A Myosin IK-Abp1-PakB Circuit Acts as a Switch to Regulate Phagocytosis Efficiency

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Actin dynamics and myosin (Myo) contractile forces are necessary for formation and closure of the phagocytic cup. In Dictyostelium, the actin-binding protein Abp1 and myosin IK are enriched in the closing cup and especially at an actin-dense constriction furrow formed around the neck of engulfed budded yeasts. This phagocytic furrow consists of concentric overlapping rings of MyoK, Abp1, Arp3, coronin, and myosin II, following an order strikingly reminiscent of the overall organization of the lamellipodium of migrating cells. Mutation analyses of MyoK revealed that both a C-terminal farnesylation membrane anchor and a Gly-Pro-Arg domain that interacts with profilin and Abp1 were necessary for proper localization in the furrow and efficient phagocytosis. Consequently, we measured the binding affinities of these interactions and unraveled further interactions with profilins, dynamin A, and PakB. Due to the redundancy of the interaction network, we hypothesize that MyoK and Abp1 are restricted to regulatory roles and might affect the dynamic of cup progression. Indeed, phagocytic uptake was regulated antagonistically by MyoK and Abp1. MyoK is phosphorylated by PakB and positively regulates phagocytosis, whereas binding of Abp1 negatively regulates PakB and MyoK. We conclude that a MyoK-Abp1-PakB circuit acts as a switch regulating phagocytosis efficiency of large particles.

INTRODUCTION

Phagocytosis is an endocytic process strictly dependent on actin polymerization and myosin (Myo) contraction (Swanson et al., 1999; Rivero, 2008). Both forces contribute to the efficient extension and closure of a circular lamella around the internalized particle. In mammalian cells, amoebae and yeasts, actin, class I myosins (M1s), and the Arp2/3 complex are involved in the generation of the burst of actin polymerization that accompanies vesicle or phagosome closure (Merrifield, 2004; Clarke and Maddera, 2006; Kaksonen et al., 2006; Krendel et al., 2007). In this study, phagocytic cup closure in the amoeba Dictyostelium was used as a model system to dissect which molecular interactions precisely link and coordinate M1s relative to actin and the Arp2/3 complex, how these molecules are spatially organized to build constriction systems, and how the uptake efficiency is regulated.

M1s have a common role in membrane deformation and localize to regions of dynamic actin turnover. They are essential for endocytic uptake in yeast and necessary for correct endocytic membrane trafficking in mammals (Soldati and Schliwa, 2006). In Dictyostelium, M1 mutants have overlapping defects in phagocytosis, macropinocytosis, chemotaxis, and cortical tension management (Dai et al., 1999; Schwarz et al., 2000; Rivero, 2008). M1s are composed of three distinct domains: an N-terminal motor domain, a neck domain, and a C-terminal tail domain. The conserved motor or head domain is responsible for ATP-dependent F-actin-binding and contributes with the neck domain to movement generation. The motor efficiency and binding affinity to actin are regulated by an acidic (Glu/Asp) or a phosphorylatable residue (Ser/Thr) at the specific TEDS site of the motor domain. The myosin heavy chain kinase PakB was first identified as a kinase for MyoD (Lee and Cote, 1995), and activation of the kinase domain was shown to impact on phagocytosis rates (de la Roche et al., 2005), but whether MyoD is the major phospho-switch for phagocytosis is not known. The activation of PakB itself depends on binding to the p21-binding domain (PBD) domain of small GTPases of the Rac1 family that causes the protein to open and release the C-terminal kinase domain from autoinhibitory interaction with the N-terminal proline-rich domain. Indeed, ablation of the central polo-box domain (PBD) and linker
domains (PakBΔPL) generates a constitutively active kinase (de la Roche et al., 2005). The tail domain is of variable length and determines the interaction partners and functional specificity of M1s. Short tail M1s contain only the lipid-binding tail homology 1 (TH1) domain, rich in basic residues. Long tail M1s contain two additional protein–protein interaction domains, a tail homology 2 (TH2) domain and a Src homology 3 (SH3) domain. TH2 domains are Gly-Pro rich and therefore a particular subtype of proline-rich domains (PRD) that has been shown to bind actin in an ATP-insensitive manner (Jung and Hammer, 1994). SH3 domains interact with PRD domains, among others. The TH2 and SH3 domains are essential to link M1s to the Arp2/3 complex and to proteins promoting actin polymerization (Soldati, 2003). In Dictyostelium, there are seven M1s of partially overlapping function. Three have long tails (MyoB, C, and D), three have short tails (MyoA, E, and F), and MyoK is divergent (Schwarz et al., 2000). The only identified link between long tail M1s and the Arp2/3 complex is indirect. The capping protein Arp2/3 and myosin I linker CARMIL bind the Arp2/3 complex and the SH3 domain of MyoB and MyoC (Jung et al., 2001).

