Hybrodoma Bank, University of Iowa) directed against LAMP1. The lower part was probed with a rabbit polyclonal antibody specific for flotillin-1. These antibodies were raised against a synthetic peptide (Chiron Technologies, Adelaide, Australia) corresponding to the C-terminus of mouse flotillin-1 (VNHNKPLRTA) with the addition of a cysteine residue at the N-terminus for coupling to carrier protein or for preparing an affinity purification column. Affinity purification was performed exactly as described previously (Parton et al., 1997).

Appropriate second antibodies coupled to HRP were then used and the membranes treated for ECL (Roche Diagnostics).

The presence of flotillin-1 and LAMP1 in phagosomes and phagosome rafts was evaluated by Western blot on the same membrane (see above). The same amount of protein was loaded for each sample. For 2-D gel Western blot, the portion of the gel corresponding to the area where flotillin-1 was identified was transferred to nitrocellulose membrane and immunoblotted with the anti-flotillin-1 antibody as described above.

Immunofluorescence

J774 macrophages were grown on coverslips to a confluency of about 80%, at least 36 h before the experiment. Cells were then fed or not with 3 µm latex beads (Sigma) (3 µm beads rather than 0,8 µm beads were used to facilitate microscopy observations) in culture medium for 30 min (1/200) or 60 min (1/400) followed by chase periods of 1, 3 and 16 hours. In some cases, cells were infected with *Leishmania donovani* strain 1S grown as described (Duclos et al., 2000) at a concentration of 2,0 and 1.0 X 10^7/ml medium for 60 min, or with an *lpg2-/-* mutant lacking surface LPG (Desjardins and Descoteaux, 1997) followed by a 3 h chase. Cells were then fixed at –20°C in methanol 80%/acetone 20% for 20 min. Fixed cells were then washed and rehydrated in PBS 2 times 5 min, 2 times 10 min and blocked 10 min with PBS 2% BSA (fraction V, Sigma, St-Louis, MO), 0,2% gelatin. Coverslips were then incubated with the rabbit anti-flotillin-1 antibody and the rat anti-LAMP1 1D4B for 1 h. In the case of *Leishmania* infection, cells were incubated with the CA7AE antibody directed against the major surface glycoconjugate of *Leishmania* (Tolson et al., 1989) to visualize the parasites within cells and with the anti-flotillin-1 antibody. After several washes in PBS 1% BSA, coverslips were incubated with an anti-rabbit IgG coupled to Alexa and with an anti-rat IgG coupled to Texas Red for 1 h. Coverslips were then washed in PBS, mounted on slides with Gelvatol and observed at the epifluorescence

or confocal microscope. Controls included the tests for interspecies cross-reaction and cells incubated only with the secondary antibodies.

High resolution 2-D gel electrophoresis

Samples destined for 2-D gel electrophoresis were prepared from cells metabolically labeled with 35S-methionine following published protocols (Desjardins et al., 1994). The various samples were first separated according to their isoelectrical point using immobilized pH-gradient strips (IPGs). Equal counts of radioactivity were loaded for each sample in a given experiment. Loading of the samples in the first dimension was performed by in-gel reswelling (Pasquali et al., 1997). At the end of the first dimension, the strips were equilibrated by a 10 min incubation in equilibration solution (urea 6M, SDS 2%, glycerol 20%, Tris-HCl 1,0 M pH 6,8) freshly supplemented with DTE (2% w/v) followed by a 5 min incubation in equilibration solution freshly supplemented with iodoacetamide (2,5% w/v), and the proteins separated according to their molecular mass using standard SDS-PAGE. At the end of the migration, gels were treated for autoradiography as described previously (Desjardins et al., 1994). In order to identify some of the lipid raft proteins according to their migration properties, protein patterns of rafts preparation were compared with a phagosome 2-D gel database in which 140 protein spots have been identified so far (Garin et al., 2001).

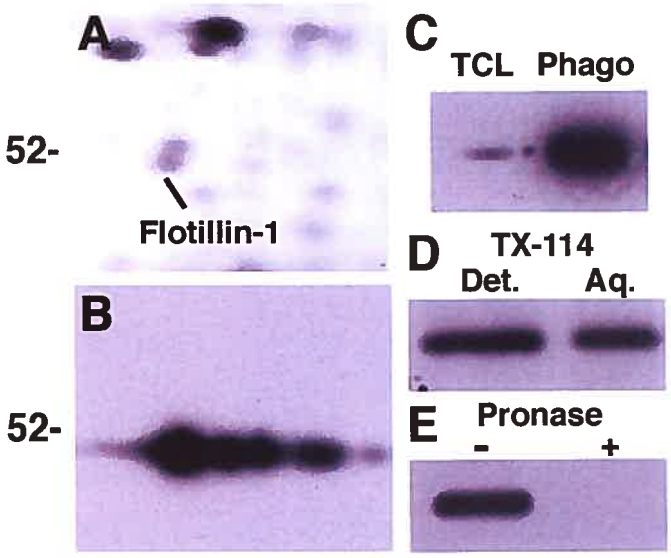
Results and discussion

In this study, we provide evidence that lipid rafts are present on the phagosome membrane, a key organelle involved in the killing and degradation of intracellular pathogens (Méresse et al., 1999). The existence of lipid subdomains on phagosomes was first suggested by a proteomic analysis indicating that proteins known to associate to lipid rafts, including flotillin-1, are present on this organelle (Garin et al., 2001). Here, we further demonstrate

the enrichment of flotillin-1 on phagosomes by Western blot analysis in both 1- and 2-D gels (Fig. 1A-C). The association of flotillin-1 to the phagosome membrane was confirmed by Triton X-114 extraction showing that a great proportion of this protein partitioned in the detergent phase (Fig. 1D), and pronase proteolysis experiments indicating that flotillin-1 (at least the C-terminal end recognized by our antibody) is exposed on the cytoplasmic side of the phagosome membrane (Fig. 1E).

Figure 1 Flotillin-1 is present and enriched on the phagosome membrane.

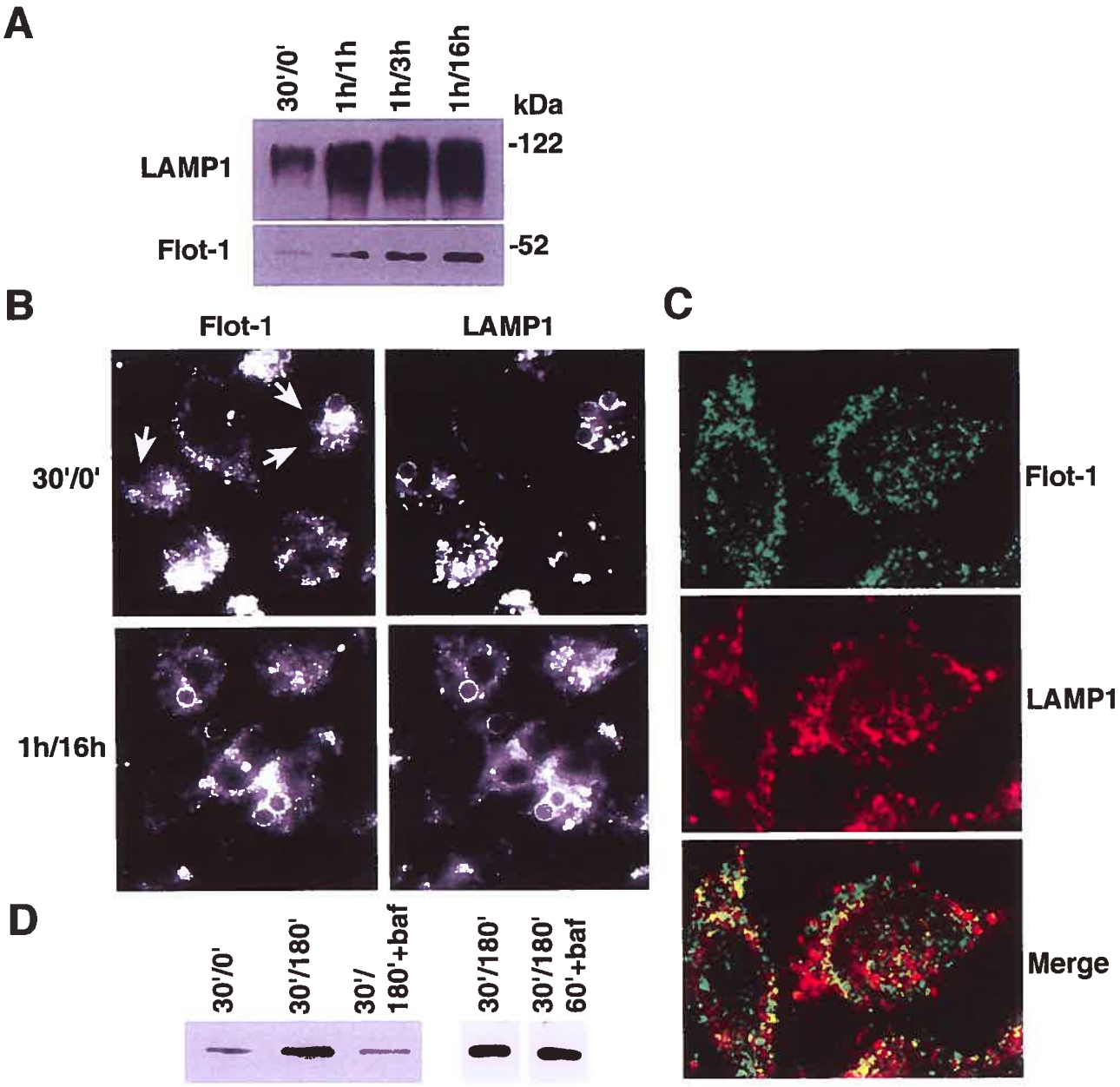
A) Phagosomes were isolated from J774 macrophages and 2-D-gel electrophoresis was performed with immobilized-pH gradients in the first dimension and SDS-PAGE in the second dimension, following standard procedures. The spot corresponding to flotillin-1 was previously identified by a proteomic approach (see 8) B) An area of a 2D gel corresponding to the location of flotillin-1 was cut off and transferred to nitrocellulose for immunoblotting with the anti-flotillin-1 antibody. Several spots at the same molecular weight with different pI were revealed. C) Western blot from SDS-PAGE gels indicates that flotillin-1 is highly enriched on phagosomes (Phago) compared to total cell lysate (TCL). In each lane, equal amounts of protein were loaded. D) Western blot analysis indicates that flotillin-1 is partially recovered in the detergent phase of a Triton X-114 phagosome extract, as expected for a membrane-associated protein. The presence of flotillin-1 in the aqueous phase could imply that this protein is loosely associated to phagosomal membrane. E) Phagosome fractions were incubated 30 min at 37°C in the presence or absence of pronase. This treatment degrades all proteins or portion of proteins exposed on the cytoplasmic side of phagosomes. The anti-flotillin-1 antibody, which recognizes the C-terminal portion of the protein, failed to reveal the protein in the fraction treated with pronase, indicating that this part of the protein is present on the cytoplasmic side of phagosomes.


A
52-
Flotillin-1
B
52-
C
TCL
Phago
D
TX-114
Det.
Aq.
E
Pronase
-
+

The latter experiment rules out the possibility that flotillin-1 is simply present within the lumen of phagosomes for degradation. Although flotillin-1 was originally shown to accumulate in subdomains of the plasma membrane of adipocytes and neurons (Bickel et al., 1997, Lang et al., 1999), our studies using immunofluorescence analysis failed to detect noticeable levels of flotillin-1 on the plasma membrane of macrophages. Furthermore, biochemical (Fig. 2A) and morphological (Fig. 2B) analyses indicated that flotillin-1 is barely detectable on early phagosomes (derived from the plasma membrane), but accumulates on maturing phagosomes. Interestingly, our results showed that flotillin-1 associates to phagosomes at later time points than LAMP1, a marker normally used to define late endocytic/phagocytic structures (Fig. 2A and B). In cells that had not internalized latex beads, observation at the confocal microscope revealed that although a small part of the flotillin-1 labeling is present on vesicles also labeled for LAMP1, most of the labeling does not colocalize to the same vesicle populations (Fig. 2C), suggesting that these markers are distributed on different vesicles of a common pathway. These results also indicate that flotillin-1 is a novel marker of late endocytic/phagocytic organelles that may accumulate on post-LAMP structures. This is supported by results showing that bafilomycin, a drug that inhibit the formation (or maturation) of lysosomes (Clague et al., 1994), also inhibits the accumulation of flotillin-1 to phagosomes (Fig. 2D).

Figure 2 Flotillin-1 accumulates on phagosomes during maturation.

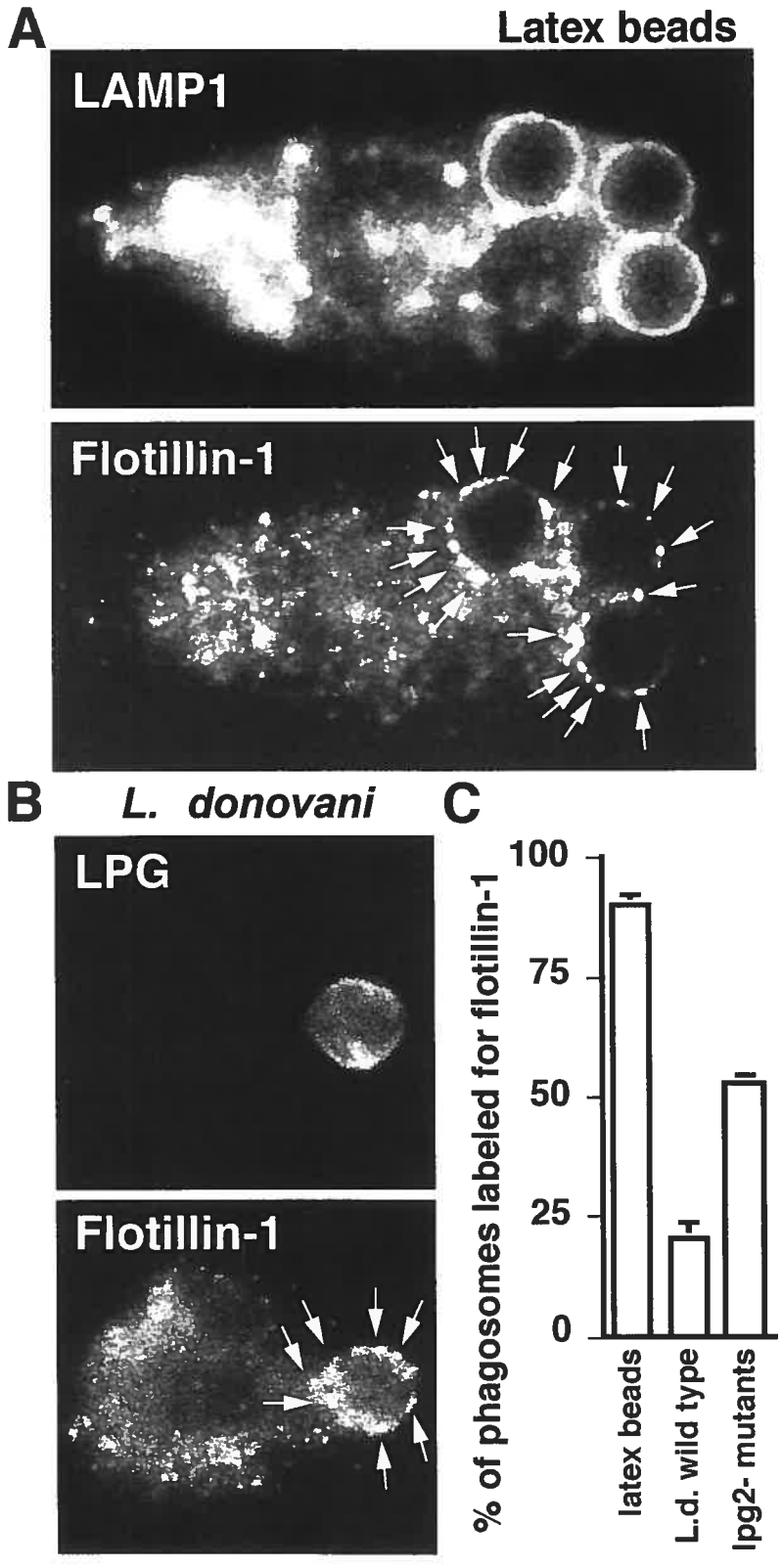
A) Western blotting was performed on purified phagosomes of increasing ages formed by the internalization of latex beads for 30 minutes internalization/ no chase (early phagosomes) to 1 hour internalization/16 hour-chase (late phagosomes). The membrane was cut in half to reveal LAMP1 and flotillin-1 on the same samples. Each lane was loaded with the same number of phagosomes determined by flow cytometry. The results indicate that flotillin-1 accumulates on maturing phagosomes and thus represents a late phagocytic marker. B) Double immunofluorescence analysis with flotillin-1 and LAMP1 antibodies confirms that flotillin-1 accumulates on late phagosomes. It also clearly shows that flotillin-1 appears to be a later marker than LAMP1, as shown by the absence of flotillin-1 labeling on most of the early phagosomes (arrows). C) J774 macrophages that had not internalized latex beads were processed as described for double immunofluorescence. Observation by confocal microscopy indicates that there is very little colocalization of flotillin-1 with vesicular structures labeled for LAMP1. D) Cells were fed with latex beads for 30 min. Phagosomes were then either isolated immediately or allowed to mature for 3 h in the presence or absence of bafilomycin A1, an inhibitor of the vacuolar H^+ ATPase, and processed for Western blotting. Inhibition of endovacuolar acidification prevented the recruitment of flotillin-1 to phagosomes, indicating that this process is pH-dependent. In contrast, treatment of cells already containing mature flotillin-1-enriched phagosomes (lane 4 and 5) did not release this protein from phagosomes, indicating that the association of flotillin-1 to phagosomes is not regulated by the lumenal pH.


A
30'/0'
1h/1h
1h/3h
1h/16h
kDa
LAMP1
-122
Flot-1
-52
B
Flot-1
LAMP1
30'/0'
1h/16h
C
Flot-1
LAMP1
Merge
D
30'/0'
30'/180'
30'/ 180'+baf
30'/180'
30'/180' 60'+baf

At high magnification, double immunofluorescence labeling clearly indicates that flotillin-1 is present on patches of the phagosome membrane while LAMP1 forms a uniform ring around the membrane of this organelle (Fig. 3A). To demonstrate that flotillin-1 is a general marker of phagosomes, and not simply associated with latex-containing compartments, we showed by immunofluorescence its presence on phagosomes housing the intracellular parasite *Leishmania* (Fig. 3B). However, we observed that only a small proportion of *Leishmania*-containing phagosomes were positive for flotillin-1. Indeed, quantitative analysis indicates that over 90% of latex bead-containing phagosomes are positive for flotillin-1, whereas only 20% of phagosomes housing *Leishmania* parasites are labeled by the antibody (Fig. 3C). We have shown previously that the promastigote form of *Leishmania* parasites are able to inhibit phagosome fusion with late endocytic organelles (14). This inhibition is caused by the lipophosphoglycan (LPG), the major surface glycoconjugate of *Leishmania*, since mutants lacking LPG fuse extensively with late endocytic organelles (Dermine et al., 2000). Accordingly, we performed additional experiments and measured the presence of flotillin-1 on phagosomes containing *Leishmania lpg2-/-* mutants. The results obtained indicate that 53% of phagosomes containing that mutant are positive for flotillin-1. This suggests that flotillin-1 might be necessary for, or acquired through, fusion with late endocytic organelles. Interestingly, LPG is a GPI-anchored molecule secreted by the parasite. Its mode of action in the inhibition of phagosome-endosome fusion was proposed to involve its insertion, through the lipidic anchor, in the phagosomal membrane (Desjardins and Descoteaux, 1997; Miao et al., 1995). Since GPI-anchors have a strong affinity for lipid rafts (Sargiacomo et al., 1993), this process could interfere with the formation of lipid rafts on *Leishmania*-containing phagosomes, or the association of flotillin-1 to these structures. Other *Leishmania* LPG-deficient mutants are currently tested in our system to further ensure the role of that molecule in the modulation of raft formation.

Figure 3 Flotillin-1 is present in sub-domains of the phagosome membrane.

A) J774 macrophages were fed with latex beads for 60 min followed by a 3 h incubation in normal medium. Double-immunofluorescence was then performed to localize LAMP1 and flotillin-1. The results indicate that while LAMP1 covers the whole surface of latex bead-containing phagosomes, flotillin-1 is present on distinct regions of the phagosome membrane (arrows). B) J774 macrophages were infected with the intracellular pathogen *Leishmania donovani* for 60 min followed by a 3 h incubation in normal medium. Double-immunofluorescence was then performed to localize flotillin-1 and the leishmanial molecule LPG. The results indicate that pathogen-containing phagosomes also display a punctate labeling for flotillin-1 (arrows), although only a very small proportion of phagosomes are labelled. C) Quantitative analysis show that 20% of *Leishmania donovani* wild type-containing phagosomes are positive for flotillin-1. In contrast, 53% of the *lpg2 -/- Leishmania*-containing phagosomes and 90% of latex beads-containing phagosomes are labeled for flotillin-1. This result represents the mean of three separate experiments.


A
Latex beads
LAMP1
Flotillin-1
B
L. donovani
LPG
Flotillin-1
C
% of phagosomes labeled for flotillin-1
100
75
50
25
0
latex beads
L.d. wild type
lpg2- mutants

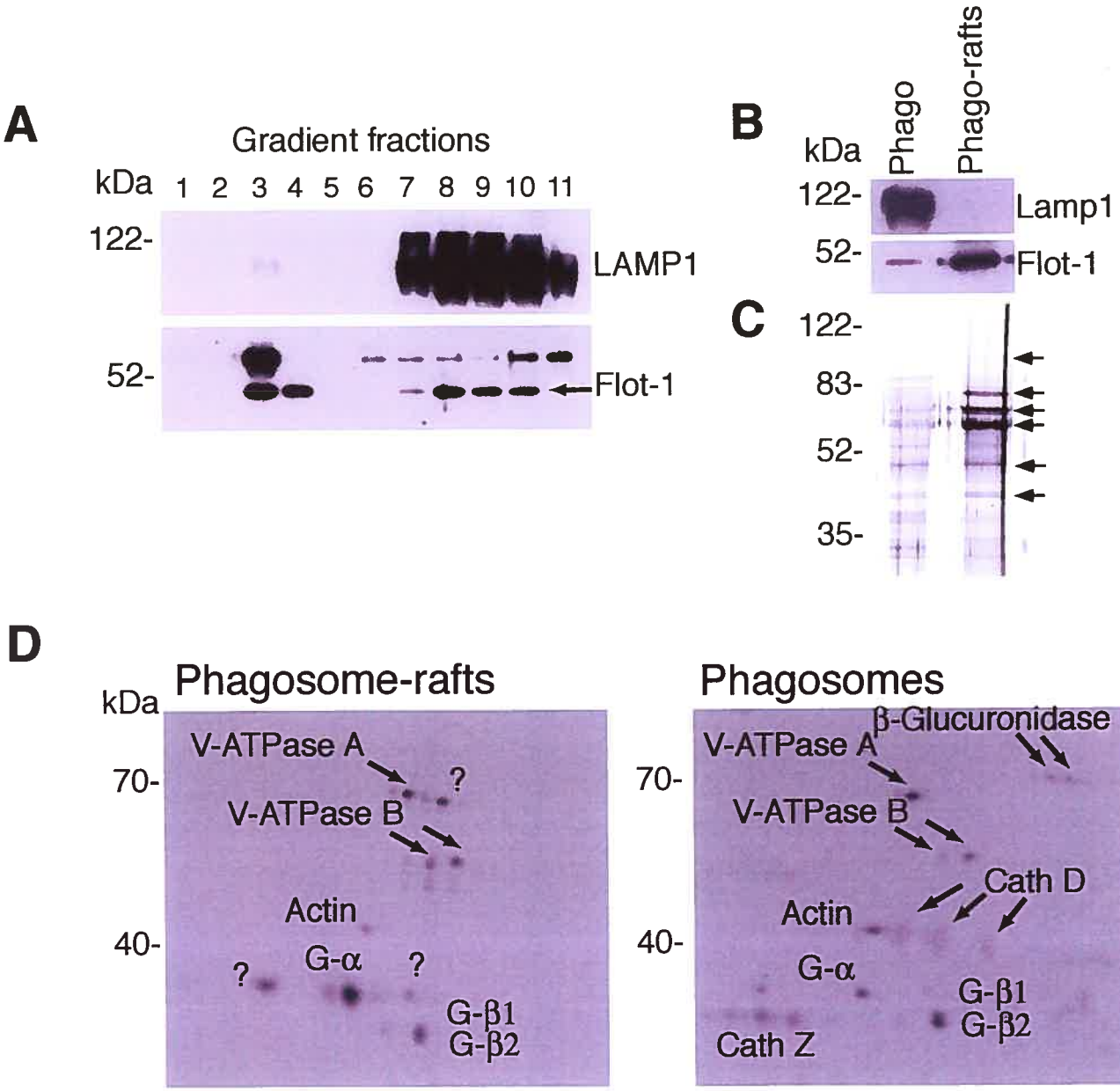
The properties governing the association of flotillin-1 to phagosome lipid rafts are unknown. The presence in its structure of a Prohibitin Homology (PHB) Domain, also referred to as Stomatin, Prohibitin, Flotillin, HflC and K (SPFH) Domain (Tavernarakis et al.,1999), might allow its association to lipid rafts. As the name implies, this domain is also present on prohibitin and stomatin, two membrane proteins shown to associate with Triton X-100-insoluble rafts (Terashima et al., 1994; Snyers et al., 1999). Interestingly, both prohibitin and stomatin have been identified on latex bead-containing phagosomes by mass spectrometry (Garin et al., 2001). Alternatively, results by Western blot analysis on 2-D gels showing that flotillin-1 migrates as a series of spots of different pI suggest that flotillin-1 might be hyperphosphorylated (Fig. 1B). This feature may also provide a potential mechanism of association to lipid rafts, as shown for $p56^{lck}$, whose segregation in lipid domains of the plasma membrane is linked to its phosphorylation state (Rodgers and Rose, 1996).

The presence of lipid rafts on phagosomes is surprising since these structures have been described mainly in the Golgi apparatus and the plasma membrane. Despite the phagosomal distribution of flotillin-1 in raft-like structures, it was important to establish whether these structures correspond to the biochemical definition of rafts which is the insolubility in Triton X-100 and low density in sucrose gradients. To address this point we performed raft isolation from purified phagosomes and tested them for the presence of flotillin-1 by Western blotting (Fig. 4A). Our results clearly showed an enrichment of flotillin-1 in the phagosome-rafts fraction compared to total phagosomes (Fig. 4B). In contrast, LAMP1, a major membrane protein of phagosomes, was not detected in the phagosome-rafts fraction, demonstrating the specificity of our extraction procedure. Our results also showed that a significant portion of flotillin-1 present on phagosomes is solubilized by the triton X-100 treatment (Fig. 4A), suggesting that some of the phagosomal flotillin-1 is not associated with lipid rafts. It is also possible that flotillin-1-enriched rafts are partially solubilized by the detergent since it was shown that lipid rafts displaying different sensitivities to solubilization, depending on the detergent used, can coexist in the same cells (Roper et al., 2000).