Recently, a homologue of Abp1 (AbpE), another M1–Arp2/3 linker protein, has been discovered in Dictyostelium (Wang and O’Halloran, 2006). Strikingly, abp1 null mutants display no defect in pinocytosis (Wang and O’Halloran, 2006) contrary to what is observed in M1 null mutants (Rivero, 2008). However, Abp1 binding to M1s and its role in phagocytosis have not been addressed. Abp1 has an overall domain organization similar to cortactin and proteins of the drebrin family (Kessels et al., 2000; Supplementary Figure 5). It is composed of an F-actin–binding actin-depolymerizing factor domain at the N terminus, an Arp2/3 binding variable domain at the C terminus, and an SH3 domain, the most conserved part of the protein. It binds at least eight identified ligands in yeast and, among them, the M1 Myo5p but it also binds the adenyl cyclase-associated protein (Srv2p/CAP) and synaptoplakin (Sij2p) (Fazi et al., 2002). In mammals, it binds to the PRD of neuronal-Wiskott-Aldrich syndrome protein (WASP), an Arp2/3 activator, and dynamin I and II, among others (Kessels et al., 2001; Pinyol et al., 2007; Onabajo et al., 2008).

Based on previous work, MyoK was chosen as model to understand the regulation and function of M1s in membrane deformation because it has a distinctive role in cortical management. Despite its low abundance, its mere absence slows down the initial phase of phagocytic uptake, disturbs the dynamic organization similar to cortactin and proteins of the drebrin family (Kessels et al., 2001; Pinyol et al., 2007; Onabajo et al., 2008).

MATERIALS AND METHODS

Cell Culture

Dictyostelium cells of wild-type strain AX-2 were grown at 22°C in HL-5c medium (Formedium, Huntingdon, United Kingdom) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Paisley, United Kingdom). myoK null cells (Schwarz et al., 2000), abp1 null, and myoB null mutants were generated in the AX-2 background with the appropriate knock-out plasmid. After electroporation, transformants were selected with 10 μg/ml blasticidin S (Merck, Darmstadt, Germany) for 48 h and maintained in 5 μg/ml blasticidin in HL-5c medium. All green fluorescent protein (GFP) fusion protein constructs were expressed and maintained in orf3Δ::G418 (Invitrogen) in HL-5c medium. MyoK full-length (MyoK OE) or the yellow fluorescent protein (YFP)-MyoKαloop and GFP-CPR loop fusion proteins were overexpressed in myoK null cells. MyoK&αfarnesyl, GFP-Myo tail, and YFP-M and the fusion proteins were overexpressed in wild-type cells. The GFP-Myo fusion protein was overexpressed in the myoB null background. Profilin mutants W3N and K114E (Lee et al., 2000) in AX-2 background were maintained in 20 μg/ml hygromycin B (Merck) in HL-5c medium.

Vectors Construction

Full-length MyoK was overexpressed from the pDXA-MyoK-CLIQ plasmid. MyoK&αfarnesyl was cloned into pDXA-HC (Schwarz et al., 2000) in frame with a c-myc tag (TRDALEQKLLSEEDLN), therefore masking the CAAX box farnesylation motif (CLIQ). The MyoKαloop construct lacks residues 122-261, and its N terminus is fused with YFP. The GFP loop (amino acids aa 114-272) and MyoK tail (aa 801-858) constructs were expressed as GFP fusion proteins from pDXA-GFP. The GFP loop sequence was also cloned in the Escherichia coli expression vector pGEX-3X (GE Healthcare, Otelfingen, Switzerland) for expression as a glutathione transferase (GST) fusion protein as described previously (Schwarz et al., 2000). The motor domain of MyoK (K824; aa 1-824) was cloned into pDXA-3H to be expressed as a His-tag fusion protein fused with two actin repeats to serve as artificial lever arms for motor proteins (Duerwang et al., 2006). The motor domains of MyoB and MyoD (aa 1-693 and 1-694, respectively) were cloned into the pT-TFLAG plasmid and expressed with a FLAG-tag at the N terminus (Levi et al., 2000). The PRD of WASP was isolated from Dictyostelium genomic DNA and cloned in pT-Flag for expression as fusion protein–proneosin (LMB). The plasmids for the expression of profilins I and II in E. coli are described previously (Lee et al., 2000). The plasmids for Abp1 as a GST or FLAG-tag fusion and the abp1 knockout construct were gifts of Dr. M. de la Roche (Queen’s University, Kingston, ON, Canada). The myoB knockout plasmid pDTb35R and the GFP-MyoB plasmid pDTb60 were a gift of Dr. M. A. Titus (University of Minnesota, Minneapolis, MN). The myoC coding sequence (1182 aa) was amplified from Dictyostelium genomic DNA and cloned in pDXA-3HeYFP-Mc5 for overexpression of YFP-Myc (Knetisch et al., 2002).

Protein Expression in E. coli and Purification

The GST fusion proteins (GPR loop; Abp1; PRD; PakB) were expressed in E. coli BL21-DE3 and purified as described previously (Geissler et al., 2000). The GPR loop, Abp1 and PRD protein fractions were dialyzed against HEPES buffer (20 mM HEPES, pH 7.2, and 0.25 M sucrose) and concentrated with Centricon-10 (Millipore, Zug, Switzerland). The GST-Pak fusion protein was dialyzed against 20 mM NaCl, 1 mM dithiothreitol (DTT), and 20 mM HESES (pH 7.5) and the His-tagged K824AR construct was overexpressed from pTX-FLAG. The His-tagged Abp1 binds the Arp2/3 complex and the His-tagged PakB binds the Arp2/3 activator, and dynamin I and II, among others (Kessels et al., 2000; Pinyol et al., 2000).

Purification of Constructs from Dictyostelium

FLAG-tagged MyoB and Myod heads were overexpressed and purified as described previously (Crawley et al., 2006). Purified His-tagged dynamin and the head domain of Myod, used in blot overlays, were generous gifts of Dr. D. J. Manstein (Hannover Medical School, Hannover, Germany). MyoK full-length and the His-tagged K824-2R construct were expressed and purified from Dictyostelium cells and purified as described previously (Manstein and Hunt, 1995). In brief, depletion of cellular ATP by alkaline phosphatase was used to stabilize recombinant myosin into a rigor-like complex with F-actin. After cell lysis, the F-actin meshwork was precipitated by centrifugation and washed. Mg2+ATP was then added to release the recombinant myosin. His-tagged K824-2R was further purified by Ni2+-affinity chromatography (Superflow column; Qiagen, Hilden, Germany) and gel filtration (HiLoad 16/60 Superdex 200 PG column; GE Healthcare).