SDS-PAGE analysis indicated that several proteins are enriched in the phagosome-rafts fraction compared with total phagosomes (arrows in Fig. 4C). In order to identify some of the proteins present in lipid rafts, we used a proteomic approach. The proteins recovered in phagosome lipid rafts isolated from [35S] methionine-labeled cells were separated by 2-D gel electrophoresis. The gels were dried and exposed for 6 weeks, and then analyzed and compared with gels of total phagosomes. This allowed identification of phagosome proteins present in the rafts. Using our database of identified phagosomal proteins (Garin et al., 2001), we were able to show that actin, the α, β1 and β2 subunits of heterotrimeric G-proteins, as well as the A, B and possibly the E subunits of the vacuolar proton pump ATPase were among the major proteins of the Triton X-100 insoluble lipid rafts (Fig. 4D). At least 9 as yet unidentified proteins were also enriched in the phagosome lipid rafts preparations (3 of which are highlighted by question marks in Fig. 4C). Subunits of heterotrimeric G-proteins have been identified in lipid rafts in other studies (Lisanti et al., 1994). Their identification was instrumental to the proposal that rafts are specialized sites for signal transduction (Kurzchalia and Parton, 1999). Our findings suggest that signal transduction could also take place through specialized regions of the phagosome membrane. Subunits of the proton pump ATPase have also been identified previously in Triton X-100 insoluble fractions (Galli et al., 1996), in association with proteins of the SNARE complex, suggesting that control of fusion events (see below), through acidification, could involve lipid rafts.

Figure 4 Flotillin-1 and other proteins are enriched in phagosome lipid rafts of J774 macrophages.

Lipid rafts from purified phagosomes were isolated based on their insolubility in Triton X-100 and their flotation on sucrose gradients. A) 1 ml aliquots starting at the top of the gradients were collected and analyzed by Western blot. The results indicate that fractions 3 and 4, corresponding to the interface of the 5% and 35% sucrose where rafts are recovered, contain the bulk of flotillin-1 and are devoid of LAMP1. All the LAMP1, as well as a fraction of the flotillin-1, is present in the fractions at the bottom solubilized by Triton X-100. The band corresponding to the molecular weight of flotillin-1 (48 kDa) is pointed by the arrow (the identity of the high molecular weight band is not currently known). B) As shown by Western blotting, flotillin-1 is enriched in the Phago-rafts compared to total phagosomes (Phago). In contrast, LAMP1, present in the total phagosome sample, is absent from the phagosome rafts. C) SDS-PAGE analyses of total phagosomes and phagosome rafts stained with silver nitrate indicate that several proteins are enriched in the lipid raft domains (arrowheads). D) To identify some of the proteins present in lipid subdomains, phagosomes and phagosome lipid rafts were isolated from metabolically labeled cells and their proteins separated by 2-D gel electrophoresis. Analysis of the 2-D protein patterns after autoradiography indicated that only a subset of the total phagosome proteins was present in the rafts (Only a portion of the gel where proteins are clearly visible is represented). The protein spots in both fractions were identified by comparison against a 2-D gel phagosome database (see Experimental procedures). Note the absence of soluble hydrolases like cathepsins D and Z and β-glucuronidase in the raft preparations indicating, as expected, that soluble proteins are not present in the lipid rafts. Question marks indicate unknown proteins.


A
Gradient fractions
kDa
1 2 3 4 5 6 7 8 9 10 11
122-
LAMP1
52-
Flot-1
B
kDa
Phago
Phago-rafts
122-
Lamp1
52-
Flot-1
C
122-
83-
52-
35-
D
kDa
Phagosome-rafts
V-ATPase A
?
V-ATPase B
Actin
70-
40-
?
G-α
?
G-β1
G-β2
Phagosomes
β-Glucuronidase
V-ATPase A
V-ATPase B
Cath D
Actin
G-α
G-β1
G-β2
Cath Z

Although flotillin-1 was recently shown to be involved in insulin signaling at the plasma membrane of adipocytes (Bauman et al., 2000), the functions of this protein and, more generally, of lipid rafts on phagosomes are currently unknown. An interesting feature of phagosomes is that it is an organelle unable to perform its main task, the killing and degradation of microorganisms, immediately after its formation at the plasma membrane. Indeed, the acquisition of phagosome functional properties depends on complex sets of interactions with various cellular organelles, leading to the biogenesis of phagolysosomes (Desjardins, 1995). Studies of this complex process in the last few years has put forward at least two types of interaction required for phagolysosome biogenesis. First, phagosomes must bind and move along cytoskeletal elements, both microtubules and actin filaments, in order to encounter and interact with other endovacuolar organelles (Desjardins et al., 1994, Defacque et al., 2000). Second, phagosomes must recognize and fuse with these endovacuolar organelles to allow the transfer of important microbicidal molecules to the phagosome lumen. Interestingly, data from the study of phagolysosome biogenesis, as well as analyses of lipid raft composition and function support the idea that specialized subdomains of the phagosome membrane might play key roles in both types of interactions. Biochemical analyses have shown that actin and actin binding proteins are closely associated with phagosomes (Desjardins et al., 1994) and that this organelle has the ability to induce the nucleation of actin at certain foci on its membrane (Defacque et al., 2000). Interestingly, the later study demonstrated that late phagosomes are more efficient at inducing actin nucleation, in accordance with a potential role for flotillin-1 and lipid rafts in this process, which accumulate on maturing phagosomes. Proteomic analysis of phagosome lipid rafts indicated that actin is a major protein of these structures, in accordance with recent results showing that lipid rafts are the sites of actin accumulation and polymerization (Rozelle et al., 2000). Allen and Aderem (1996) have also published results clearly showing the focal recruitment of the actin associated molecules vinculin and paxillin to phagosomes.

The presence of molecules of the fusion machinery in membrane subdomains indicates that specialized regions of biological membranes might also favor membrane

fusion (Kurzchalia and Parton, 1999; Schnitzer et al., 1995). There is increasing evidence that fusion between phagosomes and endosomes might take place preferentially at certain sites on the membrane of these organelles. Stahl and colleagues (Roberts et al., 1999) have shown that phagosome-endosome fusion is initiated at "hot spots" on membranes where rab5 accumulates. Focal distribution of EEA1, a rab5 effector of endosome/phagosome fusion, was also observed at the surface of early endosomes (McBride et al., 1999). Interestingly, phagosome-endosome fusion also appears to involve transient interactions of parts of their membranes, a process referred to as kiss and run fusion (Desjardins et al., 1994; Desjardins, 1995). According to the kiss and run hypothesis, fusion between these organelles is initiated by the formation of a fusion pore that allows transient exchange of lumenal molecules. However, the expansion of the pore is limited and does not lead to the complete fusion of the organelles. Instead, the fusion pore closes allowing the separation of phagosomes and endosomes. Confirmation that transient fusions occur between phagosomes and endosomes was shown by the fact that molecules of different sizes present in the same endosomes are not transferred to phagosomes simultaneously (Wang et al., 1987). Instead, small molecules are transferred while larger molecules remain in endosomes (Desjardins et al., 1997). Similar results are also observed between endosomes along the endocytic pathway (Berthiaume et al., 1995). Remarkably, the kiss and run fusion is regulated, in part, by the small GTPase rab5, as shown by the loss of size selectivity in the transfer of solute materials from endosomes to phagosomes in Raw 264.7 macrophages expressing the active GTP-bound form of rab5 (Duclos et al., 2000). Interestingly, current models of the fusion pore predict that lipidic pores could either expand irreversibly or remain open for several seconds and then close if slight changes in the membrane lipid composition were to occur (Nanavati et al., 1992). In this context, the presence of lipidic microdomains on the phagosome membrane could rapidly provide the lipid changes required for the fusion pore closure.

This study extends current models of lipid raft microdomain formation to the membrane of phagosomes. Segregation of lipids and proteins within the phagosomal membrane may provide focal points on which complexes of signaling proteins or proteins

of the fusion machinery can assemble, and where specialized functions may occur. Phagosomes have considerable advantages in the study of the function of lipid rafts since these organelles can be formed and isolated at will under various cellular conditions, and experimentally manipulated in *in vitro* assays.

Chapitre IV. Résultats. Article 3

Dermine J.-F., Goyette G., Letarte S., Houde M., Turco S.J., Desjardins M. *Leishmania* survival strategy in macrophages relies on the disruption of phagosome microdomains by its lipophosphoglycan. Soumis Nature Immunology (Juillet 2004)

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Leishmania survival strategy in macrophages relies on the disruption of phagosome microdomains by its lipophosphoglycan

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Abstract

The rapid clearance of pathogens at sites of infection by phagocytosis and their killing in phagolysosomes is a key aspect of our innate ability to fight infectious agents. However, pathogens have evolved ways to avoid the harsh environment of phagolysosomes either by escaping phagosomes or by inhibiting their maturation. We show here that the intracellular parasite *Leishmania* has evolved a novel survival strategy in its mammalian hosts involving the use of its surface lipophosphoglycan (LPG), a virulence factor that has pleiotropic effects on host functions, to prevent the formation or disrupt lipid microdomains on the phagosome membrane. LPG acts locally on the membrane and requires its sugar repeating units to alter microdomains organization. The disruption of these foci of specialized functions, where proteins involved in key aspects of phagolysosome biogenesis assemble, confers a clear survival advantage to the parasite. *Leishmania* is thus the first pathogen shown to disorganize phagosome lipid microdomains for its survival in mammals.

Introduction

Phagocytosis, the mechanism used by a variety of cells to internalize large particles, plays a key role in our innate ability to restrict the spread of infectious diseases. Microorganisms internalized by phagocytosis are sequestered in phagosomes, an organelle formed by the invagination of the plasma membrane and the rapid recruitment of endovacuolar membranes from endosomes and the endoplasmic reticulum (Scott et al., 2003; Niedergang et al., 2003; Gagnon et al., 2002; Desjardins et al., 2003). Newly formed phagosomes are inapt at killing and degrading pathogens. They acquire their microbicidal properties through a complex maturation process, involving sequential fusion with endocytic organelles (Desjardins et al., 1994; Desjardins et al., 1997; Desjardins and Descoteaux, 1997; Jahraus et al., 1998), leading to the formation of phagolysosomes. The complexity of phagolysosome biogenesis is only beginning to be estimated, as it encompasses the coordinated activity of hundreds of proteins (Garin et al., 2001; Brunet et al., 2003).

Through evolution, pathogens have developed strategies to adapt and survive in their hosts by avoiding the harsh environment of phagolysosomes. For example, bacteria like *Listeria* and *Shigella* do so by escaping phagosomes to invade and replicate within the cytoplasm (Cossart et al., 2003; Tran Van Nhieu et al., 2000). Organisms like *Salmonella*, *Legionella* and *Brucella* rather remain in phagosomes and use either type III or type IV secretion systems, to inject virulence factors in the host cytoplasm, where they interfere with specific cell pathways influencing phagolysosome biogenesis (Gruenheid and Finlay, 2003; Nagai and Roy, 2003). These virulence factors can either be injected through the plasma membrane while the pathogen sits at the cell surface or through the phagosome membrane, after its engulfment by host cells.

Parasites of the genus *Leishmania* rely on two obligate hosts; a blood feeding insect and a mammal that is also the blood source of the insect. Within the insects, the parasites develop as extracellular flagellated promastigotes. Once delivered in the skin of a mammalian host, the parasites are rapidly cleared and phagocytozed by professional phagocytes such as polymorphonuclear cells or macrophages. The development of the parasites then depends on their ability to establish a proper intracellular niche whitin their host where they can differentiate into non-flagellated amastigotes and replicate. Remodeling of phagosomes and inhibition of phagolysosome biogenesis to avoid the harsh environment of these organelles is a key aspect of this process. However, the absence of secretion systems in *Leishmania*, allowing for the disturbance of host cellular functions through molecules secreted in the cytoplasm, implies that a direct action on the phagosome membrane must occur from within the organelle lumen where promastigote parasites are located. Evidence indicated that lipophosphoglycan (LPG), one of the key virulence factors present at the surface of *Leishmania* promastigotes (Turco et al., 2001), is used to inhibit phagolysosome biogenesis by preventing phagosome-lysosome fusion (Desjardins and Descoteaux, 1997; Dermine et al., 2000, Spath et al., 2003). The precise way by which LPG interferes with phagosome functions is still unknown. In the present study, we show that *Leishmania* promastigotes can directly alter phagosome functional properties, and evade the harsh environment of phagolysosomes, by using its LPG to prevent the formation

of lipid microdomains or disrupt these structures in the phagosome membrane. By targeting phagosome microdomains, parasites gain a major survival advantage as they alter specialized foci containing proteins involved in important aspects of phagolysosome biogenesis, such as signal transduction, actin nucleation and membrane fusion.

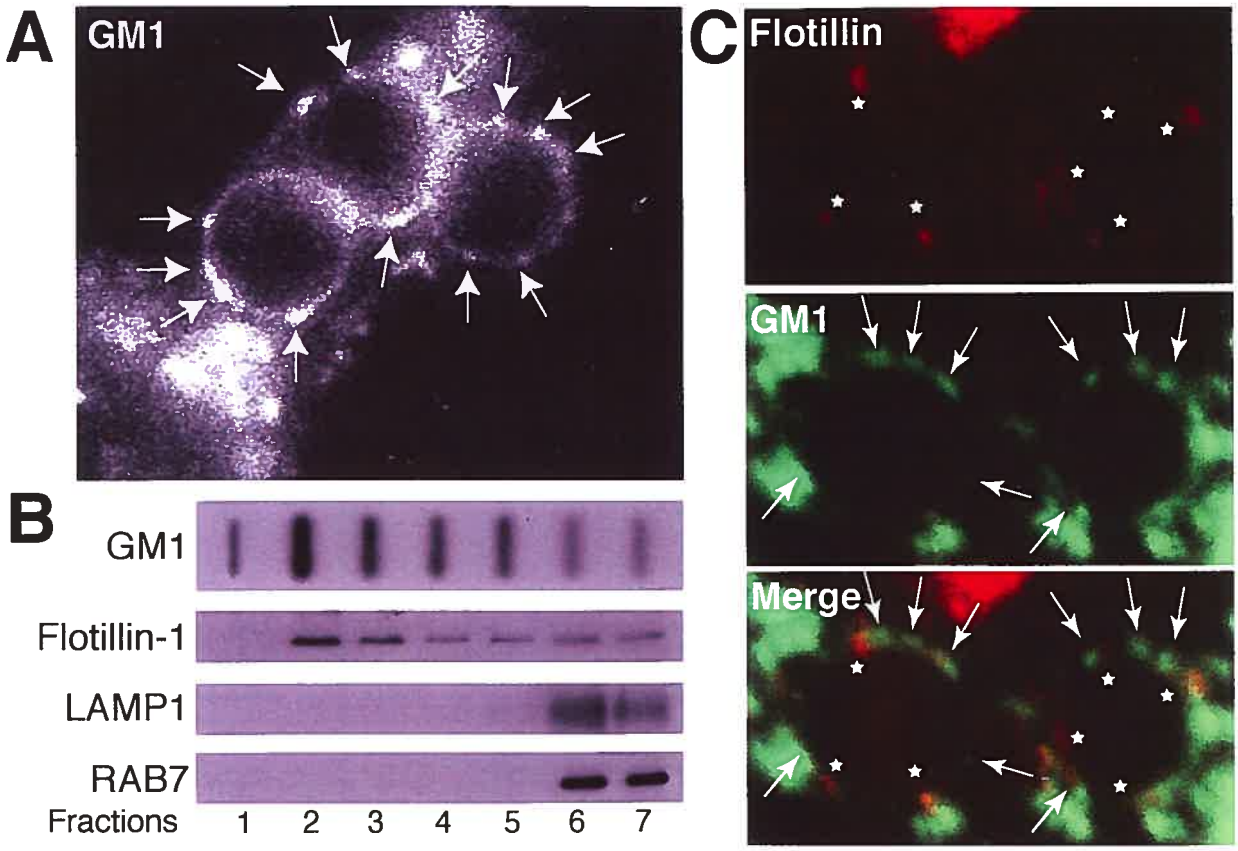
Results

Multiple microdomains on the phagosome membrane

In a previous study, we provided biochemical and morphological evidence that lipid microdomains are present on phagosomes (Dermine et al., 2001). These microdomains are enriched with the protein flotillin-1, which is present in lower abundance on newly formed phagosomes and accumulates on "maturing" phagosomes. These results indicated that flotillin-1-enriched microdomains are not acquired during the early process of phagocytosis at the cell surface but rather originate from an intracellular source. In order to further characterize phagosome microdomains, we examined whether the ganglioside GM1, a well-known marker of rafts, was also present on phagosomes. When the surface of cells already containing phagosomes was labeled for GM1 using cholera toxin coupled to FITC (CTB-FITC), further incubation allowed this lipid to reach the phagosome membrane within 30 min and accumulate on that organelle for up to 120 min (fig. 1A). The labeled GM1 was not present all over the phagosome membrane but rather observed in sub-regions of the membrane (dotted labeling), in a pattern similar to the one previously described for flotillin-1 (Dermine et al., 2001). Western blot analysis of Triton X-100 insoluble fractions

Figure 1 Distinct microdomains are present on the phagosome membrane

A) J774 macrophages were fed latex beads for 30 min followed by a 30 min chase. Plasma membrane GM1 ganglioside was then labeled with CTB-FITC at 4°C, to prevent the internalization of CTB-FITC in the fluid phase. Labeled GM1 was next chased for 2 hours at 37°C. GM1 accumulates in a dotted pattern on the phagosome membrane (arrows). B) Lipid microdomains were isolated from purified phagosomes based on their insolubility in Triton X-100 and their floatation on Optiprep^{tm} gradients. GM1 and flotillin-1 are enriched in fractions 2 and 3 where lipid microdomains are normally located. LAMP1 and Rab7 are soluble in Triton X-100 and remain in the bottom fractions. C) Phagosomes were labeled for GM1 as described in A). Cells were then fixed and labeled with the anti-flotillin-1 antibody. These 2 raft markers localize in distinct regions of the phagosome membrane, indicating that multiple lipid microdomains exist on phagosomes.


A
GM1
B
GM1
Flotillin-1
LAMP1
RAB7
Fractions
1
2
3
4
5
6
7
C
Flotillin
GM1
Merge

isolated from purified phagosomes confirmed the presence of GM1 in lipid microdomains where flotillin-1 is also present (figure. 1B). These results indicate that in addition to being able to recruit or assemble microdomains from internal sources (flotillin-1), phagosomes can also receive microdomain molecules and/or assembled microdomains from the cell surface (GM1).

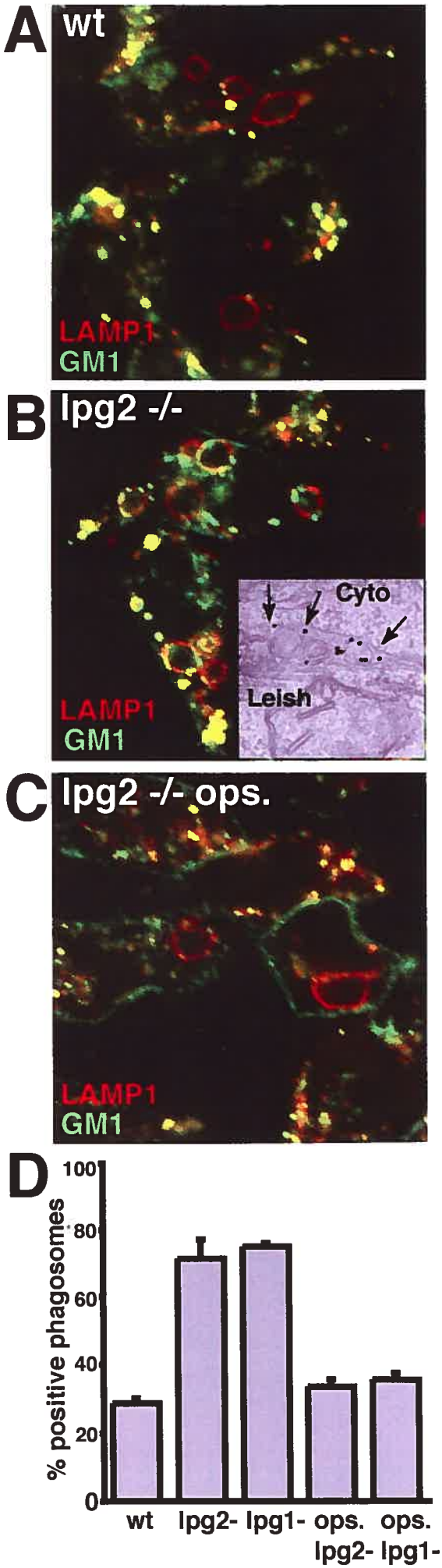
In order to determine if flotillin-1 and GM1 are present within the same domains of the phagosomal membrane, we performed double-fluorescence analysis in cells containing latex phagosomes (fig. 1C). Confocal microscopy indicated that most of the labeling for flotillin-1 and GM1 do not co-localize on the phagosome membrane, indicating that different types of microdomains are present on phagosomes.

Leishmania promastigotes use their surface lipophosphoglycan (LPG) to prevent the assembly of GM1-enriched microdomains

We have shown that *L. donovani* promastigotes, the form that initiates infection in mammals, reside in phagosome devoid of flotillin-1-enriched lipid microdomains (Dermine et al., 2001). This observation prompted us to investigate if the recruitment of GM1 from the cell surface was impaired in phagosomes containing this form of the parasite. In contrast to latex bead-containing phagosomes (fig. 1A), the phagosomes of cells loaded with *L. donovani* promastigotes were unable to acquire the labeled cell surface GM1 (fig. 2A). However, the membrane of phagosomes containing mutant parasites lacking all phosphoglycan-containing glycoconjugates, including LPG, due to a defective Golgi GDP-Man transporter (lpg2-/-) (Descoteaux et al., 1995), displayed a strong dotted signal (fig. 2B). Similar results were obtained with another LPG mutant, defective in the assembly of its glycan core region (lpg1-/-) (Ryan et al., 1993). In some cases, because of the low resolution of the confocal microscope, part of the labeling appeared as if the GM1 was

Figure 2 *Leishmania* parasites use their surface LPG to prevent the recruitment of GM1-enriched microdomains

J774 macrophages were exposed to wild-type *Leishmania donovani* promastigotes (A), lpg2-/- mutants lacking LPG (B), or lpg2-/- mutants opsonized with purified LPG (C) for 30 min followed by a 30 min chase. The plasma membrane GM1 was then labeled with CTB-FITC as in Figure 1A. The cells were fixed and labeled for LAMP1 (red labeling) to identify the phagosomes. A) Wild type parasites prevent the phagosomal acquisition of surface GM1 as shown by the exclusion of CTB-FITC labeling on phagosome membranes. B) Several GM1-enriched microdomains are formed on phagosomes containing lpg2-/- mutants. (Insert) At the electron microscope, the use of CTB-gold to label surface GM1 indicates that GM1-enriched microdomains are present within the phagosome membrane and not in vesicles surrounding phagosomes, as shown by the presence of the gold particles on the luminal side of phagosomes. Arrows indicate the phagosome membrane surrounding a *Leishmania* parasite (Leish). C) Phagosomes containing opsonized mutants display a labeling similar to the one observed for wild type parasites. D) Quantitative analysis of the percentage of phagosomes displaying GM1-enriched microdomains. Error bars indicate the mean standard error.


A
wt
LAMP1
GM1
B
lpg2 -/-
Cyto
Leish
LAMP1
GM1
C
lpg2 -/- ops.
LAMP1
GM1
D
% positive phagosomes
100
80
60
40
20
0
wt
lpg2-
lpg1-
ops. lpg2-
ops. lpg1-

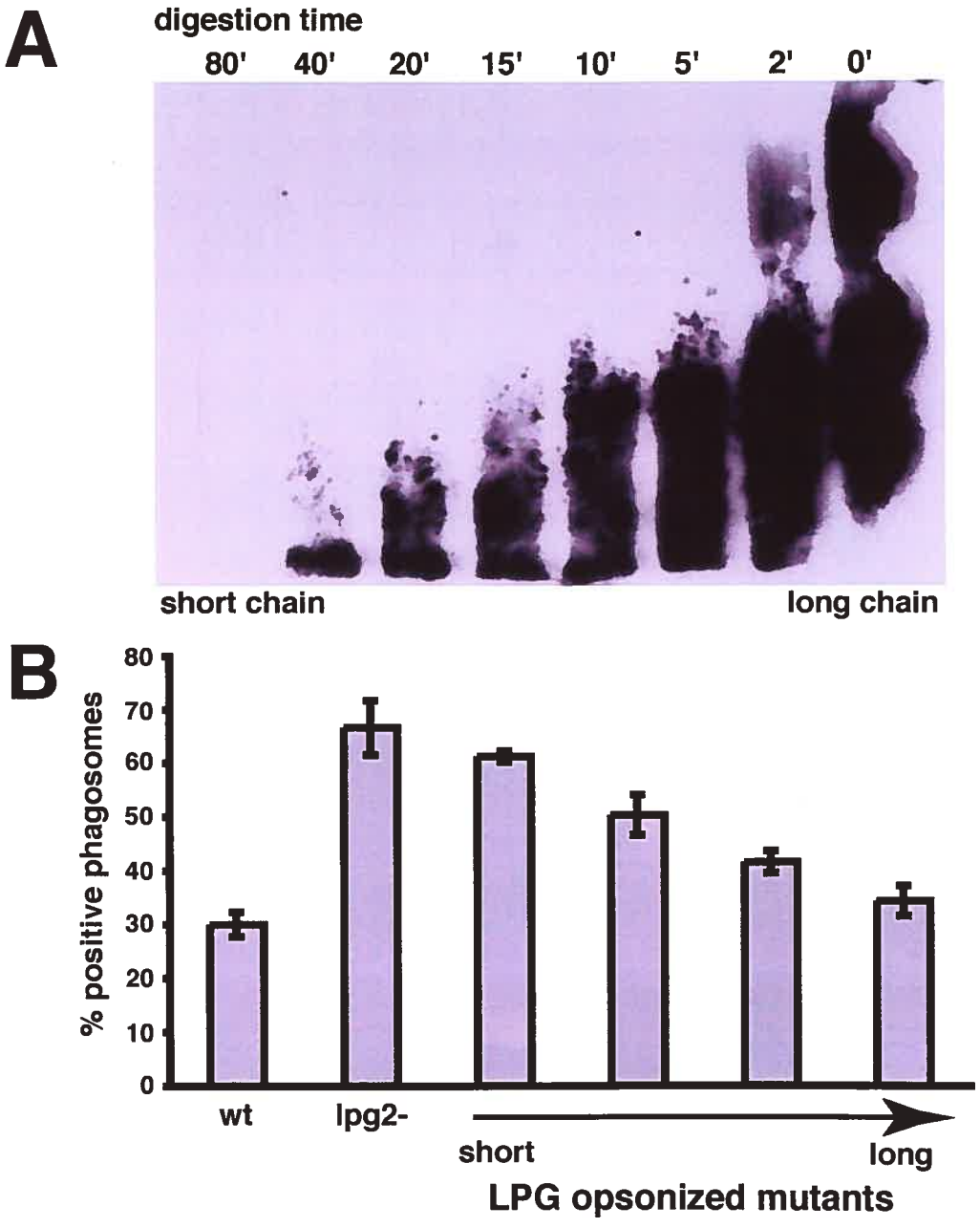
present in small vesicles in the vicinity of the phagosome membrane. Observation at the electron microscope using CTB coupled to gold particles instead of FITC demonstrated that the GM1 coming from the cell surface was clearly inserted in the phagosome membrane through fusion events as shown by the presence of the gold particles on the luminal side of the phagosome membrane (fig. 2B inset). These results strongly suggested that *Leishmania* parasites use their LPG to inhibit the formation and or acquisition of membrane microdomains on phagosomes. Opsonization of the mutant parasites with purified LPG prior to phagocytosis conferred the ability to inhibit the recruitment and/or assembly of GM1-enriched microdomains in a manner similar to wild type parasites, confirming the direct involvement of LPG in this process (fig. 2C-D).

LPG disaccharidic repeating units are required for the exclusion of GM1-microdomains from phagosomes

LPG consists of a polymer (avg. n~16) of repeating Gal(β1,4)Manα1-PO_4-6□ units, attached via a glycan core of 6 sugars to a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol anchor (Turco et al., 1987; Turco et al., 1989; Orlandi and Turco, 1987). LPG is terminated with an oligosaccharide containing galactose and mannose residues. Since lpg2-/- mutants still display the lipid anchor and the glycan core of LPG at their surface (Descoteaux et al., 1995) these two parts of the molecule are unlikely to be directly involved in the prevention of GM1 delivery to phagosome microdomains. To assess if the polymer of disaccharidic repeating units is required *per se* in the exclusion of these microdomains from phagosomes, lpg2-/- mutant parasites were opsonized with purified LPG displaying chains of various lengths, obtained by partial depolymerization of the repeating units by mild acid hydrolysis (fig. 3A). The various opsonized parasites were phagocytosed and the cells were labeled as in figure 2. The results obtained clearly demonstrated that the prevention of GM1 delivery to phagosome microdomains is related to the length of the LPG disaccharidic chain (fig. 3B). The same results were obtained with the lpg1-/- mutants (not shown).

Figure 3 LPG disaccharidic repeating units are responsible for the exclusion of GM1-lipid microdomains from phagosomes

A) Purified LPG was subjected to acid hydrolysis at 60°C for different periods of time to generate molecules with shorter chains of disaccharidic repeating units, as shown by Western blotting using the CA7A9 anti-LPG antibody. B) *Leishmania* lpg2-/- mutants were opsonized with the various shortened LPG molecules and used to infect cells. Plasma membrane GM1 was labeled with CTB-FITC as in Fig. 1A. The number of phagosomes positive for the CTB-FITC labeling was then scored as in Fig. 2D. The results show a direct relation between the length of the LPG molecule and its ability to inhibit GM1 recruitment from the cell surface. Error bars indicate the mean standard error.

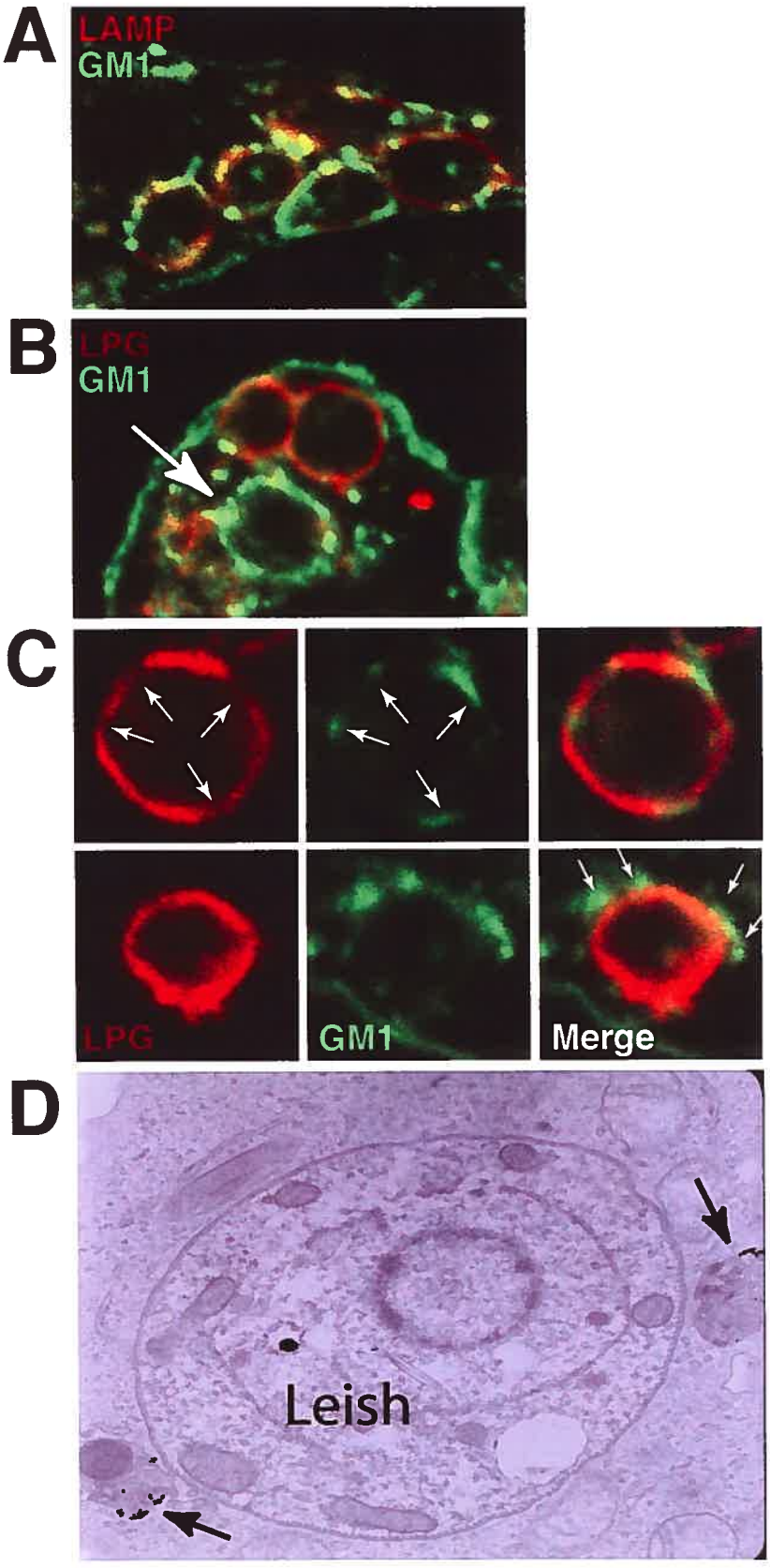

A
digestion time
80'
40'
20'
15'
10'
5'
2'
0'
short chain
long chain
B
% positive phagosomes
80
70
60
50
40
30
20
10
0
wt
lpg2-
short
long
LPG opsonized mutants

LPG prevents the recruitment of GM1-microdomains to phagosomes by a direct action on the membrane

It was shown previously that LPG molecules released from *Leishmania* promastigotes could be inserted in host cell membranes, providing a potential mechanism to alter the properties of phagosome membranes (Tolson et al., 1990). In that context, we investigated whether the delivery of purified LPG to phagosomes containing mutant parasites would then confer the ability to prevent the recruitment of cell surface GM1. While, as shown earlier, phagosomes containing mutant parasites acquired GM1 from the cell surface (fig. 2A and 4A), those that received LPG internalized by endocytosis, and delivered to phagosomes through endosome-phagosome fusion events, prevented the recruitment of GM1 (fig. 4B). In few cases, cells displayed a mixed population of phagosomes containing or not exogenous LPG. As expected, phagosomes that had not received LPG were still able to acquire GM1 from the cell surface (large arrow in fig. 4B). In some cases, we observed that the exogenously added LPG was not evenly distributed in the phagosome lumen. Remarkably, cell surface GM1 could then be detected in the regions of the phagosome membrane where LPG was absent or in low abundance (small arrows in fig. 4C). However, when LPG was present all around the phagosome membrane, the cell surface GM1 was not incorporated and remained in vesicles around the phagosome (fig. 4C, lower panel). This was also observed on some phagosomes in similar conditions at the electron microscope level (fig. 4D). These results clearly indicate that LPG exerts a direct action on the phagosome membrane to modify its properties and alter its ability to assemble or acquire membrane microdomains.

Figure 4 LPG prevents the recruitment of GM1-microdomains to phagosomes by a direct action on the membrane

A) J774 macrophages infected with lpg2-/- mutants and labeled for GM1 display phagosomes with several GM1-enriched microdomains, as shown in Fig. 2B. B) In contrast, when purified LPG was added to lpg2-/- phagosomes by endocytosis (endosome-phagosome fusion), the recruitment of surface GM1 (CTB-FITC) was inhibited specifically at sites where LPG was present (note the absence of the yellow signal indicative of a co-localization). In the cases where LPG did not reach the phagosomes, the recruitment of cell surface GM1 was not prevented (large arrow). C) GM1 is observed only at sites where LPG is absent (small arrows in upper panels). When LPG is present all around the phagosome membrane, cell surface GM1 is not incorporated and remains in vesicles around phagosomes (arrows in lower panel). D) Similar results are observed at the electron microscope. Arrows show GM1-containing vesicles.


A
LAMP
GM1
B
LPG
GM1
C
LPG
GM1
Merge
D
Leish

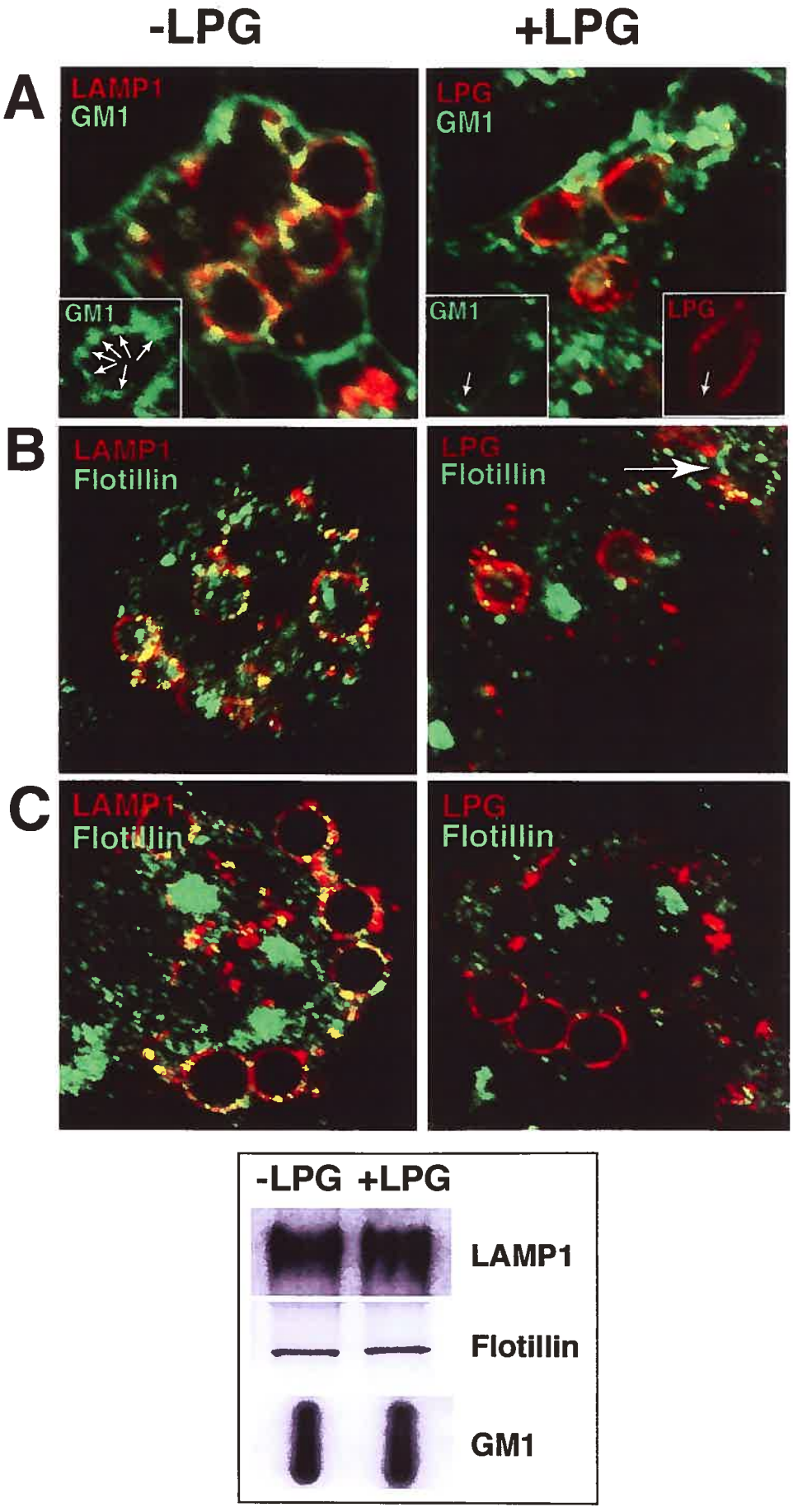
LPG disrupts preexisting phagosome microdomains

During the normal course of infection, *Leishmania* promastigotes are likely to be internalized in phagosomes displaying lipid microdomains acquired from the cell surface. Since we have shown so far that LPG can prevent the formation and/or acquisition of microdomains, we investigated next if LPG could also disrupt microdomains already assembled on phagosomes. Accordingly, we performed experiments in which phagosomes containing lpg-/- mutants were allowed to acquire CTB-FITC-labeled GM1 from the cell surface (fig. 5A, -LPG), prior to the delivery of exogenous LPG by endocytosis. The insert (fig. 5A, -LPG) shows a phagosome displaying a typical dotted labeling for GM1 after its acquisition from the cell surface. The subsequent delivery by endocytosis of exogenous LPG to these phagosomes, through endosome-phagosome fusion, resulted in the loss of the dotted GM1 labeling (fig. 5A, +LPG), which was replaced by a dim labeling around the whole surface of the phagosome membrane (inserts in fig. 5A, +LPG). These results indicate that LPG can also affect the properties of preexisting microdomains on the phagosome membrane, an argument consistent with the idea that the secretion of LPG by *Leishmania* promastigotes after their internalization might allow the disruption of these structures.

The same type of experiments was performed to determine if exogenous LPG could alter preexisting flotillin-1-enriched microdomains on phagosomes. Accordingly, cells were infected with LPG-deficient mutants for periods of time allowing the formation of phagosomes displaying high amounts of flotillin-1 (1h infection followed by the incubation of cells for 2h to allow phagosome maturation and the acquisition of flottilin-1 microdomains). The presence of flotillin-1-enriched microdomains in these conditions was confirmed by immunofluorescence (fig. 5B, -LPG). When LPG internalized by endocytosis was delivered to such phagosomes, flotillin-1 became hardly detectable

Figure 5 LPG disrupts preexisting phagosome microdomains by promoting their disassembly rather than by altering their protein composition

A) Phagosomes were formed by the internalization of *Leishmania lpg2-/-* mutants. The cell surface GM1 was then labeled as in Figure 2B. Purified LPG was then added (+LPG) or not (-LPG) to the lpg2-/- phagosomes as in Figure 4B. Phagosomes were than identified using antibodies against LPG or LAMP1. While phagosomes without LPG displayed several GM1-enriched microdomains (see also white arrows in insets), the delivery of LPG resulted in the loss of the dotted signal replaced by a faint CTB-FITC signal all around the phagosome membrane (the left inset in the +LPG panel). (B) The delivery of LPG to lpg2-/- phagosomes also resulted in the disorganization of flotillin-1-enriched microdomains, which were no longer detectable by immunofluorescence. The arrow points to a phagosome that did not received exogenous LPG and still display a strong dotted labeling for flotillin-1. (C) The delivery of LPG also disrupts flotillin-1-enriched microdomains in latex-bead-containing phagosomes. Western blot analysis for LAMP1 and flotillin-1, and dot blot analysis for GM1 indicates that the delivery of LPG had no effect on the relative amounts of these molecules on phagosomes. These results confirm that the loss of the fluorescent signal for GM1 and flotillin-1 when LPG is delivered to phagosomes is due to a redistribution of these molecules in the phagosome membrane and not their removal.


-LPG
+LPG
A
LAMP1
GM1
GM1
LPG
GM1
GM1
LPG
B
LAMP1
Flotillin
LPG
Flotillin
C
LAMP1
Flotillin
LPG
Flotillin
-LPG +LPG
LAMP1
Flotillin
GM1

(fig.5B, +LPG). Phagosomes that had not received LPG still displayed a high level of labeling for flotillin-1 (arrow in fig. 5B, +LPG).

These results strongly suggest that LPG can alter preexisting microdomains in the phagosome membrane by promoting their disassembly and the redistribution of their components to the whole membrane. This hypothesis was tested by the delivery of exogenous LPG to latex bead-containing phagosomes. After LPG endocytosis, the strong dotted labeling for flotillin-1 normally observed on these structures (fig. 5C, -LPG) was no longer detectable by immunofluorescence (fig. 5C, +LPG), confirming the results obtained with *Leishmania*. Western blot analysis of purified latex bead-containing phagosomes indicated that despite the loss of the fluorescent signal after LPG delivery, the relative level of flotillin-1, as well as GM1, was unchanged (fig. 5C, right panel). These results confirmed that the loss of fluorescent signal is due to a redistribution of the molecules in the phagosome membrane following LPG delivery rather than their removal by recycling processes.

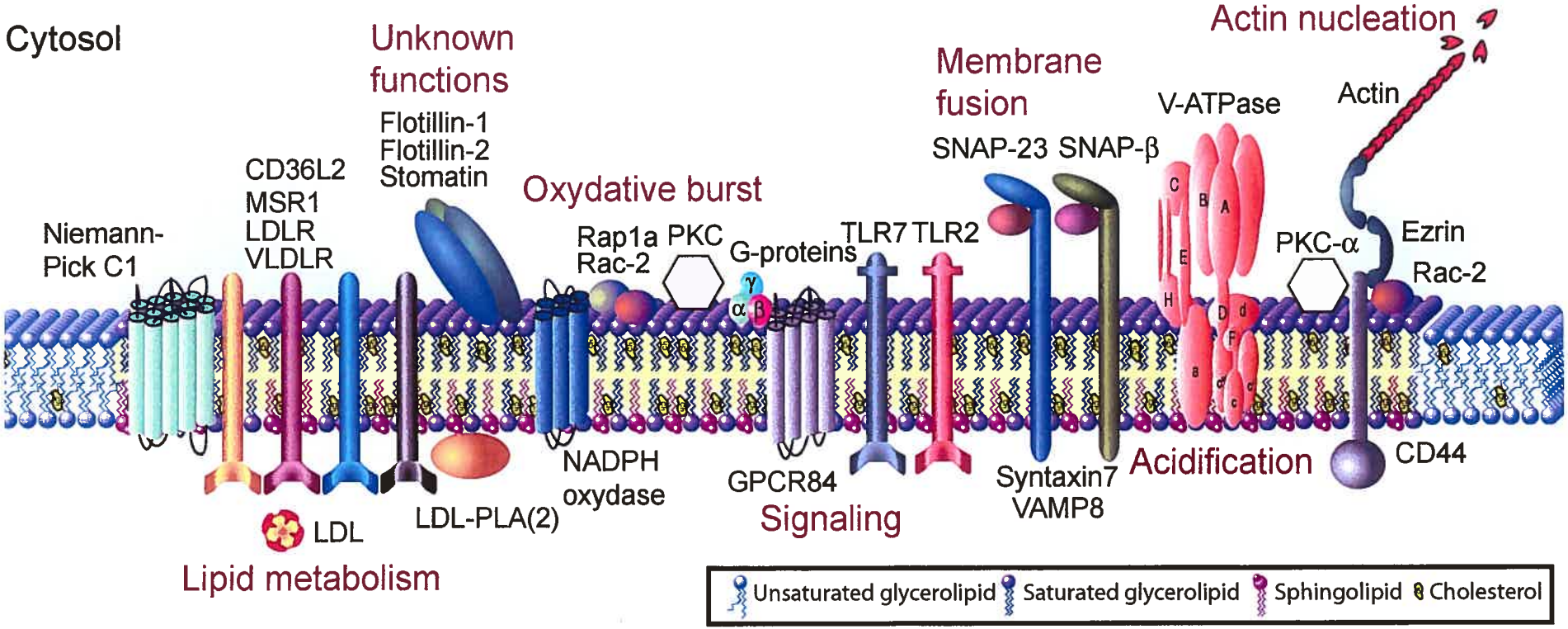
The phagosome microdomain proteome

The pleiotropic deleterious effect of *Leishmania* LPG on host cell functions, conferred by the disruption of the phagosome microdomains, implies that the assembly of a wide variety of distinct functional units must occur on these specialized foci of the phagosome membrane. In order to gain insights into the cellular functions that could be potentially perturbed by the disorganization of phagosome microdomains, we isolated their constituent using a proteomics approach. Detergent resistant membrane components were isolated by flotation on an Optiprep gradient after solubilization at 4 °C in Triton X-100 (Dermine et al., 2001). Three independent sets of tandem mass spectrometry analysis (MS/MS) led to the identification of 210 proteins present in at least two of the three phagosome microdomain samples analyzed (Goyette and Desjardins, in preparation). Partial analysis of these proteins revealed that functions related to acidification, cholesterol

Figure 6 The virtual phagosome microdomain

Proteomics analysis of phagosome microdomains isolated by flotation on an Optiprep gradient after Triton X-100 solubilization of latex bead-containing phagosomes led to the identification of X proteins. The nature of these proteins reveals that functions related to lipid metabolism, the oxydative burst, signal transduction, membrane fusion, acidification, and actin nucleation are likely to take place on specialized foci of the phagosome membrane and altered by LPG during *Leishmania* infection. CD36L2 (CD36 antigen-like 2), MSR1 (Macrophage scavenger receptor type I), LDLR (Low-density lipoprotein receptor), VLDLR (Very low-density lipoprotein receptor), LDL (Low-density lipoprotein), LDL-PLA(2) (Low-density lipoprotein associated phospholipase A2), PKC (Protein kinase C), TLR (Toll-like receptor), GPCR84 (G protein-coupled receptor 84), SNAP (Soluble N-ethylmaleimide-sensitive factor attachment protein).

The virtual phagosome microdomain



metabolism, membrane fusion, actin remodeling, oxidative burst, and intracellular signaling are likely to take place and be regulated by spatial segregation on specialized foci of the phagosome membrane (fig. 6).

Discussion

Intracellular pathogens must avoid the harsh environment of phagolysosomes in order to survive and replicate. Recently, several studies have focused on pathogenic secretion systems, capable of injecting virulence factors into the cytoplasm of host cells, where they can interfere with various cellular pathways (Gruenheid and Finlay, 2003; Nagai and Roy, 2003). This survival strategy, used by pathogenic bacteria such as *Salmonella*, *Legionella* and *Brucella,* is not applicable to *Leishmani*a parasites, since these are devoid of any secretion system. Therefore, this pathogen has to rely on virulence factors capable of interfering with host cell functions from within the phagosome. One of these factors could be the *Leishmania* surface molecule LPG, known to alter a wide variety of host cell functions as diverse as signal transduction, membrane fusion and actin organization (Descoteaux et al., 2002). LPG is also a key survival factor as LPG-deficient mutants are rapidly killed in macrophage phagosomes compared to wild type parasites (Descoteaux et al., 1992; St-Denis et al., 1999). It is thus puzzling how a single molecule, such as the LPG released from the *Leishmania* parasites in the phagosome lumen, can exert pleiotropic effects on the cells they subvert as host cells.

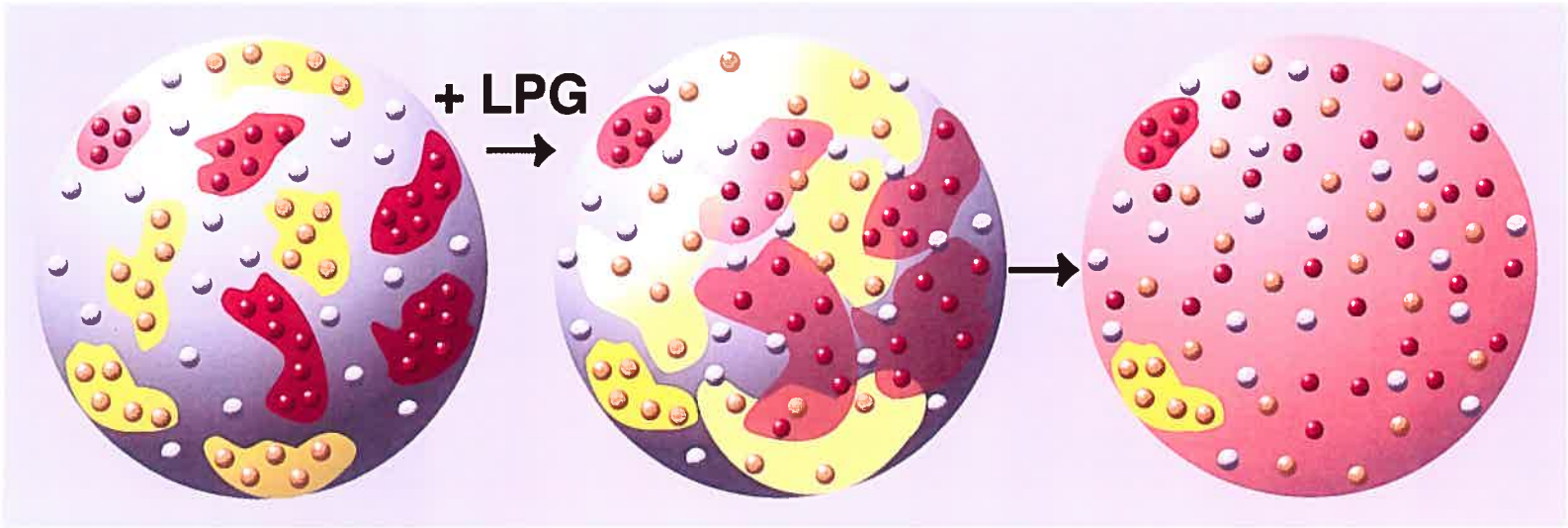
The current study provides an explanation for the diverse effects of LPG on cellular functions. Our results indicate that *Leishmania* uses its LPG to alter the assembly and/or organization of microdomains on the phagosome membrane. These structures were first identified by proteomics analyses, which revealed the presence of known raft markers, such as flotillin-1, in purified latex bead-containing phagosomes (Garin et al., 2001). Further biochemical and morphological characterization of phagosome lipid rafts showed that flotillin-1-enriched microdomains accumulate on maturing phagosomes (Dermine et al., 2001). The present study indicate that various types of microdomains are present on

phagosomes demonstrating that instead of being made of a membrane where lipids and proteins are randomly distributed, phagosomes display specialized regions where specific functions could take place, providing an additional level of organization to the organelle (see model in fig 7). Although the precise functions associated with phagosome membrane microdomains are poorly understood, proteomics analyses indicated that they might be involved in signal transduction, membrane fusion and actin organization[18]. Since all of these cellular processes play key roles in phagolysosome biogenesis, alteration of phagosome microdomains could thus represent a relevant strategy for the establishment of pathogen replicative niches in host cells. It was shown recently that two type III effectors from *Salmonella*, PipB and PipB2, were present in detergent-resistant microdomains of endovacuolar organelles upon expression in the mouse-derived RAW264.7 macrophage-like cell lines (Knodler et al., 2003). The effect of these molecules on membrane microdomains is still unknown. We show here that LPG directly inhibits the recruitment and/or formation of lipid microdomains on phagosomes, while the delivery of LPG to mature phagosomes disrupts previously formed microdomains.

Our findings shed light on several observations identifying LPG as a virulence factor important for the early establishment of *Leishmania* promastigotes in mammalian hosts. LPG was initially shown to attenuate oxidative burst in monocytes and inhibit macrophage protein kinase C (PKC) activity (Descoteaux et al., 1992; McNeely and Turco, 1990). The molecular mechanism by which LPG alters these processes is still poorly understood. Interestingly, both the NADPH oxidase, involved in the oxidative burst, and various PKC isoenzymes were recently shown to be present in lipid microdomains (Lang et al., 2002; Becart et al., 2003; Shao et al., 2003; Vilhardt and van Deurs, 2004). LPG also alters the production of Il-12 (Piedrafita et al., 1999) a cytokine that promotes cellular immunity against *Leishmania* infection (Heinzel et al., 1993; Sypek et al., 1993). The

Figure 7 LPG action on phagosome microdomains

Lipids and proteins are not randomly distributed on the phagosome membrane but rather organized in multiple types of microdomains. When LPG is added to phagosomes and, in the context of the infection when it is released by promastigote parasites after their internalization by macrophages, microdomains dissolve and their components either lipids or proteins are redistributed to the whole surface of the organelle. The disruption of these specialized foci by LPG is likely to alter a large number of functional machines, explaining the pleotropic action of this virulence factor on the mammalian hosts.


+ LPG
multiple microdomains
disrupted microdomains

production of this cytokine is dependant on the activation of CD40 and the induction of MAP kinases, a process involving lipid microdomains (Vidalain et al., 2000) LPG also inhibits signal transduction induced by LPS (Descoteaux et al., 1991) a process requiring cellular mediators like CD14, HSP 70, 90, chemokine receptor 4, growth differentiation factor 5, and Toll-like receptor 4, all of which are present within lipid microdomains (Triantafilou et al., 2002). Hence, the effects of LPG on cellular functions are consistent with the disruption by LPG of functional lipid platforms.