Antibodies

Antibodies raised against the following Dictyostelium antigens were obtained from the following sources: 1) coronin (monoclonal antibody [mAb]; 176-306-3), myosin II (mAb; 56.396.5), actin (mAb; 224-236-1), and the A subunit of the vacuolar H+ ATPase complex (Vala) (mAb; 221-335-2) (gifts of Dr. G. Gerisch, MPI for Biochemistry, Martinsried, Germany); 2) profilin I (mAb; 153-246-10), profilin II (mAb; 174-380-3), Lmb (polyclonal antibody [pAb]) (gifts of Dr. M. Schleicher, Adolf-Butenandt-Institute, Munich, Germany); 3) Arp3 (pAb; gift from Dr. R. H. Insall, Glasgow University, Glasgow, Scotland); 4) PM4C4 (mAb; gift from Dr. D. Sanz, CEA, Grenoble, France); 5) PakB (MIHCK) (pAb; gift from Dr. C. G. P. Coté, Queen’s University, Kingston, ON, Canada); and 6) dynamin A (gift from Dr. D. J. Manstein). Commercial antibodies: 1) rabbit polyclonal; Sigma-Aldrich, St. Louis, MO), and anti-GFP (mAb; GE6353) were used for immunoprecipitation and immunofluorescence, respectively. The anti-MyoK 3156NP antibody was obtained by immunizing rabbits with the synthetic MyoK peptide SARHTQYQVPQN (aa 460-472) and purified on
a peptide affinity column (Invitrogen). For immunoblotting, it was diluted 1:10,000. For immunofluorescence (IF), it was further cross-adsorbed on acetone powder from GST overexpressing Dictyostelium E. coli. The antibody against acetone powder from GST overexpressing Dictyostelium E. coli was obtained by immunizing rabbits (Eurogentec, Seraing, Belgium) against acetone powder from GST overexpressing E. coli. The secondary antibodies for IF were goat anti-mouse or goat anti-rabbit immunoglobulin (IgG) attached to Alexa 488, 594, or 633 (Invitrogen) used at 1:500 dilution. For immunoblotting, goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Bio-Rad Laboratories, Reinsch, Switzerland) were used at dilutions between 1:2000 and 1:10,000.

Fluorescence Microscopy
Phagocytosis of tetramethylrhodamine B isothiocyanate (TRITC)-labeled cells was performed as described previously (Gotthardt et al., 2002). Budded yeasts were obtained by shifting cerevisiae (RH210-1B) for 2 h at 37°C to induce budding arrest. Cells were killed by boiling in PBS for 30 min. To synchronize uptake, monodispersed yeasts (2.5 × 10⁵ yeast/ml) were centrifuged on cells 5 min at 1200 rpm (Allegre 6R; Beckman Coulter, Krefeld, Germany). After 5–10 min, excess yeast was rinsed off in HL-5c medium. Cells fixation/permeabilization in ultracold (−85°C) methanol and immunostaining were performed as described previously (Hagedorn et al., 2009). Fluorescence images were documented using an iSp2 confocal microscope (Leica, Wetzlar, Germany) with a 100× 1.4 numerical aperture oil immersion objective. Recording parameters for fields of 1024 × 1024 pixels with a digitization rate of 8 bit were 2,000×2,000 pixels. The image frame averaging with 0.1–0.32-μm vertical steps. Projections of two to three sections representing 0.2–0.7 μm in depth are shown. Where mentioned, deconvolution was performed with AutoDeblur software with default settings (Auto-Quant Imaging, Media Cybernetics, Bethesda, MD). Three-dimensional (3D) reconstruction was performed with the Imaris software (Bitplane, Zurich, Switzerland).

Electron Microscopy
Synchronized uptake of budded yeasts was performed as described above. After removal of yeast excess, cells were fixed and samples processed essentially as described previously (Hagedorn et al., 2009). Cells were allowed to adhere 20 min in 6-cm-diameter Petri dishes in HL-5c medium. As described above, a monodispersed solution of budded yeasts was centrifuged on the cells (3 × 10⁸ yeast/dish). After further 5–10 min incubation at room temperature, excess yeast was removed by three gentle washes with medium. Cells were then detached by direct resuspension in fixative (0.3% OsO₄, 0.1% glutaraldehyde, and 2% paraformaldehyde in HL-5c medium) (Marchetti et al., 2004). Cells were fixed by incubating 1 h on a rotating wheel, followed by three washes in phosphate-buffered saline (PBS). Fixed samples were dehydrated, embedded in Epon, and processed for conventional electron microscopy as described previously (Orci et al., 1973). Grids were examined with a Tecnai transmission electron microscope (FEL, Eindhoven, The Netherlands).