Further links exist between LPG and the disruption of cellular functions associated to membrane microdomains. Infection of CD4+ T cells by HIV, a process involving membrane fusion and lipid microdomains (Manes et al., 2000; Liao et al., 2001), is inhibited by LPG (Easterbrook et al., 1995). When introduced in erythrocyte ghost membranes, LPG blocks binding, as well as fusion, of Sendai and influenza viruses (Miao et al., 1995) a process occurring in lipid microdomains (Liao et al., 2001) LPG is also a potent inhibitor of host organelle membrane fusion, which also involves membrane microdomains (Lang, 2002; Chamberlain et al., 2001). Indeed, LPG was shown to inhibit phagosome-lysosome fusion, a key process for the killing and degradation of *Leishmania* (Desjardins and Descoteaux, 1997; Dermine et al., 2000) Interestingly, several fusion proteins (like SNAREs) have been identified by proteomics analyses in phagosome lipid microdomains (Fig. 6 and Goyette and Desjardins, unpublished results).

Our results clearly showed that the delivery of LPG to phagosomes already containing membrane microdomains disrupt these structures. As a consequence, proteins and lipids that were segregated to form foci of specialized functions were redistributed to the whole surface of the organelle, a process likely to impair their functions. We have shown that one of the most abundant proteins on phagosome lipid microdomains is actin (Dermine et al., 2001). Actin nucleation, a process involving lipid rafts (Rozelle et al., 2000) has been shown to occur at specialized foci on the phagosome membrane, and to be involved in phagosome movement and fusion with late endocytic organelles (Kjeken et al., 2004). Interestingly, recent data indicate that flotillin-1 might play a role in actin organization

(Haglund et al., 2004). This protein present in microdomains is clearly redistributed to the whole surface of the phagosome membrane upon LPG delivery. Accordingly, one could hypothesize that disruption of microdomains might also affect actin organization on phagosomes. In accordance with this idea, LPG has been shown to induce the accumulation of actin all around the phagosome membrane (Holm et al., 2001), a mechanisms that seems to be related with the exclusion of PKC□ from phagosomes (Holm et al., 2003). The redistribution of actin, from the focal points described by Griffiths and collaborators in normal conditions (Kjeken et al., 2004; Defacque et al., 2002), to the entire phagosome surface after exposure to LPG, could form a shield capable of specifically restricting fusion with late endocytic organelles (Dermine et al., 2000).

Insights into the molecular mechanisms by which LPG could alter phagosome membrane microdomains come from the known features of the life cycle of the parasite and of the physicochemical properties of this glycoconjugate. LPG is a glycosylphosphatidylinositol (GPI)-anchored glycolipid present within lipid microdomains at the surface of the promastigote form of the parasite (Denny et al., 2001), the flagellated developmental stage transmitted by sandflies during a blood meal on a mammalian host. After internalization in macrophages by phagocytosis, LPG is shed from the parasite surface (Kaneshiro et al., 1982; King et al., 1987) and inserted in host cell membranes (Tolson et al., 1990) modifying their physical organization (Miao et al., 1995). Accordingly, it is arguable that insertion of LPG in the phagosome membrane through its GPI anchor could alter directly the functional properties of this compartment. However, by using a genetic and biochemical approach, we were able to show that the mechanisms by which LPG alters the formation of microdomains and disrupt these structures in the phagosome membrane is not directly linked to its GPI anchor. Indeed, the membrane of phagosomes containing LPG-deficient parasites displays microdomains despite the fact that the lpg1-/- and lpg2-/- mutants possess the GPI anchor. The effect of LPG on microdomains is rather linked to its repeating disaccharide units, as indicated by the direct relation between the length of the molecule (number of disaccharide repeats) and its ability to inhibit the formation of GM1-enriched microdomains on phagosomes. These data point to a possible mechanism by

which the negatively charged sugar chain of LPG directly interferes with the clusterization of molecules into microdomains. In this regard, it has recently been proposed that a phase transition of membrane components from a liquid to a solid crystalline phase could be central to the functional role of membrane microdomains (Joly, 2004). According to this concept, the disrupting action of LPG could be due to the capacity of the heavily charged head of LPG to interfere with the assembly of such structures, or even to cause their disassembly, as we have observed for the GM1 containing structures.mains.

We have unraveled a novel mechanism enabling an intracellular pathogen to survive in its hosts by altering the formation and organization of phagosome membrane microdomains. Considering the complexity of phagosome insoluble microdomains (unpublished proteomics analyses indicate that over 150 of the slightly over 750 proteins identified so far on phagosomes are enriched in lipid microdomains), and their involvement in key cellular functions (Defacque et al., 2002), targeting of these structures explains the pleotropic effect of LPG on host cell functions and how this provides intracellular pathogens a major survival advantage.

Experimental procedures

Cell culture and phagosome formation and isolation

The murine macrophage-like cell line J774 was cultured in Dulbecco's modified Eagle medium high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were grown to approximately 80 % confluency in Petri dishes prior to each experiment, as described previously (Desjardins et al., 1994). *Leishmania donovani* wild types and mutants lpg2-/- and lpg1-/- strains (a kind gift from Dr. Albert Descoteaux) were cultured as described previously (Duclos et al., 2000).

LPG purification and digestion, and opzonisation of the *L. donovani* mutants

LPG from log phase promastigote cultures was extracted in solvent E (H2O/ethanol/diethyl ether/pyridine/NH4OH;15:15:5:1:0.017) as previously described (Orlandi and Turco, 1987). The solvent E extract was dried by N2 evaporation, resuspended in 0.1 N acetic acid/0.1 M NaCl, and applied to a column of phenyl-Sepharose (2 ml), equilibrated in the same buffer and eluted with solvent E, and further purified on octyl-Sepharose as described (McConville et al., 1987).

To shorten the length of the LPG molecule, 15 µg of lyophilized purified LPG from *Leishmania donovani* was cleaved at acid-labile phosphodiester bridges by incubation in 0,01 N HCl for 0, 2, 5 10, 15, 20, 40, and 80 min at 60 °C. The samples were then treated with 0,01 N NaOH to neutralize acidity and put on ice. To verify the proper digestion of LPG, SDS-PAGE separation of the fragments on 12% polyacrylamide gels was performed prior to Western blot analysis. The membranes were then probed with the CA7AE antibody, which recognizes the Gal-Man-P repeating units of LPG, and revealed by ECL (Tolson et al., 1989).

To opsonize LPG-deficient parasites, 1.0 x 10^6 *lpg2-/-* and *lpg1-/- Leishmania donovani* were incubated for 30 min at room temperature in 15 µl of 1 mg/ml of full length or digested LPG in PBS. Parasites were then washed 3 times in PBS by gentle centrifugation, resuspended in culture medium, and used to infect J774 cells.

Isolation of J774 phagosome microdomains

To prepare phagosome lipid microdomains, purified phagosomes were first isolated by internalizing latex beads for 90 min followed by incubation in culture medium without beads for 90 min. For each experiment, 42 Petri dishes of 10 cm were used and phagosomes were isolated as described previously (Desjardins et al., 1994). The purified phagosome pellet was resuspended in 0,3 ml of TNE-Triton buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, complete protease inhibitor (Boehringer Mannheim), pH 7.4 and 1 % Triton X-100), transferred to an Eppendorf tube and shaken gently 30 min at 4°C to solubilize phagosomal membranes. Latex beads were then pelleted by centrifugation and the supernatant collected and subjected to another round of centrifugation to ensure the removal of all beads. The final supernatant containing the solubilized and insoluble phagosome components was added to 0,6 ml 60% Optiprep^{tm} to obtain a final concentration of 40% Optiprep^{tm} which was then poured at the bottom of an Ultraclear centrifuge tube (Beckman). Finally, 2,4 ml of 30% Optiprep^{tm} and 0,9 ml of TNE buffer (with protease inhibitors) were layered on top. After a 4h centrifugation at 38 000 rpm (SW60 rotor) to float the insoluble rafts, 7 fractions of 0,6ml were collected from the top. Proteins were then precipitated with methanol/chloroform according to Wessel and Flugge (1984) and resuspended in Laemmli buffer for western blotting. In order to determine the distribution of the ganglioside GM1 in the gradient, 10 µl of each fraction were collected prior to precipitation for slot-blot analysis.

Western and slot blotting

Western blotting on the same nitrocellulose membrane was performed according to standard procedures for flotillin-1, LAMP-1 and Rab7. The total protein amount present in each 0,6 ml fraction was loaded. The membrane was cut in 3 parts between the molecular weight markers 30, 52 and 80 kDa. The upper part was probed with the 1D4B rat monoclonal antibody (Developmental Studies Hybrodoma Bank, University of Iowa) directed against LAMP1, the middle part was probed with a rabbit polyclonal antibody specific for flotillin-1 (kind gift from Dr. Gisou van der Goot) and the lower part was probed with a polyclonal antibody against Rab7 (Santa Cruz). Appropriate secondary antibodies coupled to HRP were then used and the membranes treated for ECL (Roche Diagnostics). To detect the lipid ganglioside GM1, slot blotting was used. Briefly, 10 µl from each part of the gradient was diluted in 200 µl of water and loaded in a slot blotter to adhere to a nitrocellulose membrane. After blocking in PBS milk 5%, the membrane was probed with cholera toxin B subunit coupled to HRP and revealed by ECL.

GM1-rafts labeling and immunofluorescence

J774 macrophages were grown on coverslips to a confluence of about 80%, 36 h before the experiments. Cells were infected with *Leishmania donovani* strain 1S wild type, or with LPG-mutants (*lpg2-/-* and *lpg1-/-*) deprived of the surface glycolipid lipophosphoglycan (LPG), at a concentration of 3,0 for the wild type and 2.0 $X10^7$/ml for the mutants in culture medium for 30 min[16] or fed with 3 µm latex beads (Polyscience) (1:50 dilution), followed by a 30 min chase without beads or parasites. The cell surface GM1 was then labeled by incubating cells with 5 µg/ml of CTB-FITC (Sigma) in culture medium at 4 °C to prevent internalization of the toxin in the fluid phase. After washing unbound CTB, cells were further incubated for 2 hours in normal medium, a time period that allows the delivery of cell surface GM1 to phagosomes. Cells were fixed and processed as described (Dermine et al., 2001). To reveal phagosomes within cell, cells were incubated with the rat anti-LAMP1 1D4B for 1h. In some experiments, phagosomes

were also doubly labeled for flotillin-1 and either LAMP1 or cholera toxin as described previously (Dermine et al, 2001).

Effect of purified LPG on phagosome lipid microdomains

The effect of purified LPG (Turco et al., 1984) on phagosome microdomains was tested in two distinct conditions, to see 1) if LPG can inhibit the phagosomal formation and/or acquisition of GM1-enriched microdomains from the cell surface, and flotilin-1-enriched microdomains from an internal source, and 2) if LPG can influence the organization of microdomains already present on phagosomes. In the first case, J774 cells displaying phagosomes containing either LPG-deficient *Leishmania* or latex beads were allowed to internalize purified LPG (1 mg/ml in PBS) by endocytosis for 30 min, followed by a further incubation of 30 min without LPG, allowing the delivery of LPG to phagosomes through endosome-phagosome fusion. The cells surface GM1 was then labeled as described in the previous section, and the cells observed at the confocal microscope to determine if GM1-enriched microdomains were present on phagosomes. In the second case, phagosomes were formed by the internalization of LPG-deficient *Leishmania* as above. The cell surface GM1 was then labeled with CTB-FITC and cells incubated to allow the formation of CTB-FITC-labeled GM1-enriched microdomains on phagosomes. Purified LPG was then internalized as above. The cells were then observed at the confocal microscope to determine if delivery of LPG to phagosomes already containing microdomains had an effect on these structures. Phagosomes were identified by immunofluorescence in these cells by using either the anti LPG antibody or anti LAMP1 antibody (for the control cells were LPG was not internalized). Microdomains were identified either by the intrinsic fluorescence of the CTB-FITC or using an anti-flotillin-1 antibody. Controls included tests for interspecies cross-reactions and cells incubated only with the secondary antibodies.

Following the observation that delivery of LPG to phagosomes containing microdomains resulted in the loss of the dotted fluorescent patterns for both GM1 and flotillin-1, we

performed experiments to determine if these molecules were removed from the phagosome membrane or simply redistributed on the whole surface of the organelle membrane. This was done by comparing the relative amounts of GM1 and flotillin-1 on latex bead-containing phagosomes that received LPG or not through endocytosis (as above). Phagosomes were then isolated as described previously and resuspended in Laemmli buffer for western blotting and slot blotting.

Proteomics analysis

Proteins present in phagosome lipid microdomains, isolated as described above, were solubilized in Laemmli buffer and separated by SDS-PAGE on a short (3 cm) minigel. The gel was cut into 24 equal parts, and the proteins were digested in trypsin and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Houde et al., 2003).

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Chapitre V. Discussion générale et conclusion

Discussion générale

Le parasite intracellulaire *Leishmania*, l'agent étiologique de la leishmaniose, a développé des stratégies qui lui permettent de survivre et de se reproduire dans deux environnements différents, c'est-à-dire, dans l'intestin de la mouche des sables et dans les phagolysosomes des macrophages de mammifères. Chez la mouche, le parasite existe sous une forme promastigote flagellée, tandis que chez le mammifère, il existe sous une forme amastigote aflagellée. Lors du passage de la mouche au mammifère, les promastigotes injectés sous le derme par une piqûre de l'insecte sont rapidement internalisés dans les macrophages par phagocytose. Dans ces cellules, les promastigotes résident dans des phagosomes où ils se transforment en quelques jours en amastigotes, la forme réplicative responsable de la maladie chez le mammifère. Cette transformation est déclenchée par la température plus élevée chez le mammifère et une baisse du pH à l'intérieur des phagosomes (Zilberstein et Shapira, 1994) et probablement par d'autres facteurs encore inconnus. Une étude effectuée dans notre laboratoire a démontré que les promastigotes de *L. donovani* sont internalisés dans des phagosomes qui fusionnent peu avec les endosomes (Desjardins et Descoteaux, 1997). Cette inhibition de la fusion serait produite par une molécule de surface du parasite, le lipophosphoglycane (LPG). La diminution de la fusion a pour conséquence d'empêcher la maturation des phagosomes contenant les promastigotes de *Leishmania* en phagolysosomes (Article 5 en annexe). D'autre part, plusieurs études ont montré que les amastigotes de *Leishmania* se retrouvent à l'intérieur de phagolysosomes, démontrant que la forme réplicative du parasite peut survivre en toute impunité dans l'environnement hostile des phagolysosomes, probablement en résistant à l'action des hydrolases (Alexander et Vickerman, 1975; Chang et Dwyer, 1976; Shepherd et *al.*, 1983; Rabinovitch et *al.*, 1985; Antoine et *al.*, 1990; Prina et *al.*, 1990; Russell et *al.*, 1992; Lang et *al.*, 1994). Ces résultats indiquent clairement que les promastigotes et les amastigotes ont recours à des stratégies de survie différentes dans leurs cellules hôtes.

Dans la présente thèse, nous apportons de nouvelles indications qui démontrent que les promastigotes de *Leishmania donovani* utilisent le LPG présent à leur surface pour inhiber la fusion des phagosomes avec les compartiments tardifs de la voie endocytaire. Ce processus se fait de façon active, puisque les parasites tués sont incapables d'inhiber la fusion des phagosomes avec les endosomes, malgré la présence de LPG à leur surface (article 1). Cette inhibition permet aux promastigotes de résider à l'intérieur de compartiments cellulaires inaptes à tuer les microorganismes, puisqu'ils n'acquièrent pas les hydrolases en provenance des endosomes tardifs et des lysosomes. Nous montrons aussi que le LPG altère, par une action directe, des structures spécialisées du phagosome, appelées radeaux lipidiques, dont nous avons démontré l'existence sur cet organite grâce à la présence de la protéine flotilline-1. L'analyse protéomique de radeaux lipidiques isolés de phagosomes formés par la phagocytose de billes de latex a mis en évidence la présence de protéines, telles que l'actine, des sous-unités des GTPases hétérotrimériques, des sous-unités de la pompe à proton, des protéines du complexe NADPH oxydase et des molécules impliquées dans la régulation des événements de fusion membranaire dans ces structures. Ces résultats indiquent que, contrairement à l'idée que le phagosome est constitué d'une membrane où les lipides et les protéines sont distribués aléatoirement, il existe des microdomaines où certaines protéines sont ségréguées, ce qui pourrait permettre à des fonctions spécialisées comme la fusion membranaire de se produire en des points focaux de la membrane phagosomiale.

Le LPG suffit à lui seul à inhiber la fusion phagosome-endosome.

Le LPG consiste principalement en une longue chaîne d'unités disaccharidiques phosphorylées répétitives (PO4-6Galβ1-4Man) attachée à un noyau hexasaccharidique, qui est fixé à une ancre lipidique insérée dans la membrane plasmique du parasite. Les unités répétitives, que l'on retrouve également chez d'autres macromolécules du parasite telles la phosphatase alcaline et des protéophosphoglycanes (Ilg et *al.*, 1994), sont essentielles à l'inhibition de la fusion phagosome-endosome (Desjardins et Descoteaux, 1997). L'étude

de Desjardins et Descoteaux (1997) utilisant le mutant *lpg2-/-* n'a toutefois pas permis de démontrer sans équivoque que le LPG est responsable de l'inhibition de la fusion entre les phagosomes et les compartiments de la voie endocytaire. En effet, chez les mutants utilisés dans cette étude, les unités disaccharidiques répétitives sont absentes du LPG, mais également absentes de nombreuses autres molécules qui les contiennent chez les parasites du type sauvage. Ces autres molécules, comprenant la phosphatase alcaline (Ilg et *al.*, 1994) pourraient ainsi, aussi participer au processus d'inhibition de la fusion. Dans le but de déterminer le rôle des autres molécules riches en unités répétitives, nous avons utilisé un second mutant, appelé le LPG1-, dont une galactofuranose transférase essentielle à la synthèse du noyau hexasaccharidique de la molécule du LPG est mutée (Huang et Turco, 1993). Chez ce mutant il y a ajout des unités disaccharidiques répétitives à toutes les macromolécules qui en contiennent normalement, sauf au LPG puisque les unités répétitives ne peuvent pas être ajoutées à un noyau incomplet (Huang et Turco, 1993). Les résultats de l'essai de fusion en microscopie électronique, qui permet de suivre le transfert de particules d'or contenues dans les endosomes vers les phagosomes, montrent que les phagosomes contenant les mutants LPG1- ou LPG2- sont beaucoup plus fusogéniques que les phagosomes contenant les parasites de type sauvage (article 1). De plus, puisque les phagosomes contenant les mutants LPG1- ou LPG2- ont des niveaux de fusion similaires, ces résultats confirment que le LPG est la principale molécule responsable de l'inhibition de la fusion chez *L. donovani*. Une autre indication selon laquelle le LPG est la molécule responsable de l'inhibition de la fusion provient du fait que la disparition du LPG de la surface du parasite lors de la différenciation des promastigotes en amastigotes dans les phagosomes est associée à une reprise de fusion entre les phagosomes et les endosomes (Russell et *al.*, 1992). Lors de la différenciation, le LPG disparaît de la surface des amastigotes en 1 à 2 jours probablement par un mécanisme d'internalisation endocytaire et devient complètement indétectable en 5 à 6 jours. L'utilisation de parasites amastigotes extraits de la rate de hamsters infectés dans notre essai de fusion a confirmé que cette forme du parasite, où le LPG est absent, est inapte à inhiber la fusion phagosome-endosome.

Le LPG chez les autres espèces de *Leishmania*.

Il existe un polymorphisme structural du LPG chez différentes espèces de *Leishmania* qu'il nous paraissait intéressant d'étudier dans un contexte d'inhibition de fusion phagosome-endosome. Les chaînes oligosaccharidiques latérales attachées à la position 3 du galactose des unités disaccharidiques répétitives, présentes chez *L. major*, mais absentes chez *L. donovani*, constituent un très bon exemple de polymorphisme (figure 3, Chapitre I). La présence de telles chaînes chez *L. major* rend son LPG beaucoup plus complexe que celui de *L. donovani*. Butcher et collaborateurs (1996) ont montré que les chaînes latérales de *L. major* permettent à ce parasite d'infecter spécifiquement certaines espèces de mouche, comme *Phlebotomus papatasi*, alors que *L. donovani* infecte surtout les *Phlebotomus argentipes* (revue dans Sacks, 2001). La reconnaissance hôte-parasite en lien avec la structure du LPG chez les différentes espèces de *Leishmania* serait reliée à des lectines spécifiques présentes chez les mouches (Sacks et *al.*, 2000).

Le rôle précis des chaînes latérales chez *L. major* dans l'interaction entre les parasites et les macrophages, ainsi que dans la survie du parasite à l'intérieur de ces cellules est peu connu. Easterbrook et collaborateurs (1995) ont montré que le LPG de *L. major* est plus efficace que celui de *L. donovani* dans l'inhibition de la formation de syncytia de cellules, un processus qui implique des phénomènes de fusion membranaire. Ces résultats suggèrent que le LPG de *L. major* a un effet plus marqué sur la fusion membranaire que le LPG de *L. donovani*. En accord avec cette hypothèse, nos résultats montrent que l'inhibition de la fusion phagosome-endosome par *L. major* est plus efficace que celle observée lors de l'infection par *L. donovani* (article 1). La présence des chaînes latérales oligosaccharidiques chez *L. major* pourrait expliquer pourquoi cette espèce inhibe d'avantage la fusion que *L. donovani*. Toutefois, l'analyse du taux de fusion des phagosomes contenant le mutant Spock de *L. major*, dont le LPG ne possède pas de chaînes latérales et ressemble donc au LPG de *L. donovani* (Butcher et *al.*, 1996), montre que ces phagosomes fusionnent au même niveau que les phagosomes contenant le *L. major* de type

sauvage. Ces résultats éliminent donc la participation des chaînes latérales dans l'inhibition de la fusion phagosome-endosome. L'efficacité accrue de *L. major* à inhiber la fusion phagosome-endosome pourrait s'expliquer plutôt par le fait que son LPG possède en moyenne plus d'unités répétitives (PO_4-6Galβ1-4Man) (environ une dizaine) que le LPG de *L. donovani* (McConville et *al.*, 1990 ; Turco et Descoteaux, 1992). D'ailleurs, Easterbrook et collaborateurs (1995) ont montré que l'efficacité de l'inhibition de la fusion membranaire induite par le LPG lors de la formation de syncitia de cellules infectées par le VIH s'accroît en fonction du nombre d'unités disaccharidiques présentes sur cette molécule. Le mécanisme par lequel un plus grand nombre d'unités disaccharidiques répétitives augmentent l'inhibition de la fusion n'est toujours pas élucidé. La charge ionique de la molécule pourrait avoir un rôle à jouer, mais il pourrait aussi s'agir d'un encombrement stérique plus important dans le cas d'un LPG comportant plus d'unités répétitives.

Leishmania module activement les propriétés de fusion des phagosomes.

Nos résultats indiquent clairement que le LPG permet l'inhibition de la fusion phagosome-endosome. Cependant, ils montrent aussi que, bien qu'exprimant toujours du LPG à leur surface, les parasites sauvages tués avant l'internalisation induisent la formation de phagosomes qui sont beaucoup plus fusogéniques que les phagosomes formés lors de l'internalisation de parasites vivants (article 1). Ces résultats suggèrent donc qu'un processus actif du parasite, en plus du LPG, agit sur les phagosomes de façon à changer leurs propriétés fusogéniques. N'excluant pas la possibilité que les parasites tués à la chaleur puissent avoir perdu une partie de leur LPG, rendant ainsi l'inhibition moins efficace, il est permis de proposer que le LPG doit d'abord être relâché activement par le parasite avant d'exercer son action sur les membranes. D'ailleurs, il est connu que le LPG est transféré du parasite vers les membranes de l'hôte peu de temps après son internalisation (Tolson et *al.*, 1990). En tenant compte de ces observations, il est possible que du LPG ancré solidement à des parasites morts n'aurait aucune action biologique. Nos

données montrent également que tuer les parasites après leur internalisation dans les cellules ne rétablit pas les propriétés fusogéniques des phagosomes. Ces résultats suggèrent que les propriétés fusogéniques sont déterminées peu de temps après l'interaction entre le parasite et la cellule hôte, plutôt que par une action persistante du parasite sur les phagosomes. On peut imaginer que dès le moment de son internalisation, le *Leishmania* sécrète activement du LPG qui s'insère dans les membranes et que par la suite la survie du parasite n'est plus nécessaire à l'action du LPG déjà présent dans la membrane du phagosome. La façon exacte dont s'effectue la sécrétion du LPG n'est pas encore connue. Cependant, il est connu que ce processus n'implique pas l'action enzymatique d'une phospholipase qui couperait l'ancre lipidique et relâcherait le reste de la molécule, puisque nous retrouvons la molécule complète dans le milieu de culture (Kaneshiro et Wyder, 1993). Il est aussi peu problable qu'il s'agisse d'un bourgeonnement de membrane, car la molécule peut se retrouver à l'état libre sans association membranaire dans le milieu. Il serait intéressant dans le futur d'étudier plus en détail le mécanisme par lequel s'effectue la sécrétion du LPG par le parasite.

Les phagosomes contiennent des radeaux lipidiques.

L'analyse protéomique des phagosomes contenant des billes de latex a permis d'identifier plusieurs centaines de protéines associées à ce compartiment. Une de ces protéines, la flotilline-1, avait d'abord été découverte sur des radeaux lipidiques de la membrane plasmique chez des adipocyte 3T3-L1 (Bickel et *al.*, 1997). Comme mentionné dans le Chapitre I, les radeaux lipidiques constituent des assemblages latéraux de lipides présents dans les membranes des cellules eucaryotes (Simons et Ikonen, 1997; Harder et Simons, 1997). Certaines protéines se concentrent dans les radeaux alors que d'autres en sont exclues, par conséquent la présence de telles structures sur les phagosomes permettraient à certaines fonctions spécialisées de se produire en des points focaux de la membrane phagosomiale. En immunofluorescence, nous avons remarqué que la flotilline-1 s'accumule en des points focaux de la membrane phagosomiale tandis que le marquage

pour la protéine LAMP1 épouse tout le contour du phagosome (article 2). En plus de ce résultat, la flottaison de la flotilline-1 dans des domaines insolubles au Triton X-100 sur gradient de densité, une caractéristique fondamentale des radeaux, nous a convaincu de la présence de ces structures sur le phagosome. De plus, par immunobuvardage, nous avons montré un grand niveau d'enrichissement de la flotilline-1 dans les radeaux de phagosomes par rapport au reste de la membrane phagosomiale (article 2). Il est possible que d'autres protéines soient également enrichies dans les radeaux. Des études en ce sens sont actuellement en cours dans notre laboratoire. La présence des radeaux sur le phagosome nous indique que les protéines et les lipides ne sont pas distribués au hasard, mais que le phagosome présente des régions spécialisées, ce qui dénote un niveau supérieur d'organisation.