Flow Cytometry-based Uptake Assay
Uptake was measured as described previously (Gotthardt et al., 2006), with the following modifications. Fluorescent beads of 4.5 μm in diameter (fluorescent YG-carboxylated beads; Polysciences, Warrington, PA) were added to the cell at a 1:10 ratio. Flow cytometry was performed with a FACScalibur (BD Biosciences, Allschwil, Switzerland). Data were analyzed with the FlowJo software (TreeStar, Ashland, OR).

Immunoprecipitation
Cells (10⁸) were lysed in 500 μl of radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 1 mM DTT at pH 7.4). The lysate was cleared by 12,000 × g centrifugation, and the supernatant was taken for analysis. For immunoprecipitation, the lysate was diluted 1:10,000 for immunoprecipitation was quenched by incubation of the lysate with anti-GST antibody for 1 h on a roller at 4°C. Antibody was removed by incubation with protein A-Sepharose beads for 30 min at 4°C and subsequent pelleting of the beads by centrifugation at 4°C for 10 min. The antibody was then incubated with the specific anti-Abp1 antibody for 2 h at 4°C, then 30 min with protein A beads at 4°C on a roller. Abp1 complexes bound to the antibody were precipitated with the beads at 4°C for 10 min at 4°C. The IP beads were washed, resuspended in 70 μl of SDS-polyacrylamide gel electrophoresis (PAGE) buffer and resuspended in 70 μl of SDS-polyacrylamide gel electrophoresis (PAGE) buffer. Twenty microliters were loaded per lane.

In Vitro Phosphorylation Assays
Phosphorylation assays, containing 50 μg/ml GST-PakB kinase and 0.625 mg/ml myosin, were performed at 25°C in kinase buffer [2 mM MgCl₂, 1 mM dithiothreitol, 0.25 mM ATP, and 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.0] incorporating γ-[³²P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) at a specific activity of 5000–6000 cpm/pmol. To quantify the incorporation of ³²P into the myosins, aliquots of 20 μl were removed from the reactions at 5, 10, 30, and 60 min; added to 5 μl of boiling hot 5× SDS sample buffer (5% SDS, 1% β-mercaptoethanol, 300 mM Tris-HCl, pH 6.8, and a trace of bromphenol blue) and subjected to SDS-PAGE. Gels were either subjected to autoradiograms or stained with Coomassie blue. Stained substrate bands were excised, mixed to the ScintiVerse Universal LS Cocktail (Thermo Fisher Scientific, Waltham, MA), and counted in an LS 8000 scintillation counter (Beckman Coulter).

Pull-Down Assays
Dictyostelium strain AX-2 and the profilin mutant cells (10⁴–10⁵ in exponential phase were collected by centrifugation at 800 × g for 10 min at 4°C. The cell pellet was washed once in ice-cold HEKES buffer (20 mM HEPES, pH 7.2, and 0.25 M sucrose) and resuspended in ice-cold homogenization buffer (20 mM HEPES, pH 7.2, 0.25 M sucrose, 1 mM EDTA, 10 mM ATP, 10 mM MgCl₂, 1 mM DTT, 0.1 M KCl, and Complete EDTA-free protease inhibitors [Roche Diagnostics, Rotkreuz, Switzerland]). Cells were lysed on ice in a ball homogenizer (Q 8.020 mm; Isobiotec, Heidelberg, Germany). The lysate was centrifuged at 4°C for 10 min at 4°C and the resulting cleared cytosol fraction was centrifuged again at 400,000 × g for 40 min at 4°C and snap-frozen in liquid nitrogen. GST-GPR was coupled to glutathione-Sepharose (GE Healthcare) at a concentration of 1 mg protein/ml beads. Cleared cytosol was cycled over the GPR loop column. After extensive washing of the column with HEPES buffer, proteins were eluted with −100 μl poly-l-Pro (mol. wt. 5000) and 1 M NaCl. Equal proportions of eluates were separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250, and bands excised or proteins were transferred to nitrocellulose (Protran; Whatman Schleicher and Schuell, Dassel, Germany) for immunoblot analysis.

Protein Analysis by Mass Spectrometry Analysis
Excised bands were washed, “in gel” trypsin digested, and identified by peptide mass fingerprinting based on mass spectra obtained by matrix-assisted laser desorption ionization/time of flight mass spectrometry (Ultraflex; Bruker, Bremen, DE) or liquid chromatography/mass spectrometry (LTQ Orbitrap; Thermo Fisher Scientific) as described previously (Gotthardt et al., 2006).

Phagosome Isolation
Latex bead-containing phagosomes were isolated by filtration on sucrose step gradients and processed as described previously (Gotthardt et al., 2006; Dieckmann et al., 2008).

Immunoblots and Blot Overlays
Immunoblots were performed as described previously (Gotthardt et al., 2006). For blot overlays, 0.2–1 μg of protein was loaded per lane. The nitrocellulose membrane was blocked overnight at 4°C with PBS, 0.1% Tween 20, and 4% low-fat (LF milk). Filters were incubated with ~100 μg of GST-Abp1, GST-GPR, or profilins in 10 ml of PBS with or without 0.1% Tween 20 and 4% LF milk for 4 h at 4°C. Overlaid proteins were detected using specific primary and HRP-coupled secondary antibodies and washed membranes were developed using enhanced chemiluminescence reagents (GE Healthcare).

Surface Plasmon Resonance
Surface plasmon resonance experiments were performed on a Biacore 3000 apparatus (Biacore, Uppsala, Sweden), following recommendations of the supplier. Analyte binding to GST in the reference chamber was subtracted from experimental data to account for weak GST-GST interactions and unspecific binding. Proteins were immobilized via amine coupling to a carboxymethyl dextransphere (CM5 chips) after 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide activation according to manufacturer’s instructions. Chips were blocked by successive 1 M ethanolamine washes. Optimal pH for protein immobilization (lymphoprotein in 10 mM sodium acetate at 10 μg/ml protein concentration. Optimal pH was pH 4.0 for GST-GPR and GST-Abp1 and pH 3.5 for profilin II. Chip regeneration was achieved in 0.2% SDS in HBS (20 mM HEPES, pH 7.4, 1 mM DTT, 150 mM NaCl, and 0.005% Tween 20) to reduce damage to the immobilized protein. Sensorgrams were subtracted with respect to the reference and experimental chambers. Data were analyzed with Evaluation software (Biacore). The observed binding curves were fitted assuming a bivalent binding model with GST-GPR in the soluble phase. A 1:1 binding stoichiometry was fitted for all other injected analytes.
Table 1. Interactions monitored by SPR and their respective binding affinities