En plus de la flotilline-1, un lipide de surface connu comme étant enrichi dans les radeaux lipidiques, le ganglioside GM1, s'accumule en des points focaux du phagosome et flotte également sur gradient de densité à la fraction correspondant aux radeaux lipidiques. Toutefois, les marquages en immunofluorescence pour la flotilline-1 et le GM1 ne colocalisent pas à la membrane du phagosome. Les radeaux enrichis de flotilline-1 rejoignent le phagosome à partir d'une source intracellulaire et non pas de la membrane plasmique, puisque cette protéine est peu présente sur les phagosomes nouvellement formés dont la membrane provient en partie de la membrane plasmique, mais s'accumule avec une cinétique tardive similaire à celle de la protéine LAMP1, une protéine surtout intracellulaire. Le lipide de radeaux GM1 semble au contraire provenir essentiellement de la membrane plasmique puisqu'on l'y retrouve en abondance et que le GM1 pré-marqué à la surface pénètre dans les cellules par endocytose pour se retrouver par la suite sur les phagosomes. Toutes ces données suggérent qu'il existe plusieurs types de radeaux présents à la membrane phagosomiale. La présence de différentes sortes de radeaux lipidiques sur les phagosomes suggèrent que ces microdomaines puissent exercer des fonctions différentes dépendamment de leur composition biochimique. Cette hypothèse est d'autant plus probable qu'il existe plusieurs études qui ont démontré l'existence d'une hétérogénéité parmi les radeaux lipidiques. Par exemple, chez les lymphocytes T, il semble que la

protéine LFA-1, s'associe à un sous-type spécifique de radeaux et que cette association régule l'activité de cette molécule (Marwali et *al.*, 2003). Il existe de nombreux autres exemples d'hétérogénéités discutés plus longuement ailleurs (revue dans Pike, 2004).

La protéomique des radeaux lipidiques de phagosomes.

Afin de mieux comprendre la fonction des radeaux lipidiques sur les phagosomes, nous avons entrepris l'analyse de la composition protéique de ces structures en utilisant, dans un premier temps, une approche d'électrophorèse en deux dimensions. Cette approche est beaucoup plus résolutive que l'électrophorèse en une dimension, puisqu'elle permet la séparation des protéines selon leur point isoélectrique et par la suite, la séparation selon leur poids moléculaire. Quant à l'électrophorèse en une dimension, cette technique ne permet que la séparation des protéines selon leur poids moléculaire. Cependant, l'électrophorèse en deux dimensions est limitée par rapport à l'isolement des protéines membranaires, puisque des dernières pénètrent peu dans la première dimension.

En comparant le patron bidimensionnel des protéines obtenu à partir d'échantillons de radeaux de phagosomes au patron de protéines phagosomiales totales déjà identifiées par spectrométrie de masse, nous avons identifié plusieurs protéines sur les radeaux. Parmi celles-ci, nous avons retrouvé l'actine, les sous-unités α, $\beta 1$ et $\beta 2$ des protéines G hétérotrimériques ainsi que les sous-unités A, B et possiblement E de la pompe à proton vacuolaire (article 2). Des sous-unités des protéines G hétérotrimériques avaient déjà été identifiées dans les radeaux lipidiques dans d'autres études (Lisanti et *al.*, 1994). Ces résultats indiquent que la transduction de signaux pourrait se produire en des régions spécialisées de la membrane phagosomiale. Certaines sous-unités de la pompe à proton ont également été identifiés précédemment dans des fractions insolubles aux détergents (Galli et *al.*, 1996) et plus récemment dans les radeaux lipidiques de la membrane plasmique chez la levure (Bagnat et *al.*, 2001). Il est donc possible que les radeaux soient nécessaires au bon fonctionnement de la pompe à proton, qui permet l'acidification de la lumière du

phagosome, un processus essentiel à l'action bactéricide de ce compartiment. La présence de l'actine sur les radeaux de phagosomes permet de suggérer que ces microdomaines puissent agir en tant que site de nucléation de l'actine. En appui à cette hypothèse, une étude à montré que les radeaux servent de site de polymérisation de l'actine sur de petites vésicules et induisent la formation de comètes d'actine afin de favoriser la motilité vésiculaire (Rozelle et *al.*, 2000). Le précédent point sera discuté plus en détails plus bas. L'analyse protéomique des radeaux des phagosomes par spectrométrie de masse a, jusqu'à maintenant, permis d'identifier près de 150 protéines enrichies sur ces structures (Goyette et Desjardins, résultats non-publiés). Quelques-unes de ces protéines ont été représentées dans le modèle du radeau virtuel de l'article 3. Ces résultats démontrent la complexité des radeaux lipidiques de phagosomes. La prochaine étape sera de découvrir le rôle de ces différentes protéines afin de mieux comprendre les fonctions de ces plates-formes fonctionnelles sur le phagosome.

Le LPG de *L. donovani* altère les radeaux lipidiques des phagosomes

Le troisième article de cette thèse présente des résultats indiquant que le LPG prévient l'assemblage des radeaux sur le phagosome ou en altère directement leur organisation. Cet effet se produit sur les deux types de radeaux identifiés chez cet organite, c'est-à-dire les radeaux enrichis de flotilline-1 et les radeaux enrichis de GM1. Il est probable que les radeaux s'accumulent sur le phagosome par des événements de fusion avec des endosomes riches en flotilline-1 provenant de l'intérieur de la cellule ou avec des endosomes riches en GM1 provenant de la membrane plasmique. Puisque le LPG inhibe la fusion phagosome-endosome, il est possible que cette molécule empêche l'accumulation des radeaux lipidiques sur les phagosomes en inhibant la fusion avec les endosomes riches en molécules composant les radeaux. Cependant, en internalisant du LPG purifié dans des cellules où des phagosomes riches en radeaux lipidiques ont été préformés, nous avons constaté que le LPG rejoint les phagosomes et redistribue les composants des radeaux uniformément autour du phagosome. Certains phagosomes, pour une raison inexpliquée,

ne reçoivent pas de LPG. Ces phagosomes conservent alors une distribution en pointillés de GM1 et de flotilline-1. Dans d'autres cas, le LPG est bien livré aux phagosomes, mais ne se distribue pas uniformément sur les membranes et semble laisser des trous à certains endroits. On remarque alors que le GM1 de surface s'accumule dans ces régions. Toutes ces données démontrent que le LPG exerce une action directe et locale sur la membrane phagosomiale pour modifier ses propriétés et compromettre sa capacité à assembler des radeaux lipidiques.

Actions du LPG dépendantes de la modulation des radeaux lipidiques

De nombreuses observations identifient le LPG comme un facteur de virulence chez les promastigotes de *Leishmania*, surtout dans un contexte de début d'infection. La découverte que le LPG affecte les radeaux lipidiques pourrait expliquer plusieurs de ces constatations. D'abord, il a déjà été démontré que le LPG diminue la flambée oxydative chez les monocytes (Descoteaux et *al.*, 1992; McNeely et Turco, 1990). Les mécanismes moléculaires reliés à ces effets du LPG ne sont pas encore connus, mais récemment, la NADPH oxydase, impliquée dans la flambée oxydative a été retrouvée dans les radeaux lipidiques (Shao et *al.*, 2003; Vilhardt et Van Deurs, 2004). Différentes isoenzymes de la PKC, comme la PKCα récemment impliquée dans la maturation du phagosome (Ng Yan Hing et *al.*, 2004), et d'autres jouant aussi un rôle dans la fonction oxydative du phagosome ont également été retrouvées dans les radeaux (Lang et *al.*, 2002; Becart et *al.*, 2003). Puisque les radeaux forment des complexes supramoléculaires favorisant les interactions protéines-protéines nécessaires à l'activité de complexes de signalisation, on peut imaginer qu'une atteinte à l'intégrité de ces structures puisse grandement perturber la fonction signalitique des protéines qui y sont localisées, comme par exemple la PKCα., et par conséquent les fonctions normales du phagosome.

Le LPG diminue également la production d'Il-12 (Piedrafita et *al.*, 1999), une cytokine qui favorise l'immunité cellulaire contre les infections par *Leishmania* (Heinzel et

al., 1993, Sypek et *al.*, 1993). Or, la production de cette cytokine dépend de l'activation de CD40 et de l'induction des MAP kinases, un processus qui dépend des radeaux lipidiques (Vidalain et *al.*, 2000). Le LPG inhibe aussi la transduction du signal induit par le LPS. Cette activité requiert normalement des médiateurs cellulaires, tels que le CD14, les HSP70 et 90, le récepteur 4 à chimiokine, le facteur 5 de différenciation et le récepteur 4 du type Toll-like (TLR4), tous présents dans les radeaux lipidiques (Triantafilou et *al.*, 2002). De plus, il a récemment été démontré que le TLR4 contribue au contrôle de l'infection par *Leishmania major* et que le TLR2, également présent dans les radeaux lipidiques (Soong et *al.*, 2004; Triantafilou et *al.*, 2004), serait essentiel à la maturation adéquate du phagosome afin de contrôler l'infection (Blander et Medzhitov, 2004). On peut comprendre alors qu'une perturbation du signal induit par ces récepteurs puisse nuire au fonctionnement du phagosome et favoriser ainsi la survie de *Leishmania* (Kropf et *al.*, 2004).

Il existe d'autres liens entre le LPG et la perturbation de fonctions cellulaires associées aux radeaux lipidiques. Par exemple, le LPG prévient l'infection des lymphocytes T CD4+ par le VIH, un processus impliquant la fusion membranaire et les radeaux lipidiques. De plus, si on introduit du LPG dans des membranes fantômes d'érythrocytes, cette molécule prévient la liaison de même que la fusion des virus Sendai et Influenza avec la membrane plasmique des érythrocytes. Le LPG de *L. major* prévient également la migration cellulaire de cellules de Langerhans (Ponte-Sucre et *al.*, 2001), un processus démontré récemment comme étant dépendant des radeaux lipidiques chez les leucocytes (Gomez-Mouton et *al.*, 2004). Cette action du LPG empêcherait les cellules dendritiques spécialisées dans la présentation d'antigène de migrer vers les ganglions lymphatiques afin de stimuler une réponse immunitaire spécifique à *Leishmania*. Finalement, tel que discuté plus haut, le LPG est également un inhibiteur puissant de la fusion membranaire des organites de l'hôte en particulier entre les phagosomes et les endosomes, un processus essentiel à la formation de phagolysosomes dans lesquels les promastigotes de *Leishmania* ne survivent pas. Or, certaines recherches font état de l'implication des radeaux lipidiques dans la fusion membranaire (Lang et *al.*, 2002; Chamberlain et *al.*, 2001; Chapitre I). De plus, l'approche protéomique a permis

l'identification de plusieurs protéines de fusion comme les SNARES sur les radeaux lipidiques de phagosomes, ce qui sous-tend un rôle pour les radeaux dans la fusion membranaire au niveau du phagosome. Également en accord avec cette hypothèse, le groupe de Mayer a récemment proposé un rôle pour le secteur V0 de la pompe à proton, retrouvée dans les radeaux de phagosomes, dans la fusion membranaire chez la levure (Peters et *al.*, 2001; Bayer et *al.*, 2003). Ce secteur formerait des paires en trans entre des membranes s'apprêtant à fusionner pour ensuite former un pore de fusion pouvant prendre de l'expansion de façon radiaire. Toutes les données précédentes concernant les effets du LPG sur certaines fonctions cellulaires s'accordent bien avec une altération de plates-formes lipidiques fonctionnelles.

Dans l'article 2, nous avons montré que l'actine constitue un composant majeur des radeaux lipidiques. La nucléation de cette protéine, un processus dépendant des radeaux (Rozelle et *al.*, 2000) se produit en des points focaux de la membrane des phagosomes et permet le mouvement des phagosomes ainsi que la fusion avec les organites endocytaires tardifs (Kjeken et *al.*, 2004). Or, des données récentes indiquent que la flotilline-1, ainsi que la flotilline-2, une protéine également présente dans les microdomaines de phagosomes (Goyette et al., résultats non-publiés), pourraient jouer un rôle dans l'organisation de l'actine (Haglund et *al.*, 2004; Neumann-Giesen et *al.*, 2004). De plus, la flotilline-1 pourrait être régulée par phosphorylation par la PKC (une protéine inhibée par le LPG), puisqu'elle possède plusieurs sites de phosphorylation potentiels par cette protéine (Bickel et *al.*, 1997). Nos résultats présentés dans l'article 3 montrent que la flotilline-1 présente dans les radeaux de phagosomes se redistribue à toute la surface du phagosome lors de l'addition du LPG. Par conséquent, il est possible que l'altération des radeaux lipidiques de phagosomes affecte l'organisation de l'actine sur cet organite. En accord avec cette idée, il a déjà été montré que le LPG induit une accumulation de l'actine tout autour de la membrane du phagosome, ce qui permettrait l'exclusion de la PKCα des phagosomes (Holm et *al.*, 2003). Après l'exposition au LPG, la redistribution de l'actine, présente en temps normal, c'est-à-dire en l'absence de LPG, en des points focaux (Kjeken et *al.*, 2004; Defacque et *al.*, 2002), à toute la surface du phagosome pourrait former une sorte de

barrière capable de restreindre la fusion avec les organites endocytaires tardifs. Cette hypothèse s'accorde bien avec les résultats présentés dans l'article 1 qui démontrent que l'inhibition de la fusion des phagosomes produite par *Leishmania donovani* est beaucoup plus importante envers les endosomes tardifs et les lysosomes qu'avec les endosomes précoces. Les endosomes tardifs et les lysosomes étant riches en hydrolases, les leishmanies auraient donc avantage à inhiber la fusion avec ces compartiments plutôt qu'avec les endosomes précoces pauvres en hydrolases.

L'étude présentée dans l'article 5 en annexe corrobore également la diminution de la fusion des phagosomes avec les endosomes tardifs mais non envers les endosomes précoces. En effet, nous avons démontré que les phagosomes formés par l'internalisation de mutants LPG2- ou de *Leishmania* de type sauvage acquièrent en quantité similaire les marqueurs d'endosomes précoces, tels que le TFR et EEA1. Par contre, l'acquisition de marqueurs tardifs comme LAMP1 et Rab7 est beaucoup moins importante chez les phagosomes contenant le type sauvage que chez les phagosomes contenant des mutants dépourvus de LPG. Il a été montré que Rab7 régule la fusion entre les endosomes tardifs (Feng et *al.* ,1995). L'exclusion de cette molécule pourrait avoir comme conséquence l'inhibition de la fusion des phagosomes avec les endosomes tardifs, mais la possibilité que ce soit d'abord l'inhibition de la fusion qui empêche l'acquisition de cette molécule est aussi valable. Une étude a rapporté que certaines petites GTPases de la famille des rabs sont présentes dans les radeaux lipidiques extraits à partir de membranes totales chez les J774 (Li et *al.*, 2003). Nous avons également retrouvé certaines rabs (Goyette et *al.*, résultats non-publiés), par exemple Rab7 dans les radeaux de phagosomes par spectrométrie de masse, mais cette protéine est absente par immunobuvardage (article 3). Afin d'expliquer cette apparente contradiction, il est possible que cette protéine ne s'associe aux radeaux de phagosome que de façon transitoire, par exemple lors de la fusion, et qu'elle s'en dissocie par la suite. Le fait que la spectrométrie de masse soit beaucoup plus sensible que l'ummunobuvardage pourrait également expliquer la non-détection de rab7 par cette dernière technique. Des recherches futures seront nécessaires afin de préciser le lien qui existe entre les rabs, les radeaux lipidiques et la fusion membranaire.

Mécanisme d'altération des radeaux lipidiques par le LPG.

Le LPG de *Leishmania donovani* est présent dans le feuillet cytoplasmique de la membrane du parasite et est distribuée sur toute sa surface (Ilg, 2000). Il s'agit d'une molécule complexe contenant plusieurs parties notamment, une ancre lipidique, 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol (GPI), un noyau hexasaccharidique et un polymère d'unités disaccharidiques phosphorylées, (PO4-6Galβ1-4Man) appelé aussi le phosphoglycane (PG). Cependant, le mécanisme d'action du LPG et surtout laquelle de ses parties lui confère la capacité de modifier plusieurs fonctions des cellules hôtes ne sont pas encore connus.

Le LPG est relâché par les parasites peu de temps après leur internalisation et s'insère en quelques minutes dans les membranes du phagosome en une distribution quasi uniforme (Kaneshiro et *al.*, 1982; King et *al.*, 1987; Tolson et *al.*, 1990; article 3). Toutefois, ni la cinétique, ni la façon exacte dont s'effectue cette insertion dans les membranes de l'hôte et si celle-ci est nécessaire au mécanisme d'action du LPG ne sont encore connues. Puisque le LPG contient une ancre GPI, il est possible que cette partie de la molécule permette l'ancrage dans les membranes de l'hôte. Cet ancrage, qui pourrait se faire directement dans les radeaux lipidiques puisque que les ancres GPI sont reconnues pour leur forte affinité envers ces structures, pourrait être nécessaire à l'action du LPG. En appui à cette hypothèse, il a été démontré que le LPG se retrouve enrichi dans les radeaux lipidiques à la surface même du parasite (Denny et *al.*, 2001). Par contre, par une approche génétique, nous avons montré que la capacité du LPG d'altérer les radeaux lipidiques n'est probablement pas relié à l'ancre GPI puisque les mutants *lpg1-/-* et *lpg2-/-* possèdent toujours cet ancre (figure 3, Chapitre I), mais n'ont pas d'effet sur les radeaux lipidiques de phagosomes. Les effets du LPG sur les radeaux seraient plutôt reliés aux unités répétitives (PO4-6Galβ1-4Man) tel qu'indiqué par la relation directe entre la longueur du polymère d'unités répétitives et sa capacité à inhiber la formation des radeaux lipidiques enrichis de GM1 sur le phagosome. Toutes ces données orientent plutôt vers un

mécanisme où les charges négatives de la chaîne glycosylée du LPG interfèreraient avec le regroupement de molécules en radeaux.

L'importance des unités disaccharidiques répétives a également été mis en relief par des approches expérimentales où on observe l'effet cellulaire de différents fragments de la molécules du LPG, tels que la portion phosphoglycane (PG), la portion lipidique (GPI) ou le LPG complet. Par ces approches, il a été démontré que le PG de la molécule, c'est-à-dire le LPG sans l'ancre lipidique ni le noyau hexasaccharidique, peut provoquer des effets sur les cellules avec lesquelles il est mis en contact. Par exemple, l'inhibition de la fusion virale avec des membranes d'érythrocytes dépend de la longueur de la chaîne d'unités répétitives du PG (Miao et *al.*, 1995). Dans le cas de l'inhibition de la production d'IL-12 mentionnée plus haut, c'est également la portion PG du LPG qui exerce un effet et non pas la portion GPI, puisque l'incubation des cellules avec cette ancre n'a pas d'effet sur la synthèse de l'Il-12 contrairement à l'incubation avec du PG (Piedrafita et *al.*, 1999). Il a également été montré que la portion PG est nécessaire à la régulation de l'expression de la protéine synthétase de l'oxyde nitrique chez le macrophage (Proudfoot et *al.*, 1996), à l'inhibition de la formation de syncytium induite par le VIH (Easterbrook et *al.*, 1995), et à l'inhibition de l'activité des monocytes humains (Frankenburg et *al.*, 1990; Giorgione et al. 1996). Dans tous les cas énumérés précédemment, la présence de l'ancre GPI attachée au PG n'avait pas d'effet additionnel.

Il existe des études où l'on montre la nécessité de l'ancre GPI. En effet, le GPI seul inhiberait de façon plus importante la PKCα que les unités répétitives (McNeely et *al.*, 1989) et ces dernières seraient incapables d'inhiber la migration transendothéliale des monocytes en l'absence de l'ancre GPI (Lo et *al.*, 1998). Ces données semblent contradictoires avec celles énumérées plus haut, mais étant donné la complexité de la molécule et la myriade d'effets qu'on lui attribue, il n'est pas impossible que chacun de ces effets soit attribuable à des parties différentes de la molécule. Dans certains cas, l'insertion membranaire pourrait ne pas être nécessaire à produire des modifications chez les cellules hôtes. En effet, puisque que le LPG constitue une grosse molécule encombrante, elle

pourrait agir directement sur des protéines spécifiques ou des composants lipidiques par sa partie chargée résidant dans le polymère d'unités disaccharidiques répétitives. Des études plus approfondies seront nécessaires afin de déterminer avec exactitude la façon dont le LPG exerce ses fonctions, en particulier des études faisant appel à des approches biophysiques où on peut analyser à un niveau moléculaire l'effet du LPG sur la bicouche lipidique. En ce qui concerne les radeaux lipidiques de phagosomes, d'autres études protéomiques et peut-être même des études de lipidomique seront importantes afin d'obtenir une meilleure compréhension de ces structures.

Conclusion

Le *Leishmania* est un pathogène important chez l'humain puisqu'il cause une maladie grave contre laquelle il existe peu de traitements efficaces. Ce parasite a développé des stratégies afin de survivre non seulement dans la mouche des sables, mais également dans les phagosomes de macrophages chez le mammifère. Une des stratégies de survie du parasite est reliée à la production d'un glycoconjugué de surface, le LPG, qui a des effets pléiotropiques chez les macrophages. Les résultats présentés dans cette thèse, qui démontrent clairement un effet du LPG dans l'inhibition de la fusion phagosome-endosome et la biogenèse du phagolysosome, ont permis de mettre en évidence l'importance de cette molécule lorsque les promastigotes initient l'infection. Les résultats ont aussi montré l'existence de radeaux lipidiques sur le phagosome. Par une approche protéomique, plusieurs protéines ont été identifiées sur ces structures suggérant que les radeaux exercent des rôles essentiels au bon fonctionnement du phagosome. Le fait que le LPG perturbe les radeaux lipidiques pourrait expliquer les multiples effets connus de cette molécule sur la cellule hôte et sur le phagosome qui abrite *Leishmania*.

Étant donné l'intérêt de la communauté scientifique envers les radeaux lipidiques (au-delà de 800 articles sur PUBMED), la découverte d'une molécule microbienne capable de perturber ces structures ouvrira la voie à de nouvelles hypothèses et à de la recherche

excitante dans le domaine des interactions hôtes-pathogènes et des interactions pathogènes-radeaux lipidiques.

Chapitre V. Bibliographie

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Chapitre VI. Annexes

Annexe I. Article 4

Dermine J.-F., Desjardins M. (1999) Survival of intracellular pathogens within macrophages. Protoplasma **210**, 11-24

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PROTOPLASMA

Survival of intracellular pathogens within macrophages

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Summary. The threat caused by intracellular pathogens increases as conventional drug treatments are less and less effective against a wide range of microorganisms. Understanding the molecular mechanisms used by intracellular pathogens to avoid killing and degradation in their host cells is likely to point at new ways to threat infectious diseases. We discuss some of the strategies used by various microorganisms to avoid killing and degradation in phagolysosomes. Interestingly, it appears that microbes have a lot to teach us about the cell biology and molecular mechanisms of organelle sorting in macrophages.

Keywords: Infectious disease; Phagocytosis; Phagosome fusion; *Leishmania*; Natural-resistance-associated macrophage proteins.

Introduction

Humans and microorganisms have coevolved to benefit from symbiotic relationships. A good example is the interaction existing between humans and the intestinal bacterial flora. However, some microbes pose a real threat to human health and are the causative agents of serious diseases like tuberculosis, salmonellosis, and leishmaniasis to name just a few. The danger posed by microorganisms is even more acute as conventional drug treatments are less and less effective against a wide variety of intracellular pathogens. Thus, in order to develop new ways to fight intracellular pathogens, it becomes imperative to understand at the molecular cell biology level the processes by which microorganisms evade our natural cellular defenses.

Paradoxically, several intracellular parasites can invade and replicate within cells of the immune system specialized in ingesting and killing microorganisms, like neutrophils and macrophages. In these cells, microbes are taken up through phagocytosis and reside in phagosomes, organelles originating from invaginations of the plasma membrane. The newly formed phagosomes will then engage in a complex process of maturation involving the rapid recycling of membrane components to the plasma membrane (Pitt et al. 1992a), binding to and movement along cytoskeletal elements (Blocker et al. 1998), and a series of fusion events with endocytic organelles, giving rise to lysosome-like organelles referred to as phagolysosomes (Desjardins et al. 1994a, Desjardins 1995, Beron et al. 1995). At that point, phagolysosomes are filled with hydrolytic enzymes and toxic oxygen metabolites that contribute to the destruction of microbes. Once degraded, microbial peptides are presented to effector cells of the immune system in order to trigger specific immune responses (Harding et al. 1995, Germain 1995). Unfortunately, intracellular pathogens have evolved strategies to evade killing and degradation within their host cells. Basically, three main survival strategies can be distinguished.

1. Some microorganisms like *Leishmania* spp. amastigotes and *Coxiella burnetii* reside and proliferate with impunity in the harsh environment of mature phagolysosomes.

2. Microorganisms like *Listeria monocytogenes* and *Shigella* spp. avoid their delivery to phagolysosomes by synthesizing lytic enzymes allowing the degradation of the phagosome membrane and their escape to the nutrient-rich cytoplasm.

3. Pathogens like *Toxoplasma gondii*, *Brucella* spp., *Legionella* spp., *Mycobacterium* spp., and the pro-

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cytosis, although a recent report indicates that coiling phagocytosis could be involved (Rittig et al. 1998). Early studies have shown that phagosomes containing leishmanial amastigotes display the markers and characteristics of lysosome-like organelles. Indeed, several groups have shown that vacuoles containing *L. mexicana* or *L. amazonensis* are accessible to endocytic tracers and display activity for the late-endosome and lysosome enzyme acid phosphatase (Alexander and Vickerman 1975, Chang and Dwyer 1976, Shepherd et al. 1983, Rabinovitch et al. 1985). Moreover, recent studies have shown that vacuoles housing amastigotes of *L. amazonensis* maintain an acidic pH (Antoine et al. 1990) and that *L. donovani*-containing phagosomes have hydrolases and membrane markers typical of late-endosome and lysosome compartments (Prina et al. 1990, Russell et al. 1992, Lang et al. 1994a). These data are consistent with the proposal that leishmanial amastigotes reside and multiply within phagolysosomes. The molecular machinery allowing amastigotes to survive in phagolysosomes is poorly known. The finding that they do not inactivate phagolysosomal hydrolases suggests that amastigotes are resistant to their action. Unlike the promastigote form, amastigotes do not form a glycocalix capable of protecting them from hydrolases (Pimenta et al. 1991). Instead, they might synthesize proteases involved in the degradation of host molecules in phagolysosomes (Pupkis et al. 1986). An approach to analyze those proteins that are specifically expressed by *Leishmania* spp. within host phagosomes is currently developed in our laboratory to address this issue.

Coxiella burnetii

The rickettsia *Coxiella burnetii* is the causative agent of the Q fever. This bacterium was shown to reside within vacuoles staining for acid phosphatase and containing accumulation of membranes characteristic of late-endocytic or lysosome-like organelles (Burton et al. 1978, Heinzen et al. 1996), and displaying an acidic pH (Maurin et al. 1992). Interestingly, it was shown that *L. amazonensis* amastigotes and *C. burnetii* can be present in the same vacuoles after coinfection, further confirming the ability of these organisms to survive within the same type of phagolysosomes (Rabinovitch and Veras 1996). The mechanisms allowing *C. burnetii* to survive in the hostile environment prevalent in phagolysosomes are poorly understood. Recent reports indicate that *C. burnetii* may produce enzymes eliminating or preventing the formation of oxygen metabolites (Y. Li et al. 1996). Its surface-expressed lipopolysaccharide (LPS) could also be used as a virulent factor (Baca et al. 1994). Interestingly, surface-expressed molecules like LPS, or lipophosphoglycan in the case of *Leishmania* spp., are also used as virulence factors by other microorganisms (see below). Obviously, there is still a lot to learn about the intracellular trafficking of *C. burnetii* and its mechanisms of survival in mammalian cells.