<table>
<thead>
<tr>
<th>Ligand immobilized on chip</th>
<th>Analyte injected in the flow chamber</th>
<th>Used concentration range of the analyte</th>
<th>Dissociation constant (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Abp1</td>
<td>Dynamin A</td>
<td>12.5 nM–100 nM</td>
<td>2.6 nM (^p)</td>
</tr>
<tr>
<td>GST-Abp1</td>
<td>Profilin II</td>
<td>15 nM–1.0 (\mu)M</td>
<td>11 nM (^p)</td>
</tr>
<tr>
<td>GST-Abp1</td>
<td>GST-GPR</td>
<td>145 nM–1.16 (\mu)M</td>
<td>27 nM (^c)</td>
</tr>
<tr>
<td>GST-Abp1</td>
<td>GST-PRD</td>
<td>0.5 (\mu)M</td>
<td>No binding (^c)</td>
</tr>
<tr>
<td>Profilin I</td>
<td>GST-GPR</td>
<td>72 nM–2.3 (\mu)M</td>
<td>~500 nM (^p)</td>
</tr>
<tr>
<td>GST-GPR</td>
<td>Profilin II</td>
<td>125 nM–1.0 (\mu)M</td>
<td>108 nM (^p)</td>
</tr>
<tr>
<td>GST-GPR</td>
<td>GST-GPR</td>
<td>145 nM–2.3 (\mu)M</td>
<td>1.71 M (^c)</td>
</tr>
<tr>
<td>Profilin II</td>
<td>GST-Abp1</td>
<td>0.5 (\mu)M, 1.0 (\mu)M</td>
<td>Not quantitated</td>
</tr>
<tr>
<td>Profilin II</td>
<td>Dynamin A</td>
<td>0.5 (\mu)M</td>
<td>Not quantitated</td>
</tr>
</tbody>
</table>

\(^a\) The analyte was serially diluted within the indicated concentration range to determine binding affinities.

\(^b\) Dissociation constants were calculated by fitting the binding curves with a 1:1 binding model. Due to unspecific hydrophobic adsorption of profilin I to the experimental setup, experimental curves could not be properly fitted and the dissociation constant was only approximated.

\(^c\) Because the GST-GPR polypeptide forms homomers, a bivalent binding model was chosen to compensate for the additional mass transfer to the chip surface when GST-GPR was used as the analyte.

RESULTS

MyoK Is Phosphorylated by PakB and Is Competent for Membrane and Protein Binding

Analysis of the MyoK sequence revealed a conventional TEDS site and two unique features, a 150-residue GPR-rich insertion in loop 1 of the motor domain and a CAAX box farnesylation motif at the C terminus (Figure 1, A and B). Detailed sequence analysis of the GPR loop revealed a class I SH3 binding motif overlapping with a potential profilin-binding poly-Pro stretch and four putative profilin-binding ZPP\(_\phi\) motifs (Witke et al., 1998) (Figure 1B). To test for profilin binding, a GST fusion with the GPR loop was immobilized on beads, incubated with cell lysate, and gently eluted with 100 \(\mu\)M polyl-1-Pro (PLP) to specifically disrupt interactions with poly-Pro stretches. Compared with the GST control, significant profilin and actin binding to the GST-GPR construct was observed (Figure 1C). No profilin remained on the beads after PLP elution, indicating quantitative dissociation of the profilin–G–actin complex. Traces of actin were still bound to the GPR loop after PLP elution, reflecting direct binding of the GPR loop to F-actin (Schwarz et al., 2000). Subsequent studies with cells expressing a profilin II point mutant defective for PLP binding indicated that interaction of profilin II with the GPR loop was probably mediated not only by poly-Pro stretches but also by unconventional (ZPP\(_\phi\)) motifs and electrostatic interactions (Supplemental Figure 1, A and B).

Potential binding partners of the SH3 binding motif were also identified in GST-GPR pull-downs (Figure 1D). The main bands enriched after PLP and salt elutions contained Abp1 and the elongation factor 1α. CAP and the large ribosomal subunit 3, RL3, were enriched after salt elution and identified in other gels (unpublished data). Immunoblots indicated that Arp3 was efficiently eluted by PLP together with Abp1 (Supplemental Figure 1C). The interaction network is similar in yeast where Abp1 binds Myo5p and CAP/Srv2p via its SH3 domain (Freeman et al., 1996; Fazi et al., 2002) and the Arp2/3 complex via two acidic motifs (Goode et al., 2001), and CAP binds EF1α, RL3, and profilin (Yanagihara et al., 1997; Bertling et al., 2007). Demonstrating that Abp1 can bind the GPR domain in the context of MyoK full-length in vivo, Abp1 cofractionated with affinity-purified MyoK (Supplemental Figure 1D) and MyoK was coimmunoprecipitated with Abp1 (Figure 1E). The myosin I heavy chain kinase PakB was also partially coimmunoprecipitated with Abp1.