Microorganisms that escape phagosomes

An efficient way for microorganisms to prevent their killing and degradation in phagolysosomes is to escape phagosomes and gain entry to the more cozy environment of the cell cytoplasm. This is the strategy used by microorganisms such as *Listeria monocytogenes*, *Shigella* spp., and *Trypanozoma cruzi.*

Listeria monocytogenes

Listeria monocytogenes is a food-borne pathogen that infects mostly epithelial cells of the intestine causing listeriosis, a disease that can also affect the nervous and reproductive systems (Cooper and Walker 1998). As mentioned above, the main survival strategy used by *L. monocytogenes* is to escape from the phagosome before the environment becomes too hostile and to invade the cytoplasm. It does so by synthesizing listeriolysin O, an enzyme that lyses the phagosomal membrane (Gaillard et al. 1987, Portnoy et al. 1988). The finding that listeriolysin O is activated at an acidic pH (Geoffroy et al. 1987) suggests that phagosomes housing *L. monocytogenes* have to mature to a certain degree allowing the acidification of the compartment. *Listeria monocytogenes* also produces two phospholipases that help degrade the phagosomal membrane (Leimeister-Wacher et al. 1991, Mengaud et al. 1991, Vasquez-Boland et al. 1992). Recent evidence suggests that *L. monocytogenes* might be able to control the degree of maturation of the phagosomes in which they reside (Alvarez-Dominguez et al. 1997). With listeria mutants lacking active listeriolysin, it was recently reported that early-endosomal markers (mannose and transferrin receptors) and proteins necessary for early-fusion events (N-ethylmaleimidine-sensitive membrane fusion protein and soluble N-ethylmaleimidine-sensitive factor attachment protein) accumulate on phagosomal membranes enclosing *L.*

forms of LPG containing few repeating units are ineffective in modifying the fusogenic properties of membranes (Miao et al. 1995). The observation that *L. donovani* RT5, a mutant expressing truncated LPG with three to five repeating units (McNeely and Turco 1990), is unable to inhibit phagosome-endosome fusion (Desjardins and Descoteaux 1997) supports the requirement for complete LPG molecules.

The partial inhibition of fusion between phagosomes and endocytic organelles induced by *L. donovani* promastigotes has potential importance in the establishment of an effective infection. First, the hydrolase-poor environment encountered in the early phagosome may be more suited to allow the transformation of promastigotes into amastigotes, which are adapted to the conditions prevailing inside phagolysosomes. Second, the limited transfer of hydrolases into the promastigote-containing phagosomes might alter the efficiency with which this cell generates and presents, at its surface, microbial peptides linked to MHC (major histocompatibility complex) class II molecules, a process requiring the hydrolytic action of enzymes present in lysosomes. Several studies have shown that leishmania-containing phagosomes display MHC class II molecules after stimulation with gamma interferon in macrophages (Antoine et al. 1991; Russell et al. 1992; Lang et al. 1994a, b) and in Langerhans cells (Flohe et al. 1997). Antoine et al. (1991) provided evidence that infected macrophages are able to process and present leishmanial antigens together with MHC class II molecules. Furthermore, they showed that macrophages infected with the promastigote form of the parasite were more suited to stimulate T cells than those infected with amastigotes, suggesting that differentiation of *L. amazonensis* was part of the strategy to evade the immune system (Prina et al. 1996). The finding that *L. amazonensis* can internalize and degrade some of its host MHC class II molecules also indicates a potential way by which the parasite could circumvent its host immune system (De Souza Leao et al. 1995).

Mycobacterium spp.

Mycobacterium spp. enter macrophages through phagocytosis and reside in compartments that fail to fuse with lysosomes (Fig. 1) (Armstrong and Hart 1971, de Chastellier et al. 1993, Xu et al. 1994, Clemens and Horwitz 1995, Barker et al. 1997). However, the idea that mycobacterium-containing phagosomes are totally fusion incompetent has been challenged in the past few years. It is now commonly accepted that these phagosomes are dynamic and fusion-competent compartments. Using electron microscopy and pulse chase experiments with horseradish peroxidase, de Chastellier et al. (1995) have clearly shown that transfer of horseradish peroxidase to phagosomes occurs from early endosomes. Clemens and Horwitz (1995) have shown that *M. tuberculosis*-containing phagosomes acquire endosomal markers like CD63, Lamp1, Lamp2, and cathepsin D, albeit to a lesser extent than fully fusogenic phagosomes containing dead mycobacteria or latex beads. Moreover, Russell et al. (1996) have elegantly demonstrated that phagosomes housing *M. tuberculosis* are accessible by glycosphingolipids from the plasma membrane. Further investigations from this laboratory confirmed that mycobacterium-containing phagosomes display a composition similar to that of early endosomes and may stop their maturation at a transitional state in phagolysosome biogenesis (Sturgill-Koszycki et al. 1996). This study has also shown the presence of endosomal markers such as Lamp1, the immature form of cathepsin D, and cathepsin B and L on these phagosomes. Since cathepsins are normally processed to their mature forms in lysosomes, these results confirm that mycobacterium-containing phagosomes fail to transform into phagolysosomes. Further similarities with early-endocytic organelles have been described. Mycobacterium-containing phagosomes are accessible to exogenous transferrin (Clemens and Horwitz 1996) and are enriched with the small GTPase Rab5, but devoid of the late-endosome marker Rab7 (Via et al. 1997, Deretic et al. 1997). Since Rab5 is known to regulate early endocytic events whereas Rab7 is involved in late events (Chavrier et al. 1990, Gorvel et al. 1991, Méresse et al. 1995, Feng et al. 1995), these results suggest that mycobacterium-containing phagosomes display the machinery required to engage in fusion events with Rab5-enriched early endosomes, but lack molecules allowing subsequent fusion with late-endocytic organelles. This lack of fusion with late-endocytic organelles is likely to explain the absence of proton pump ATPases from these phagosomes and their improper acidification (Sturgill-Koszycki et al. 1994). In turn, lack of acidification may interfere with the recruitment of key fusogenic molecules from the host cell cytoplasm (Aniento et al. 1997). Systematic characterization of the composition of mycobacterium-containing phagosomes by high-resolution

two-dimensional gel electrophoresis might indicate the nature of proteins involved in the alteration of their fusion properties (Sturgill-Koszycki et al. 1997, Hasan et al. 1997).

The mechanisms used by *Mycobacterium* spp. to inhibit phagosome-lysosome fusion are still poorly understood. Early studies indicated that it may involve the production of ammonia interfering with movement of lysosomes and their potential interaction with phagosomes (Gordon et al. 1980, Hart et al. 1983). Virulence factors such as glycolipids present at the bacterial surface (Ehlers and Daffe 1998), in contact with the inner phagosomal membrane after internalization, may also affect the properties of phagosomes.

Salmonella spp.

Members of the genus *Salmonella* infect nonphagocytic cells of the intestinal epithelium by mechanisms involving ruffling of the plasma membrane (Finlay 1994). They also enter macrophages either through conventional phagocytosis, which gives rise to closely fitting phagosomes, or through induction of macropinocytosis, which gives rise to spacious phagosomes (Alpuche-Aranda et al. 1994). Spacious vacuoles are thought to enhance intracellular survival by diluting toxic compounds present in phagolysosomes. The subsequent trafficking and properties of these vacuoles have been the subject of intense research yielding conflicting results. It was reported that, in macrophages, *S. typhimurium* reside in compartments that fuse poorly with lysosomes (Ishibashi and Arai 1990, Buchmeier and Heffron 1991), although the latter study showed a phagosome-lysosome fusion level of about 40% in peritoneal and J774 macrophages. Both studies indicated that the surface-expressed LPS was not involved in this inhibition of fusion. A partial inhibition of fusion was also shown to occur in epithelial cells (Garcia-del Portillo and Finlay 1995). In this case, around 30% of the phagosomes containing *S. typhimurium* also contained horseradish peroxidase previously internalized and chased to lysosomes. These phagosomes were shown to display lysosomal glycoproteins but to lack M6PR and high level of cathepsin D. Similar patterns of labeling were reported in macrophages (Rathman et al. 1997). These two studies suggest that *S. typhimurium* is internalized in phagosomes that interact poorly with M6PR-positive compartments. In contrast, a recent study reported the complete fusion of lysosomes with phagosomes containing *S. typhimurium* indicating its ability to survive within mature phagolysosomes displaying Lamp1 and the lysosomal enzyme cathepsin L (Oh et al. 1996). This discrepancy was suggested to be caused by the use of peritoneal macrophages by Oh et al. (Rathman et al. 1997).

Although further studies are required to define clearly the intracellular routing of salmonella-containing phagosomes, it is apparent that these phagosomes engage in a maturation process resulting in their rapid acidification (Alpuche-Aranda 1992, Rathman et al. 1996), an essential step for the activation of bacterial survival genes.

Legionella pneumophilia

Legionella pneumophilia, the causative agent of a pneumonia known as Legionnaires' disease, is a facultative intracellular parasite that infects alveolar macrophages (McDade et al. 1977). It enters its host cells through a specialized form of phagocytosis referred to as coiling phagocytosis (Horwitz 1984) and resides in phagosomes that fail to acidify and fuse with lysosomes (Horwitz 1983a, Horwitz and Maxfield 1984). Following entry, phagosomes sequentially associate with smooth vesicles, mitochondria, and endoplasmic reticulum (Horwitz 1983b, Swanson and Isberg 1995). Phagosomes eventually relocate near the nucleus where they appear as vacuoles covered with ribosomes due to their close association with the rough endoplasmic reticulum (Horwitz 1983b). These final compartments, referred to as replicative phagosomes, display similarities with autophagic vacuoles (Swanson and Isberg 1995). They lack transferrin receptors and fail to acquire late-endosomal and lysosomal markers like Lamp1, Lamp2, CD63, and cathepsin D (Clemens and Horwitz 1995). These results indicate that maturation of these vacuoles toward phagolysosomes is

Fig. 1 A–D. Interaction of endocytic organelles with phagosomes containing various microorganisms. Phagosomes containing brucellas (**A**), the promastigote *Leishmania donovani* (**B**), or mycobacteria (**D**) interact poorly with endocytic organelles. As a result, all these microorganisms reside in phagosomes that do not transform into phagolysosomes. In contrast, the amastigote form of *Leishmania donovani* (**C**) resides in phagosomes that fuse with endocytic organelles and display the characteristics of phagolysosomes

smooth LPS contribute to the resistance, but smooth LPS seems to be more efficient.

Besides LPS, there are other factors that contribute to resistance of *B. abortus* to killing, namely the stress response proteases of the high-temperature requirement A (HtrA) family that represents a defense system against oxidative killing (Elzer et al. 1996). Evidence implicating the HtrA family as a defense against killing were also found for other bacteria including *S. typhimurium* (Bäumler et al. 1994, Boucher et al. 1996, Johnson et al. 1991, S. Li et al. 1996). All of these elements contribute to the survival and replication of the parasites.

Toxoplasma gondii

Toxoplasma gondii is the etiological agent of toxoplasmosis, a disease that can affect many organs and is encountered mainly in immunocompromised individuals. The parasites enter macrophages and other nucleated cells through an active process that differs from conventional phagocytosis. Internalization is driven by the parasite's ATP and actin cytoskeleton and does not depend on host cell endocytic machinery (for a detailed review, see Sinai and Joiner 1997). As a result, *T. gondii* reside in parasitophorous vacuoles incapable of fusion with host endocytic organelles (Jones and Hirsch 1972, Sibley et al. 1985, Joiner et al. 1990). The parasitophorous vacuole has little in common with normal phagosomes. It arises from a mechanism of entry that does not involve the specific receptors required for phagocytosis (Morisaki et al. 1995, Dobrowolski and Sibley 1996), and it excludes host plasma membrane-associated proteins (de Carvahlo and de Souza 1989, Pacheco-Soares and De Souza 1998). This exclusion may account for the fusion incapacity of the vacuole which apparently lacks the molecular machinery required for fusion. However, when parasites enter macrophages through receptor-mediated phagocytosis, after opsonization with immunoglobulin G, the vacuoles formed are fusion competent and mature normally into phagolysosomes (Joiner et al. 1990, Mordue and Sibley 1997). This suggests that the mechanism of entry determines the fate of parasites within macrophages. This is further demonstrated by the finding that killing of parasites inside phagosomes, after entry, does not restore the fusion competence of vacuoles (Joiner et al. 1990).

Immunocytochemical studies indicate that *T. gondii*-containing vacuoles lack detectable levels of cell surface markers, transferrin receptors, Igp, and M6PR (de Carvalho and de Souza 1989, Joiner 1992, Joiner et al. 1990, Mordue and Sibley 1997), indicating that traffic of these vacuoles does not follow the phagolysosome pathway. Rather, they associate with the endoplasmic reticulum and mitochondria (Sinai et al. 1997). The significance of this association is unknown. It could serve as a nutrient delivery system or could provide building material to expand the vacuole membrane as parasites replicate. The role of endoplasmic reticulum and mitochondria in lipid synthesis goes along with this idea (Trotter and Voelker 1994, Vance and Shiao 1996).

Resistance to infection

It is well documented that killing of intracellular parasites, for instance mycobacterium to name just one, is correlated with the level of activation of macrophages by various cytokines, including gamma interferon (Rook et al. 1986, Flesch and Kaufmann 1987, Denis 1991, Via et al. 1998, Schaible et al. 1998). However, besides the ability to become activated and more efficient at killing, some populations of macrophages have a genetically based natural resistance to intracellular infection. A well-known genetic determinant controlling infection is the *Nramp1* gene product which confers resistance to a series of unrelated intracellular pathogens including *Mycobacterium* spp., *L. donovani*, and *S. typhimurium* (Plant and Glynn 1976, Bradley 1977, Gros et al. 1981). Interestingly, the strategy used by those three microbes to survive within their host cells is to inhibit to different extents phagosome-endosome fusion. This suggested that Nramp1 might play its role within phagosomes. The recent findings that Nramp1 is expressed only in polymorphonuclear leukocytes and macrophages and is recruited to the membrane of phagosomes confirm this hypothesis (Cellier et al. 1997, Gruenheid et al. 1997). The recruitment of Nramp1 to the phagosomal membrane has been shown to follow kinetics similar to that of Lamp1 (Gruenheid et al. 1997). Interestingly, phagosomes containing one of the three pathogens whose infection is controlled by Nramp1 also acquire Lamp1 at some point during their maturation. In contrast, *Listeria* spp. and *Legionella* spp., which are not under genetic control of Nramp1, fail to acquire this marker (see above).

Nramp1 is a member of a family of metal ion transporters (Cellier et al. 1995, 1996), although its sub-

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Annexe II. Article 5

Scianimanico S., Desrosiers M., Dermine J.-F., Meresse S., Descoteaux A., Desjardins M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. Cell Microbiol. **1**(1), 19-32

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Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes

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Summary

We have shown recently that one of the survival strategies used by *Leishmania donovani* promastigotes during the establishment of infection in macrophages consists in inhibiting phagosome–endosome fusion. This inhibition requires the expression of lipophosphoglycan (LPG), the predominant surface glycoconjugate of promastigotes, as parasites expressing truncated forms of LPG reside in phagosomes that fuse extensively with endocytic organelles. In the present study, we developed a single-organelle fluorescence analysis approach to study and analyse the intracellular trafficking of 'fusogenic' and 'low-fusogenic' phagosomes induced by an LPG repeating unit-defective mutant (*lpg2* KO) or by wild-type *L. donovani* promastigotes respectively. The results obtained indicate that phagosomes containing mutant parasites fuse extensively with endocytic organelles and transform into phagolysosomes by losing the early endosome markers EEA1 and transferrin receptor, and acquiring the late endocytic and lysosomal markers rab7 and LAMP1. In contrast, a majority of 'low-fusogenic' phagosomes containing wild-type *L. donovani* promastigotes do not acquire rab7, wheres they acquire LAMP1 with slower kinetics. These results suggest that *L. donovani* parasites use LPG to restrict phagosome–endosome fusion at the onset of infection in order to prevent phagosome maturation. This is likely to permit the transformation of hydrolase-sensitive promastigotes into hydrolase-resistant amastigotes within a hospitable vacuole not displaying the harsh environment of phagolysosomes.

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Introduction

Phagolysosome biogenesis, a key process in the killing and degradation of a variety of pathogenic microorganisms, involves regulated interactions between phagosomes and early endosomes, late endosomes and lysosomes (Mayorga *et al.*, 1991; Desjardins *et al.*, 1994a; 1997; Jahraus *et al.*, 1994; de Chastellier and Thilo, 1997), allowing the modulation of phagosome composition and properties (Pitt *et al.*, 1992; Desjardins, 1995; Desjardins *et al.*, 1997). In order to survive in their host cells, intracellular microorganisms have evolved diverse strategies to evade killing and degradation within phagolysosomes (reviewed by Finlay and Falkow, 1997). One strategy involves inhibiting the fusion of phagosomes with endocytic organelles. This is the case for pathogens such as *Mycobacteria*, *Legionella* and *Salmonella* (Horwitz, 1983; Horwitz and Maxfield, 1984; Clemens and Horwitz, 1995; Garcia-del Portillo and Finlay, 1995; Roy *et al.*, 1998) and, in certain situations, for *Toxoplasma gondii* (Jones *et al.*, 1972; Sibley *et al.*, 1985; Joiner *et al.*, 1990; Mordue and Sibley, 1997) and *Brucella abortus* (Pizzaro-Cerdá *et al.*, 1998). The molecular mechanisms used by these microbes to alter the fusion properties of phagosomes are, in most cases, poorly understood.

The promastigote form of the protozoan parasite *Leishmania donovani* also inhibits phagosome–endosome fusion during the early phase of infection in macrophages (Desjardins and Descoteaux, 1997). This inhibition requires the repeating disaccharide–phosphate unit moiety of lipophosphoglycan (LPG), the major surface glycoconjugate of promastigotes (Turco and Descoteaux, 1992). Indeed, phagosomes containing wild-type (wt) *L. donovani* promastigotes displayed poor fusogenic properties, whereas phagosomes containing LPG-defective mutants fused extensively with endocytic organelles (Desjardins and Descoteaux, 1997). The consequences of this lack of fusion on the intracellular trafficking of phagosomes containing wt *Leishmania* promastigotes are unknown. However, the possibility of inducing the

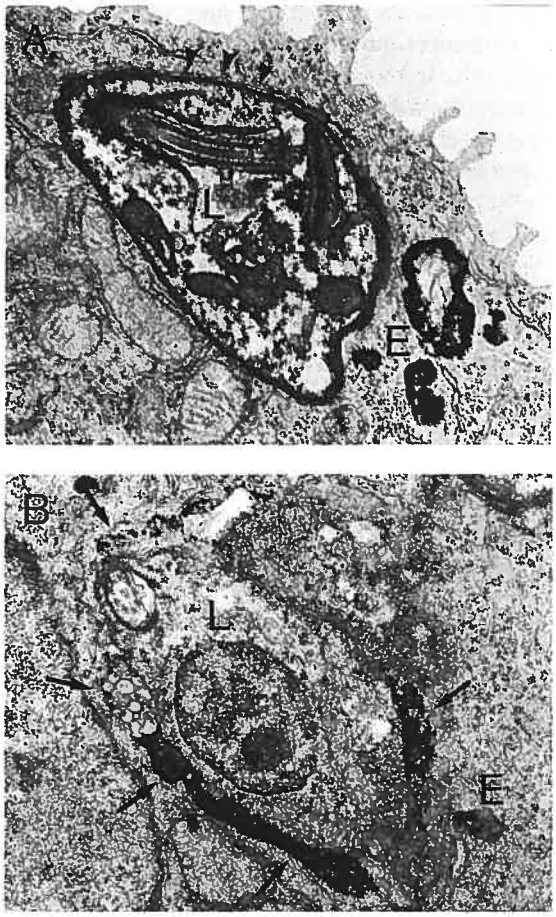

Fig. 2. Fusion properties of phagosomes containing wild-type *Leishmania donovani* promastigotes or *lpg2* KO mutants. Parasites were internalized for 60 min in J774 macrophages followed by a 60 min chase in culture medium. Cells were then fed 16 nm BSA–gold particles for 30 min followed by a 60 min chase, allowing interaction between phagosomes and endosomes. Cell were fixed with glutaraldehyde and prepared for electron microscopy.
A. A phagosome containing a wild-type *L. donovani* promastigote.
B. A phagosome containing a *lpg2* KO mutant. Gold particles present in this phagosome indicate that fusion occurred with endocytic organelles. L, Leishmania; E, endosomes; arrowheads, region in which the phagosome membrane is well distinguished; arrows, gold particles in the phagosome.

60 min followed by a 60 min chase in culture medium. We then loaded the macrophage endosomes with 16 nm BSA–gold for 30 min followed by a 60 min chase. This period of time allowed extensive interaction between phagosomes and BSA–gold-filled endosomes. Observation at the electron microscope indicated that gold particles were transferred to only a few of the wt-containing phagosomes, compared with the *lpg2* KO-containing phagosomes (Fig. 2). The frequency and extent of fusion events occurring between endosomes and either wt-containing or *lpg2* KO-containing phagosomes were comparable with those described previously (Desjardins and Descoteaux, 1997). Indeed, the viability of the two types

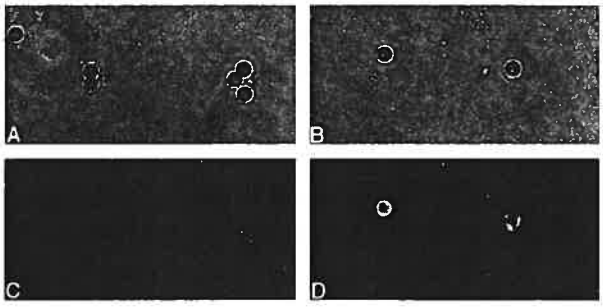

Fig. 3. The cytoplasmic side of isolated phagosomes can be labelled with antibodies. Latex beads were internalized in J774 for various periods of time. Cells were then gently broken, and phagosomes were concentrated by centrifugation. The phagosome preparations were incubated with antibodies against the luminal part of LAMP1 or antibodies against the cytoplasmic tail of LAMP1. Labelling was revealed with a second antibody tagged with FITC. Phase-contrast (A) and fluorescence signal (C) shows no labelling of phagosomes with antibodies against the luminal part of LAMP1, indicating that phagosomes are intact after cell breakage.
B and D. Phagosomes can be labelled with antibodies against the cytoplasmic tail of LAMP1, indicating the efficiency of the approach to detect proteins on the cytoplasmic side of phagosomes. Phagosomes shown here have internalized latex beads for 60 min followed by a 60 min chase.

of parasites differs greatly. While 10% of the wt are still alive 24 h after macrophage infection, only 0.1% of the *lpg2* KO are still living (A. St-Denis and A. Descoteaux, unpublished results). In the present study, phagosomes containing wt *L. donovani* promastigotes are referred to hereafter as 'low-fusogenic' phagosomes, while phagosomes containing the *lpg2* KO mutant are referred to as 'fusogenic' phagosomes.

Single organelle fluorescence analysis (SOFA)

To study the intracellular trafficking/maturation process of 'fusogenic' and 'low-fusogenic' phagosomes, we developed an immunofluorescence assay to analyse the association and dissociation of known proteins on the cytoplasmic side of phagosomes. Our antibodies against LAMP1 and rabs cross-react with *Leishmania* epitopes (not shown). For this reason, standard immunofluorescence techniques could not be used, as the fluorescent signals originating from *Leishmania* organelles could not always be distinguished from the signal of the surrounding host phagosomes (not shown). Consequently, to label proteins specifically on the cytoplasmic side of phagosomes, we developed a SOFA approach involving gently breaking the host cells without fixation or permeabilization steps. A first requisite for this approach was to allow the labelling of proteins with antibodies on the cytoplasmic side of phagosomes, while restricting access to the parasite in the phagosome lumen. We first developed the SOFA approach on latex bead-containing phagosomes, using antibodies recognizing either the luminal domain or the

(Desjardins *et al.*, 1994a; 1997). Indeed, Western blot and GTP overlay analyses revealed that rab7 appears gradually on young phagosomes and disappears from maturing phagosomes, while LAMP1 appears and remains associated with mature phagolysosomes.

Latex bead-containing phagosomes isolated at three different time points were analysed. After a pulse of 10 min without chase (10–0), some labelling for rab7 could be detected on phagosomes by fluorescence microscopy (Fig. 4, left). Rab7 was detected on a higher proportion of phagosomes in the 30–30 min preparations. As expected, labelling for rab7 was not detected on 30–180 min phagosomes. For LAMP1 (Fig. 5), labelling was not detected on the 10–0 min phagosome preparations but was detected on the 30–30 min and the 30–180 min phagosomes (Fig. 5, left). No signal was observed for negative controls when labelling was performed in a PNS solution to which free latex beads were added (not shown), indicating that antibodies did not bind non-specifically to latex beads.

Quantitative analysis by flow cytometry

Analysis of samples by fluorescence microscopy is time consuming, can only be performed on relatively small phagosome populations, and some antibodies yield weak signals that can generate ambiguous results. Thus, a third requisite for the SOFA approach was that the measurements should be performed by flow cytometry to analyse rapidly, efficiently and unambiguously large populations of phagosomes. The efficiency of the approach was also determined with latex bead-containing phagosomes. The

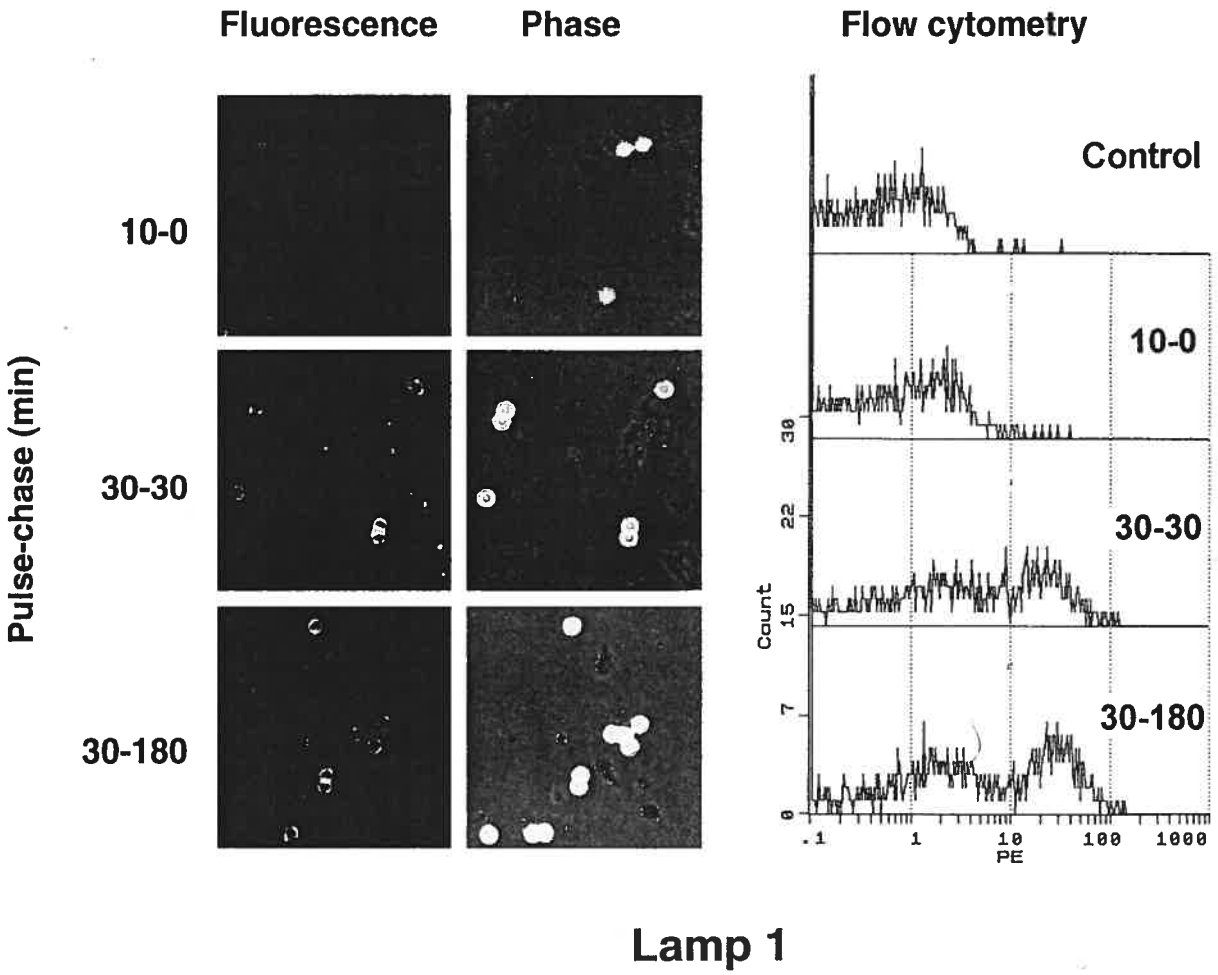

Fig. 5. Single organelle fluorescence analysis of the kinetic association of LAMP1 with latex bead-containing phagosomes. Latex beads were internalized and chased for increasing periods of time in J774 macrophages. Cells were then broken, and the concentrated phagosomes were incubated with antibodies against LAMP1. The first antibody was revealed with a second antibody tagged with FITC. Left. The fluorescent signal on concentrated phagosomes.
Middle. The phagosomes in the phase contrast.
Right. Measurement of the percentage of labelled phagosomes determined by flow cytometry (see Fig. 8).

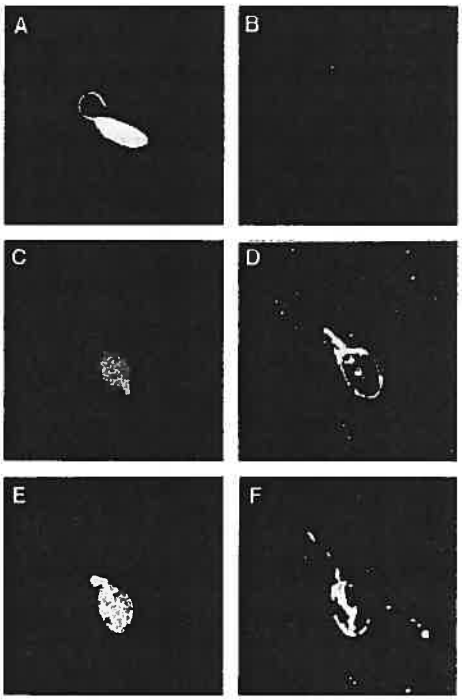

Fig. 8. Labelling of the cytoplasmic side of phagosomes containing *Leishmania donovani*. Parasites were internalized in J774 macrophages for 60 min followed by a 60 min chase in culture medium. Cells were then broken, and the concentrated phagosomes were incubated with antibodies against LPG, rab7 or LAMP1. The first antibodies were revealed with a second antibody tagged with rhodamine.
A, C and E. GFP-*Leishmania*.
B. Results obtained with antibodies against LPG, indicating that the parasite surface is not accessible to antibodies added to the phagosome preparation.
D. Labelling of a phagosome with anti-rab7.
F. Labelling of a phagosome with anti-LAMP1.

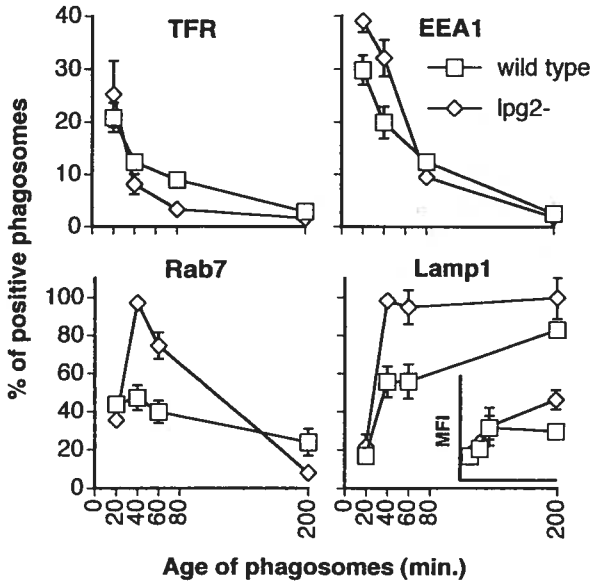

Fig. 9. Kinetic association of markers to the cytoplasmic side of *Leishmania*-containing phagosomes measured by flow cytometry. Wild-type *Leishmania* or *lpg2* KO mutants were internalized in J774 macrophages for various periods of time. Concentrated phagosomes were then incubated with antibodies against the transferrin receptor, EEA1, rab7 or LAMP1. The first antibodies were revealed with a second antibody tagged with phycoerythrin. Each sample was analysed by flow cytometry to determine the percentage of phagosomes labelled at each time point for each antibody. Inset in the LAMP1 graph shows the mean fluorescence intensity of labelling for the corresponding time points.

the SOFA approach in conjunction with flow cytometry to analyse the association and dissociation of key proteins with 'fusogenic' phagosomes (containing *lpg2* KO-GFP) or 'low-fusogenic' phagosomes (containing wt-GFP) isolated at various time points after their formation. The proteins analysed were the early markers, transferrin receptor and EEA1, and the late markers, rab7 and LAMP1. Results of the kinetics of association of these markers with the various phagosome populations are presented in Fig. 9. The transferrin receptor was present on early phagosomes containing either the wt-GFP or the *lpg2* KO-GFP (Fig. 9). The proportion of phagosomes displaying this marker decreased with time. The early endosome marker EEA1 (Fig. 9) was also present on the early *Leishmania*-containing phagosomes, and the proportion of labelled phagosomes decreased during phagosome ageing. The proportion of phagosomes positive for EEA1 was consistently higher for *lpg2* KO-containing phagosomes than for wt-containing phagosomes. EEA1 has been localized to endosomes (Mu *et al.*, 1995). This is the first localization of EEA1 to phagosomes, a result that emphasizes the idea that phagosomes and endosomes share the same functional proteins (Desjardins, 1995; Storrie and Desjardins, 1996).

The kinetics of rab7 association to the 'fusogenic' *lpg2* KO-containing phagosomes is characterized by an increase in the proportion of phagosomes labelled at early time points, followed by a decrease at the later time points. In contrast, the proportion of wt-GFP phagosomes labelled for rab7 remained constant at all time points, at levels that were significantly lower than those observed for *lpg2* KO-GFP 'fusogenic' phagosomes. For LAMP1, the percentage of labelled *lpg2* KO-GFP phagosomes increased during the early time points and reached a plateau as phagosomes matured. The association of LAMP1 with the wt-GFP phagosomes was consistently lower than with *lpg2* KO-GFP phagosomes. The mean of fluorescence intensity for LAMP1 was also measured on all phagosomes (see insert in Fig. 9). The results show that the intensity of the fluorescent signal at the later time points was higher for *lpg2* KO-GFP phagosomes compared with wt-GFP phagosomes. This also suggested that the maturation process of wt phagosomes into phagolysosomes is impaired.

effector of late endosome/lysosome transformation (Feng *et al.*, 1995; Méresse *et al.*, 1995; Mukhopadhyay *et al.*, 1997; Vitelli *et al.*, 1997), could explain why these phagosomes are fusing poorly with endocytic organelles. It would be interesting to determine in future studies whether rab7 is recruited to phagosomes at later time points after infection when promastigotes transform into amastigotes in macrophage phagosomes. Indeed, the group of Antoine has shown that phagosomes housing amastigotes of *Leishmania* display the rab7 marker (Lang *et al.*, 1994). As these phagosomes are known to be fusogenic (for example, see Russell *et al.*, 1992), this suggests that rab7 is recruited to *Leishmania*-containing phagosomes as parasites transform into amastigotes (see below). The molecular mechanisms responsible for the failure to recruit rab7 to promastigote-containing phagosomes are still unknown. However, excluding or avoiding the recruitment of rab7 to phagosomes appears to be a process used by several microorganisms to survive in their host cells.

Toxoplasma gondii is present within a vacuole that does not interact with endocytic organelles and does not acquire rab7 or LAMP1 molecules (Joiner *et al.*, 1990; Mordue and Sibley, 1997). The particular mechanism of entry of this pathogen into its host cells, referred to as active penetration, appears to modulate the fusion properties of phagosomes by restricting the association of molecules from the plasma membrane to the nascent vacuoles during internalization (Mordue and Sibley, 1997). *Legionella pneumophila* reside within a phagosomal compartment that does not interact with late endocytic organelles (Horwitz, 1983) and fails to acidify (Horwitz and Maxfield, 1984). As a result, *L. pneumophila* is present in rab7-negative and LAMP-negative compartments (Clemens and Horwitz, 1995; Swanson and Isberg, 1996; Roy *et al.*, 1998) that display a rough endoplasmic reticulum-like or autophagosome morphology (Horwitz, 1984; Swanson and Isberg, 1995). Although *Mycobacteria*-containing phagosomes can fuse with early endosomes, they do not seem to fuse with lysosomes, as shown in numerous studies (Armstrong and Hart, 1971; Frehel *et al.*, 1986; Sibley *et al.*, 1987; Xu *et al.*, 1994; Clemens and Horwitz, 1995; de Chastellier *et al.*, 1995; Russell *et al.*, 1996). These results are supported by the recent finding that these phagosomes accumulate the small GTP-binding protein rab5 at their surface but fail to acquire detectable levels of rab7 (Via *et al.*, 1997). Still, the mechanisms whereby *Mycobacterium* species alter some of the phagosome properties remain to be determined.

Our results, together with recent observations by other laboratories, provide a better understanding of the strategy used by *Leishmania* to establish infection within their host cells and allow us to propose the following model (Fig. 10). At the onset of infection, LPG from promastigotes

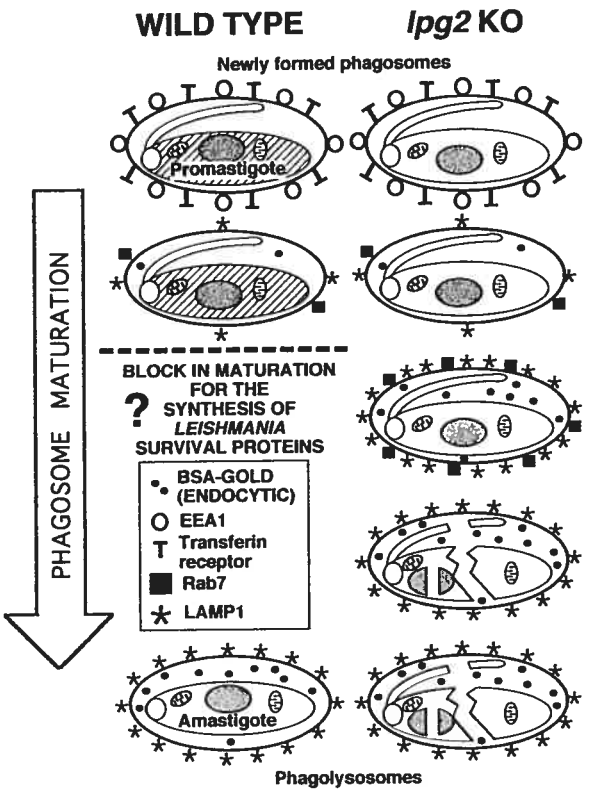

Fig. 10. Intracellular trafficking of phagosomes containing *Leishmania* promastigotes. Phagosomes containing *Leishmania* mutants lacking LPG repeating units mature into phagolysosomes by displaying sequential markers of early endosomes, late endosomes and lysosomes. These phagosomes fuse extensively with endocytic organelles. In contrast, the presence of LPG on wild-type *L. donovani* promastigotes alters phagosome fusion properties and interferes with the phagosome maturation process. This allows promastigote parasites to initiate their transformation into amastigotes within a hospitable compartment. The disappearance of LPG at the surface of parasites during this transformation process lifts the phagosome–endosome fusion inhibition. Phagosome maturation resumes, and hydrolase-resistant amastigotes survive with impunity in the phagolysosomes.

plays a crucial role by inhibiting phagosome–endosome fusion. It does so by inserting itself, within minutes, into the macrophage membrane at the immediate area of internalization of the promastigote (Tolson *et al.*, 1990) and within the phagosomal membrane. Insertion of LPG stabilizes the bilayer against the formation of an inverted hexagonal structure (Miao *et al.*, 1995). As a consequence, LPG gives rise to an effective 'steric repulsion' between phagosomal and endosomal membranes or reduces the negative curvature strain in bilayers, increasing the energy barrier for forming highly curved fusion intermediates, and thereby preventing fusion. In turn, the lack of fusion with late endocytic organelles prevents the acquisition of key molecules, such as rab7, and results in the inhibition of phagosome maturation, allowing the hydrolase-sensitive promastigotes to transform into amastigotes in

incubated for 60 min in culture medium to allow complete internalization of bound parasites. These macrophages were subsequently fed 16 nm BSA–gold particles for 30 min and incubated further in culture medium for 60 min, as described previously (Desjardins and Descoteaux, 1997). After all internalization steps, cells were washed thoroughly with cold PBS three times for 5 min. At the end of each experiment, cells were fixed in 1% glutaraldehyde, post-fixed in OsO_4, processed for embedding in Epon 812 resin as described previously (Desjardins *et al.*, 1994a) and observed by electron microscopy.

The level of fusion between phagosomes and endocytic organelles was determined by measuring the presence of BSA–gold particles in *Leishmania*-containing phagosomes. The presence of a single gold particle inside a phagosome was scored as a fusion event. Each experiment was done three times, and a minimum of 100 phagosomes per sample was recorded, while extreme care was taken to avoid counting the same phagosome on serial sections.

Preparation of phagosomes for immunofluorescence studies

J774 macrophages were grown to subconfluency in DMEM in 10 cm Petri dishes. For each determination, one 10 cm dish was used (between 5 and 10 million cells). Phagosomes were formed by the internalization of either 3 μm of latex beads (Sigma) diluted 1:200, or stationary phase promastigotes at a parasite-to-host cell ratio of 10:1 for wt and 5:1 for the *lpg2* KO mutant in culture medium. Internalization took place at 37°C for various lengths of time (pulse). Uninternalized particles were then removed by several washes with cold PBS. Cells were then incubated further in culture medium for various time periods (chase). At the end of the chase period, cells were washed with cold PBS, followed by one last wash in cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EDTA, 0.05% gelatin, pH 7) (Chakraborty *et al.*, 1994) containing protease inhibitors (Desjardins and Scianimanico, 1998) and 0.1% NaN_3 to neutralize parasite movement and avoid breakage of phagosomes by promastigote flagella. After washes, cells were scraped with a rubber policeman in 600 μl homogenization buffer per dish at 4°C. Cells were then broken by passages through a 22G gauge emulsifying needle connected to two syringes (Chakraborty *et al.*, 1994; Desjardins and Scianimanico, 1998). Great care was taken to avoid air bubbles and breakage of the host cell nuclei. A post-nuclear supernatant was obtained after two low-speed centrifugations at $200 \times g$ for 5 min in 15 ml Falcon tubes. Phagosomes were concentrated by a final centrifugation at $700 \times g$ for 10 min, gently resuspended in 50 μl of homogenization buffer and immediately processed for immunofluorescence as described below. This approach yields enriched phagosome preparations suitable for fluorescence analysis.

Immunolabelling of proteins on the cytoplasmic side of phagosomes

Presence of the transferrin receptor, EEA1, rab7 and LAMP1 on phagosomes was investigated using immunofluorescence by directly labelling the cytoplasmic side of phagosomes with antibodies against these proteins. The absence of permeabilization or fixation steps allowed us to label only the molecules present on the cytoplasmic side of the phagosome membrane, therefore restricting access of the antibodies to the phagosome lumen and any non-specific binding to *Leishmania* proteins. For labelling, fresh phagosome preparations were incubated for 30 min at 4°C in the presence of the first antibody in a 10–20 μl aliquot. The transferrin receptor was detected with a monoclonal antibody raised against the human receptor and that cross-reacts with the mouse receptor (Zymed Laboratories). EEA1 was detected with a human polyclonal antibody (Mu *et al.*, 1995). Rab7 protein was detected with an affinity-purified rabbit polyclonal antibody against a C-terminal peptide (Méresse *et al.*, 1997). LAMP1 was revealed using a rabbit polyclonal antibody specific to the cytoplasmic portion of the molecule (KLVGRKRSHAGYQTI). Secondary antibodies were added directly without washes for another 30 min at 4°C. The secondary antibodies were conjugated to either FITC or rhodamine for observation at the fluorescence microscope or to FITC or R-phycoerythrine (PE) (Jackson Immunology, Bio-Can) for analysis by flow cytometry (see figure legends). Observation at the fluorescence microscope was performed immediately by placing 1 μl of the labelled samples under a coverslip.

A high proportion of the phagosomes remained intact after breaking the cells, as the phagosome lumen was not accessible to a rat monoclonal antibody directed against the luminal portion of LAMP1 (Chen *et al.*, 1988). The same approach was used with a monoclonal antibody directed against the LPG repeating units (CA7AE; see Tolson *et al.*, 1989) to evaluate the proportion of intact phagosomes containing wt *L. donovani* promastigotes. For LPG labelling, phagosome preparations were incubated for 30 min at 4°C with ascites fluid containing CA7AE. A secondary antibody against mouse IgM was then added directly without washes for another 30 min at 4°C. Secondary antibodies were either conjugated to rhodamine for observation at the fluorescence microscope or to cyanin (Cy5) (Jackson Immunology, Bio-Can) for analysis by flow cytometry.

Analysis of phagosomes by flow cytometry (SOFA)

For single organelle fluorescence analysis (SOFA), labelled samples were diluted in 400 μl of ice-cold PBS and filtered through a 30 μm nylon mesh. Fluorescence measurements were performed on an Epics Elite flow cytometer (Coulter Electronics) equipped with an argon laser emitting at 488 nm for the excitation of GFP and PE (emission at 525 nm and 575 nm) and an HeNe laser emitting at 633 nm for the excitation of Cy5 (emission at 675 nm). Different gating strategies were elaborated to optimize the analysis of phagosomes. First, the latex bead-containing phagosomes produced a distinctive pattern of light scattering allowing their discrimination against cell debris for the fluorescence measurements (not shown). The light-scattering pattern of the phagosomes containing *Leishmania* overlay in part the scattering of cell debris compromising fluorescence measurements. Accordingly, we have generated *Leishmania* expressing GFP and designed another gating strategy taking advantage of this additional signal. Briefly, the light-scattering gate was designed based

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Annexe III. Article 6

Garin, J., Diez, R., Kieffer, S., Dermine, J.-F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., Desjardins, M. (2001). The Phagosome Proteome : Insight into Phagosome Functions. *J.Cell Biol.* 152, 165-180.

The Phagosome Proteome: Insight into Phagosome Functions

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Abstract. Phagosomes are key organelles for the innate ability of macrophages to participate in tissue remodeling, clear apoptotic cells, and restrict the spread of intracellular pathogens. To understand the functions of phagosomes, we initiated the systematic identification of their proteins. Using a proteomic approach, we identified >140 proteins associated with latex bead–containing phagosomes. Among these were hydrolases, proton pump ATPase subunits, and proteins of the fusion machinery, validating our approach. A series of unexpected proteins not previously described along the endocytic/phagocytic pathways were also identified, including the apoptotic proteins galectin3, Alix, and TRAIL, the anti-apoptotic protein 14-3-3, the lipid raft-enriched flotillin-1, the anti-microbial molecule lactadherin, and the small GTPase rab14. In addition, 24 spots from which the peptide masses could not be matched to entries in any database potentially represent new phagosomal proteins. The elaboration of a two-dimensional gel database of >160 identified spots allowed us to analyze how phagosome composition is modulated during phagolysosome biogenesis. Remarkably, during this process, hydrolases are not delivered in bulk to phagosomes, but are instead acquired sequentially. The systematic characterization of phagosome proteins provided new insights into phagosome functions and the protein or groups of proteins involved in and regulating these functions.

Key words: phagosome • proteome • matrix-assisted laser desorption/ionization–time-of-flight–mass spectrometry • membrane fusion • apoptosis

Introduction

Professional phagocytes, such as macrophages, internalize large particulate material by phagocytosis. This constitutive process, initiated by binding of the particle to cell surface receptors, triggers a reorganization of the plasma membrane and its cortical cytoskeletal elements, leading to particle engulfment and formation of the phagosome. Phagosomes are pivotal organelles in the ability of macrophages to perform several of their key functions, such as the handling of apoptotic cells, tissue remodeling, and the restriction of the establishment and spread of intracellular pathogens (see Méresse et al., 1999). The functional properties of phagosomes are acquired through a complex remodeling process involving regulated interactions with a series of endovacuolar organelles. Indeed, during phagolysosome biogenesis, phagosomes intersect the biosynthetic pathway and fuse sequentially with early endosomes, late endosomes, and lysosomes (Pitt et al., 1992; Desjardins et al., 1997; Jahraus et al., 1998). These interactions, facilitated by phagosome binding and movement along cytoskeletal elements (Desjardins et al., 1994a; Defacque et al., 2000), and controlled in part by small GTPases of the rab family (Alvarez-Dominguez and Stahl, 1999; Roberts et al., 1999; Duclos et al., 2000), allow the acquisition of groups of molecules conferring new functions to maturing phagosomes. Despite our current knowledge, the molecules and mechanisms coordinating the various steps involved in phagolysosome biogenesis are still mostly unknown. A significant part of our ignorance originates from the fact that few of the phagosome proteins are currently known or studied, despite the presence of hundreds of polypeptides on this compartment (Desjardins et al., 1994b; Haas, 1998). Identification of these molecules is likely to provide insights into the complex mechanisms governing phagosome functions.

In this study, we present a comprehensive analysis of phagosomes from which >140 proteins have been identified. Furthermore, we built a two-dimensional (2-D)[1] phago-

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[1]*Abbreviations used in this paper:* 2-D, two-dimensional; ARF, ADP-ribosylation factor; EST, expression sequence tag; MHC, major histocompatibility complex; MS, mass spectrometry; PDI, protein disulfide isomerase; PMF, peptide mass fingerprinting.

of the functional properties required to generate a microbicidal environment: they (a) fuse with endocytic organelles (Desjardins et al., 1994a, 1997), (b) mature into phagolysosomes (Desjardins, 1995), and (c) display degradative molecules such as hydrolases (Claus et al., 1998).

With the migration conditions used in the present study, the phagosome preparations loaded on 2-D gels yielded patterns displaying a few hundred spots between 15 and 100 kD in size with pI values ranging from 3.0 to ~9.0. Most of these protein spots were not observed in 2-D gels of total cell lysates, demonstrating the ability of our approach to enrich phagosome proteins (not shown). A representative silver-stained gel of phagosomal proteins was used to display the identified proteins and build our database (Fig. 1). Since only a handful of the spots present on phagosome 2-D gels were known at the beginning of the present study (see Desjardins et al., 1994b), we systematically excised the visible spots from zinc acetate–stained gels for mass spectrometry analysis. These analyses allowed the recognition of >140 phagosomal proteins identified, for the most part, by peptide mass fingerprinting (PMF) (Table I).

Protein Identification by Mass Spectrometry

To identify a protein from its PMF, several criteria were considered. The most important of these was the coverage of the full-length protein. When exceeding 15%, the coverage was considered to be sufficient unless there was some obvious conflicts such as discrepancies between the experimental molecular weight and that of the identified protein. At between 10 and 15% of coverage, the identification was validated when the matching peptides versus input peptides ratio exceeded 75% and when the molecular weight and species were not too distant. The mass accuracy of our analyses was always set at 100 ppm, but was usually better than 50 ppm. Matching peptides with one missed cleavage were validated only when there were two consecutive basic residues (KK, KR, RK, or RR) or an RP sequence inside the peptide amino acid sequence.

MS/MS analyses were conducted on proteins not identified by the PMF approach. In most cases, several peptide sequence tags were easily obtained and the protein was identified by mining protein databases with Pepfrag (PROWL). As an example, the lysosomal acid lipase (10%

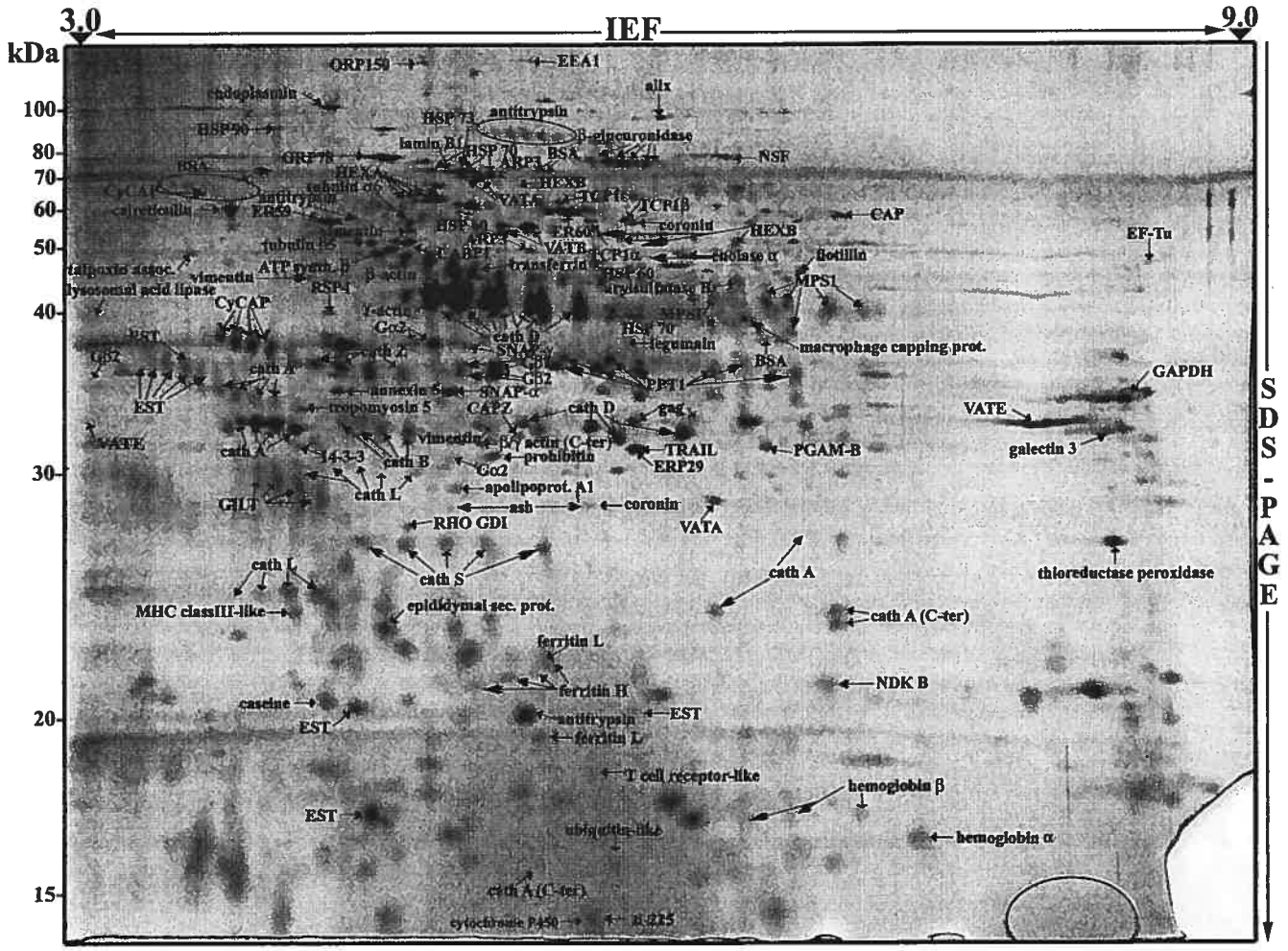

Figure 1. Phagosome protein 2-D gel map. Latex beads were internalized by J774 macrophages for 60 min followed by a 60-min incubation without bead. After cell breakage, phagosomes were isolated on sucrose gradients and their proteins separated by high-resolution 2-D gel electrophoresis. Proteins were separated according to their isoelectric point on immobilized pH-gradients 3-10, and then by standard SDS-PAGE. The major spots were excised and analyzed by mass spectrometry. The image used here is a representative gel stained with silver.

Table I. (continued)

Protein	Accession number*	Prot Param		Observed		Remarks	MALDI-TOF-MS		MS/MS	Triton X-114	Endo Phago
		Mol Wt	*pI*	*Mr*	*pI*		Peptides	Coverage			
Glucosylceramidase	P17439	57621.8	7.64	27000	ND	β-glucocerebrosidase. Lysosomal. Membrane bound.		9%		×	×
β-glucuronidase	P12265	74239.2	6.16	MF		Lysosomal.		16%		×	×
GRB2	P29354	25206.3	5.89	MF		Associates with tyrosine-phosphorylated proteins. Also interacts with Ras in the signaling pathway leading to DNA synthesis.		25%			
GRP 78	P20029	72422	5.07	77000	4.6	BIP. An ER chaperone.		37%		×	×
β-hexosaminidase α	P29416	60599	6.09	MF		*N*-acetyl-β-glucosaminidase, β-*N*-acetylhexosaminidase. Lysosomal.		14%			×
β-hexosaminidase β	P20060	61115.7	8.28	MF				19%			×
HSC70T	P16627	70695.3	5.82	MF		Heat shock-related protein. Usually an ER or mitochondrial protein.					
HSC71	P08109	70804.9	5.24	MF		Heat shock cognate protein		30%		×	
HSP-60	P19226	60955.4	5.91	MF		Mitochondrial matrix protein P1. Chaperonin. Interacts with P21RAS. Usually an ER or mitochondrial protein.	6/9	27%			×
HSP-70	A45935	70837	5.37	MF		Cytoplasmic chaperone.		27%			×
HSP-70 precursor	A48127	73461.2	5.81	MF		Cytoplasmic.		31%			
HSP-70 protein2	P17156	69740.8	5.58	MF				23%			
HSP-73	P08109	70871	5.37	70900	5.0	Heat shock cognate 71 kD protein.	24/32	46%			
HSP-90b (HSP-84)	P11499	83194	4.97	90000	4.0	Molecular chaperone. Has ATPase activity. Cytoplasmic. Interacts with the cytoskeleton as well.		13%		×	×
Lactadherin	P21956	51465.2	6.02	MF		Milk fat globule-EGF factor 8 (MFG-E8). Antiviral activity.		17%		×	
Lamin B1	P14733	66884.7	5.11	MF		Component of the nuclear lamina.	13/13	25%			
LAMP-1	P11438	43865.1	8.66	135000	ND	Lysosome-associated membrane glycoprotein 1. Type I membrane protein.			×	×	×
"LAMP-2, type B"	P17047	45647.0	7.05	120000	ND	Lysosome-associated membrane glycoprotein 2.			×	×	×
Legumain	O89017	49372.9	5.92	37600	6	Lysosomal cysteine endopeptidase.		14%	×		×
LIMP II	P27615	53959.7	4.91	95000	ND	Lysosome membrane protein II. May act as a lysosomal receptor. Type II membrane protein. Lysosomal. Belongs to the CD36 family.		18%		×	×
Lysosomal acid lipase/cholesteryl ester hydrolase	P38571	45415	6.42	43000	ND	Crucial for the intracellular hydrolysis of cholesteryl esters and triglycerides. Lysosomal.		15%	×	×	×
Lysosomal membrane glycoprotein-type B	P17046	43127.2	7.16	120000	ND	Very similar to LAMP2 (P17047).			×	×	×
lysozyme C, type M (LYCM)	P08905	16688.9	9.11	17000	ND	1,4-β-*N*-acetylmuraminidase C. Bacteriolytic. Enhances the activity of immunoagents.		25%		×	×
Macrophage capping protein	P24452	39240.4	6.73	MF		Actin-capping protein GCAP39; myc basic motif homolog-1.	6/10	17%			
MHC class Ib, mature α chain	CAA06194	33419	5.08	27000	ND	Expected to be secreted in soluble form due to absence of exon 5, which encodes the transmembrane domain.		18%		×	×
MPS1	I52603	73166.6	5.91	MF		Macrophage-specific protein. Upregulated during monocyte to macrophage differentiation.		13%‡			
Myosin heavy chain-A	O89055	18177.4	5.34	155000	ND	Non muscle form.				×	×
Napsin	CAB82907	45544.3	7.13	55000	ND	Membrane-anchored aspartyl protease.			×	×	
NDK B	Q01768	17363	6.97	21100	6.9	Nucleotide diphosphate kinase B.		61%			
NSF	P46460	82565.4	6.52	77000	6.4	Vesicular-fusion protein. *N*-ethylmaleimide-sensitive factor.		29%		×	×

(continues)

Table I. (continued)

Protein	Accession number*	Prot Param		Observed		Remarks	MALDI-TOF-MS		MS/MS	Triton X-114	Endo Phago
		Mol Wt	*pI*	*Mr*	*pI*		Peptides	Coverage			
v-ATPase α (catalytic subunit)	P50516	68268	5.62	MF		Involved in phagosome acidification.		44%			×
v-ATPase β	P50517	56584.9	5.57	MF			9/15	28%			×
v-ATPase ε	P50518	26588	9.28	MF				34%			×
VDAC1	Q60932	30624.3	8.63	28500	ND	Voltage-dependent anion-selective channel protein 1. Mitochondrial. Also found on the plasma membrane and endosomes.		25%		×	×
Vimentin	P20152	53687.6	5.06	MF		Class III intermediate filaments.		54%			
Antitrypsin	P34955	46103.9	6.05	MF		Serum proteins adsorbed on latex beads during internalization. Highly enriched in our preparations. Endocytic cargo.		18%			
Apolipoprotein A-I	P15497	30276.3	5.71	29300	5.0						
BSA	P02769	69293.4	5.82	MF			16/22	32%			
α-S1 caseine	P02662	24528.9	4.98	20600	4.3			35%			
Hemoglobin α	P01966	15053.1	8.19	16300	7.4			50%			
Hemoglobin β	P02081	15859.2	6.51	MF			6/11	46%			
Putative gag	P23090	60511.7	7.63	MF		Duplan murine leukaemia virus.					

Proteomic analysis of phagosome proteins. The 1- and 2-D gel methods combined allowed the identification of 116 phagosomal proteins directly from existing databases (including seven cargo molecules probably originating from the serum). In addition, 17 spots analyzed could not be matched to any entry in databases, while seven corresponded to ESTs (not shown), suggesting that they are novel proteins.

*Whenever possible, the SWISS-PROT or TrEMBL accession numbers are listed. Otherwise, NCBI entries are used. MF, multiple forms. Observed pI values could not be evaluated for proteins identified by the 1-D gel method.

‡MPS1 coverage is only NH_2 terminal, and when this region is considered alone, the match percentage is very satisfactory; MALDI-TOF-MS, peptides, ratio of peptides that matched the expected mass of the theoretical trypsin digests. Coverage, percentage of the full-length sequence covered by the matching peptides; MS/MS, proteins further identified my MS/MS; Triton X-114, proteins that were identified from the detergent phase samples; Endo/phago, proteins previously reported to be present in endosomal/phagosomal compartments.

coverage) was confirmed by tandem MS. In other cases, when the mouse protein was not present in the protein and EST databases, we had to generate amino acid sequences by MS/MS and use BLAST software to look for proteins exhibiting high amino acid sequence homology (for example, GILT was identified from human databases). We could also reach some EST sequences using peptide sequence tags (e.g., ESTAA445025, which shows an homology with cathepsin A) or peptide mass fingerprints (only in the case of low molecular weight proteins; e.g., EST 1447369, similar to CU-Zn SOD), and even reconstruct one protein amino acid sequence by matching a first EST, and then clustering several ESTs [e.g., an ADP-ribosylation factor (ARF)–6 isoform].

Based on these different criteria, we identified 116 proteins by the PMF approach and 7 ESTs. 19 were further analyzed by MS/MS to get unambiguous identifications. All of these were confirmed. Furthermore, 17 protein spots yielded good MS spectra that could not be matched to any entries in current databases. Identification of these potentially novel proteins will require further analysis and sequencing.

Phagosome Membrane-associated Proteins

Separation of membrane proteins is still problematic with 2-D gels (Santoni et al., 2000). Although some of the proteins identified on 2-D gels from our samples are clearly membrane associated, such as flotillin-1, other expected proteins, such as those of the LAMP family, were not identified. Accordingly, to identify as many membrane-associated proteins as possible, we complemented our 2-D gel studies with SDS-PAGE analysis of phagosome membrane proteins obtained after phase partition in Triton X-114. Using this approach, we resolved 24 major bands visualized after zinc acetate staining (Fig. 2). From these bands, we were able to identify 36 proteins, 5 of which had previously been identified from the 2-D gels. Among these proteins were seven members of the ras superfamily: rab2, 3c, 5c, 7, 10, 11, and rab14, as well as rap1b. Interestingly, these included the small GTPases previously shown to be associated to phagosomes; namely, rab5, 7, 11, and rap1 (Desjardins et al., 1994a; Pizon et al., 1994; Cox et al., 2000). Transmembrane proteins were also identified in our phagosome preparations, including LAMP1, LAMP2, lgp110, and LIMPII. LAMP1 was also identified by MS/MS analysis in a band at ~44 kD. Other identified membrane proteins included stomatin, Tmp21, and VDAC1.

Western Blotting and Immunofluorescence Analysis

Several proteins not previously reported to be associated to phagosomes were identified in our preparations. It was impossible to systematically test all the new proteins by Western blotting or immunofluorescence to confirm their enrichment on phagosomes and their localization to this organelle since antibodies against these molecules were often not available. Nevertheless, we were able to further investigate some of these proteins and demonstrate the association of LAMP1, a well-known phagosome protein used here as a control, alix, flotillin-1, GAPDH, and 14-3-3 to phagosomes (Fig. 3). Western blot analysis indicated that all these proteins are enriched in phagosomes compared with total cell lysates. Furthermore, immunofluorescence analysis showed that the staining of these molecules is localized to the periphery of latex beads, as well as to other cellular organelles not further investigated in the present study. GAPDH, a cytosolic enzyme involved in

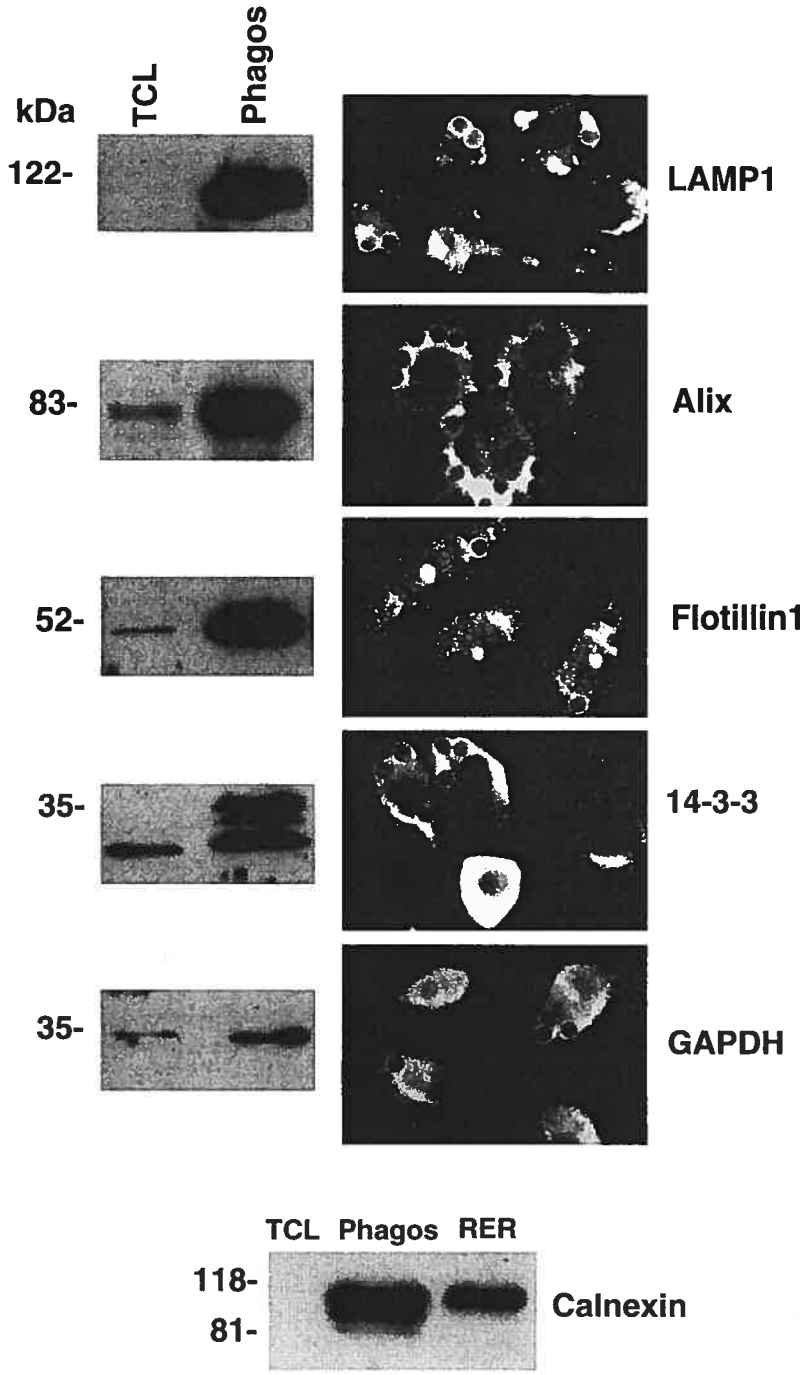

Figure 3. Immunolocalization of novel proteins to phagosomes. To demonstrate the phagosomal association of the new proteins identified in our study, we performed Western blot and immunofluorescence analyses of some of these proteins in macrophages that had internalized latex beads for 60 min, followed by a 60-min chase. LAMP1, a well known phagosomal protein, was used as a control. For Western blot analysis, the same amount of protein from total cell lysates or isolated phagosomes was loaded on SDS-PAGE. A clear enrichment of each protein on the phagosomes is observed. Immunofluorescence analysis clearly demonstrate the localization of the protein around compartments containing latex beads. In the case of 14-3-3 and GAPDH, cells were permeabilized before the labeling to get rid of most of the cytosolic proteins. Note that one of the cells has not been permeabilized in the case of 14-3-3. For calnexin, an antibody against the cytosolic portion of the molecule was used. The same amount of protein was loaded for total cell lysate (TCL), the phagosomes (Phagos), and a microsome preparation (ER) as a positive control.

5, arrowheads), including a variety of hydrolases (Table II). Our results clearly indicate that hydrolases are not acquired simultaneously by phagosomes. While some hydrolases, such as cathepsin A and β-hexosaminidase are already present in a high amount in early phagosomes, others, such as cathepsin S and the cleaved form of cathepsin D, appear at later time points during phagolysosome biogenesis (Fig. 6). Others, such as the recently cloned cathepsin Z (Santamaría et al., 1998), are present at early time points but disappear during phagosome maturation, suggesting that they are either recycled or degraded and that they play a role in the processing of peptides at the early stages of phagolysosome biogenesis.

Discussion

At the subcellular level, the observation through high-resolution 2-D gel electrophoresis that organelles are made up of hundreds of proteins, several of which are extensively post-translationally modified, highlights the complexity of the mechanisms associated with the functions of these compartments (Scianimanico et al., 1997; Rabilloud et al., 1998). It also emphasizes the overwhelming task of understanding how organelles work using conventional approaches consisting of studying each of their proteins, one by one. Hopefully, the refinement of technical approaches enabling us to separate thousands of proteins by 2-D gel electrophoresis,

and analyze them from minute amounts of material by mass spectrometry, has allowed us to envisage the possibility of identifying most, if not all, proteins associated with a given organelle and understand how they are modulated in various conditions (Lamond and Mann, 1997). This holistic approach is likely to provide new insights into the function of complex organelles and their regulation.

In the present study, we identified >140 of the proteins present on or within latex bead–containing phagosomes. Several of these proteins are expected constituents of an organelle that moves along cytoskeletal elements, interacts and fuses with endovacuolar organelles, acidifies its lumen, and degrades its content. Among these are a series of hydrolases, subunits of the vacuolar proton pump ATPase, molecules of the fusion/fission machinery, various GTPases of the Ras superfamily, as well as coat proteins and cytoskeletal-related proteins. Remarkably, a series of novel proteins, not previously reported to be associated with phagosomes were also identified. The confirmation of the enrichment of several of these proteins on phagosomes by various approaches, including differential 2-D gel display against a macrophage total cell lysate, Western blot analysis, and immunofluorescence localization, allowed us to propose new concepts regarding the molecular mechanisms of phagolysosome biogenesis.

Membrane Fusion and Small GTPases

Phagosomes were shown to fuse sequentially with subsets of endosomes (Desjardins et al., 1997). Thus, it was not surprising to find SNARE molecules and regulatory small GTPases, including Rab4, Rab5, Rab7, Rab11, and Rap1b, previously described on phagosomes (Desjardins et al., 1994a; Pizon et al., 1994; Hackam et al., 1996; Cox et al., 2000). Rab5 and Rab7 allow phagosomes to interact with early and late endosomes, respectively (see Méresse et al., 1999), while the association of Rab4 and Rab11 with recycling endosomes (Sheff et al., 1999; Sonnichsen et al., 2000) suggests that recycling processes might also be important for phagolysosome biogenesis. Three other Rab proteins, Rab 3c, Rab10, and Rab14, have been identified on phagosomes in the present study. Rab3, a small GTPase involved in Ca^{2+}-dependent exocytosis (see Geppert and Sudhof, 1998), was also localized to endosomes (Slembrouck et al., 1999). The functions for Rab10 and Rab14 still remain to be elucidated. A novel GTPase of the ARF family was also identified by tandem MS and searches in EST databases (AI006608). Based on the alignment of two overlapping ESTs, this sequence was predicted to code for a 21.6-kD protein (189 amino acids) displaying 64% homology to human and mouse ARF6. Western blot analysis using a polyclonal antibody raised against the COOH-terminal region of the protein indicated that this ARF6-like protein is highly enriched on phagosomes compared with a total cell lysate of macrophages (not shown). Previous studies have shown that ARF6 is involved in actin cytoskeleton organization and recycling process (D'Souza-Schorey et al., 1998; Franco et al., 1999). A similar role on phagosomes remains to be established.

Maturation of Phagosomes Is Accompanied by the Sequential Acquisition of Hydrolases

We have highlighted in the present study the presence of several hydrolases in phagosomes, including six members

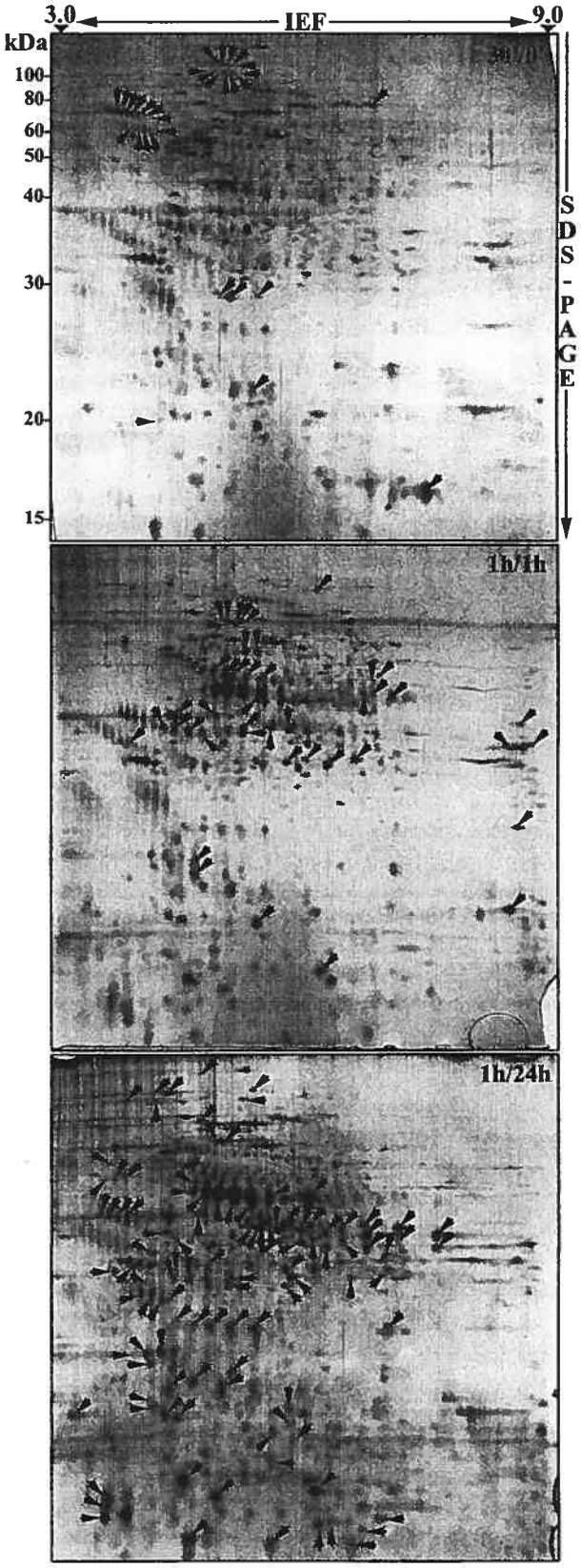

Figure 5. Modulation of phagosome composition during maturation. Latex bead–containing phagosomes were isolated at different stages of maturation and their proteins separated by 2-D gel electrophoresis. The various gels were then compared with each other using the software PD-Quest. Arrows point to peaking proteins at their respective time points.

like most approaches relying on the isolation of organelles based on their intrinsic density, isolation of latex bead–containing phagosomes is facilitated by the low buoyant density of latex. Thus, phagosomes are floated in a region of the sucrose gradient where other cellular organelles are not detected. Previous morphological and biochemical analysis of latex bead phagosome preparations indicated the virtual absence of contamination by mitochondria, Golgi vesicles, endosomes, and the plasma membrane, while low levels of ER elements were found (Desjardins et al., 1994b). Based on all these observations, it is worthwhile to consider that the presence in our preparations of molecules previously identified in other compartments is perhaps representative of more than a simple contamination.

Four mitochondrial proteins were identified in our phagosome preparations, which are ATP synthase, prohibitin, HSP60, and VDAC-1. Interestingly, two of these proteins have been shown to be present on other compartments in the cell. Prohibitin was found to be associated with receptors at the cell surface (see below), while VDAC-1, a type 1 porin molecule of the outer mitochondrial membrane, was shown to be present on endosomes by immunofluorescence and immunogold electron microscopy (Reymann et al., 1998). VDAC-1 was also shown to be present at the cell surface, where it is targeted through the expression of an alternative first exon (Buettner et al., 2000). The potential roles of these molecules on extramitochondrial compartments are not known. An additional finding suggesting that the detection of prohibitin and VDAC-1 in our phagosome preparation is not due to mitochondrial contamination is the fact that these proteins were not identified as major constituents of mitochondria in a recent in-depth proteomic study (Rabilloud et al., 1998). Furthermore, at least two of the most prominent spots present on 2-D gels of mitochondria, fumarate hydratase and aconitase, are not present at their migration coordinates in our phagosome gels. If mitochondria were contaminating our preparations, one would assume that these spots would also be present in our gels. These data support the concept that at least some of the mitochondrial proteins present in our preparations might be genuine constituents of phagosomes.

Insights into New Phagosome Functions

ER Recruitment to Phagosomes. Our phagosome analysis allowed us to identify several molecules normally associated with the endoplasmic reticulum. Although this might represent a contamination, further data suggest that ER elements could interact directly with phagosomes. The pronase experiments have shown that all the lumenal ER proteins identified in our 2-D gels were not affected by the pronase treatment, indicating, at least, that they are not simply associated with ER elements entrapped in the cytoskeletal matrix associated to the phagosomes and floated during the isolation procedure. A further indication of the close association of ER with phagosomes came from Western blot analysis showing the enrichment of calnexin in our phagosome preparations compared with the total cell lysate. Furthermore, pre-embedding immunocytochemical analysis using an antibody against the cytosolic portion of calnexin demonstrated the presence of this protein on the phagosomal membrane, while an antibody against the lumenal portion of the molecule gave no signal (Gagnon, E., and M. Desjardins, manuscript in preparation). The presence of ER molecules on phagosomes is not surprising. Recent studies have shown that microorganisms such as *Legionella* and *Brucella* reside within their host cells in compartments displaying ER features (see Méresse et al., 1999). In our experiments, it was not uncommon to observe macrophages that had engulfed >25 latex beads. Despite the important surface of plasma membrane needed for the engulfment of these particles, the cell does not consume itself, but rather maintains a relatively stable size. This suggests that while plasma membrane recycles rapidly back to the cell surface (Pitt et al., 1992), membrane from the ER could be recruited to keep the particles within closed compartments.

Another interesting link between phagolysosomes and ER elements comes from the finding that calreticulin, a chaperone of the ER identified in our phagosome preparations, is also present in the lysosome-like lytic granules of T lymphocytes (Dupuis et al., 1993). There, it controls the lytic activity of perforin by stabilizing membranes to prevent polyperforin pore formation (Fraser et al., 2000). Although perforin has not been identified in our phagosome preparations, a newly identified protein, MPS1, was shown to display a certain homology with perforin (Spilsbury et al., 1995). The MPS1 gene was first identified as being upregulated during monocyte-to-macrophage differentiation (Spilsbury et al., 1995), as well as during prion infection, along with other lysosomal hydrolases (HEXA and HEXB) (Kopacek et al., 2000). The coded protein appears to share distant ancestry to perforin, although its property to form pores, yet alone to polymerize or span a membrane, has not been shown. Further analyses should clarify whether the ER is a contaminant or if it is recruited to form phagosomes.

Lipid Rafts. Among the new phagosomal proteins found in our study is flotillin-1. This protein was reported to be associated with caveolae or other subdomains of the plasma membrane (Bickel et al., 1997; Lang et al., 1998). Its identification by mass spectrometry on phagosomes, further confirmed by Western blot and immunofluorescence analyses, suggests that lipid rafts could also be present on phagosomes, and not solely on the Golgi apparatus or the plasma membrane (Simons and Ikonen, 1997). A second molecule recently shown to be associated to lipid rafts, stomatin (Snyers et al., 1999), is also present on phagosomes. Although stomatin was first proposed to be involved in the maintenance of the structural integrity of the red blood cell membrane, this function was ruled out after the observation that red blood cells from stomatin knockout mice were not altered (Zhu et al., 1999). Of further interest, flotillin-1 and stomatin share a homologous domain with prohibitin, a molecule also present on lipid rafts (Terashima et al., 1994), identified in our phagosome preparations. Although prohibitin is present in mitochondria (Ikonen et al., 1995), reports indicate that it may associate with certain receptors present at the cell surface (Terashima et al., 1994), a phenomenon that could explain its presence on phagosomes. The potential involvement of prohibitin in the regulation of mitochondrial membrane protein degradation (Steglich et al., 1999) may be relevant to some of the phagolysosome degradative functions. Altogether, these results strongly suggest that specialized

the emergence of TUNEL-positive cells is prevented in *Caenorhabditis elegans* Ced-7 mutants, in which engulfment is impaired (Wu et al., 2000). These observations suggest that phagolysosomes may induce apoptosis within macrophages through an unknown mechanism. Intraphagosomal TRAIL binding to its receptor on the phagocytosed cell could be part of such a mechanism. A role for lysosomes in cell-autonomous apoptosis has also been suggested since autophagy or cathepsin D translocation to nonlysosomal structures can be instrumental during the death of nonphagocytic cells (see Ferri and Kroemer, 2000). Our findings showing that proteins with a demonstrated function in apoptosis like galectin 3, Alix, VDAC1, GAPDH, or 14-3-3 are enriched in phagosomes reinforces this hypothesis and may open new avenues in understanding the role of phagolysosomal compartments in apoptosis.

Phagolysosome biogenesis is obviously a complex process made possible by the contribution of a large number of molecules. In the last few years, in-depth studies of some of the phagosome proteins have allowed us to significantly increase our knowledge of this organelle's functional properties. In the present study, we used a proteomic approach to gain a global view of the composition of phagosomes and their potential functions. The confidence level attained by the identification of several key phagosomal components and the immunofluorescent localization of some of the unexpected proteins to phagosomes, together with the low contamination of our preparations by other organelles, allows us to consider most of the proteins identified in this study as genuine constituents of phagosomes. This wide body of data provides new insights into the molecular mechanisms governing phagosome functions and phagolysosome biogenesis (Fig. 7). This proteomic approach is likely to become extremely powerful as we learn to fully use all of its strengths.

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