The TEDS site of MyoK is functional for phosphorylation in vivo (Figure 1C). A phosphorylated threonine residue was identified by tandem mass spectrometry sequencing of two partially overlapping peptides (HTQYQVPQNDQSAGLRLDALAK, \(m/z = 839.73, 3+\); QIQSGSARHTQYQVPQNFPSQ AGLR, \(m/z = 949.44, 3+\)), resulting from the trypsin digestion of the purified His-tagged MyoK motor domain. The mass of the parent ion of both peptides corresponded to the mass of the phosphorylated peptide. Several neutral loss of H\(_2\)PO\(_4\) characteristic for the fragmentation of serine/threonine phosphorylated ions, were identified in the corresponding fragmentation spectra. A potential candidate for MyoK phosphorylation in vivo was the myosin I heavy chain kinase PakB. This kinase was shown previously to phosphorylate MyoD (Lee and Cote, 1995) and coimmunoprecipitated with Abp1 and MyoK. Indeed, in vitro PakB efficiently phosphorylated MyoK but not MyoB (Figure 1, G and H). Kinetics of phosphorylation of MyoD and MyoK heads were comparable. Thus, PakB is expected to enhance MyoK motor activity via phosphorylation at its regulatory TEDS site as it does for MyoD (Lee and Cote, 1995).

Finally, a fusion of GFP to the tail domain of MyoK ending with a farnesylation motif was targeted to the plasma membrane, functionally replacing a conventional TH1 polybasic domain (Figure II). In conclusion, MyoK contains a membrane-anchoring and a protein–protein interaction domain that functionally replace the respective polybasic TH1 stretch and the TH2 domain found in M1 tails. Phosphorylation of MyoK by PakB seems specific and might regulate the myosin motor efficiency and binding affinity for actin. Binding of Abp1 to MyoK and PakB also suggest a potential regulatory loop leading to MyoK phosphorylation. Based on the similarity to protein–protein interaction networks in yeast, it is also likely that Abp1 functions as a linker between MyoK and the Arp2/3 complex.

The Direct Partners MyoK and Abp1 Are Embedded in a Complex Interaction Network

To understand the physiological relevance and regulation of M1s in phagocytosis, it was essential to identify MyoK direct interaction network and to measure binding affinities. Furthermore, we focused on collecting data not available in yeast or mammals. Confirming cofractionation of Abp1 with MyoK in pull-downs and immunoprecipitations, Abp1 bound strongly to the immobilized GPR loop in blot overlays (Figure 2A). Reciprocally, the GPR loop bound to immobilized Abp1 (Supplemental Figure 1, E and F). Profilin overlays confirmed that the interaction between the GPR loop and both profilin I and II was also direct (Figure 2C). Binding was highly specific, because no binding was observed with the WASp PRD in any overlays, although its sequence contains SH3 binding motifs and potential profilin binding sites like poly-Pro stretches and one ZPP\(_\phi\) motif (Supplemental Figure 5). Immunobilized dynamin A bound to Abp1 (Figure 2B) as well as profilin I and II (Figure 2C). All direct interactions observed by blot overlay were confirmed by SPR and their respective binding affinities determined (Table 1, Figure 3, and Supplemental Figure 2). In SPR
experiments, GST-Abp1, GST-GPR, and profilin II were immobilized on a chip for analysis. Absence of binding of GST-PRD to immobilized GST-Abp1 confirmed blot overlays and demonstrated that measurement of GST-GST interactions were negligible and efficiently subtracted (Supplemental Figure 2A). Measurement of dissociation constants showed that the GPR loop preferentially binds immobilized GST-Abp1 (27 nM; Figure 3A) compared with profilin I (500 nM; Supplemental Figure 2B) and profilin II (108 nM; Supplemental Figure 2C). Higher affinity for GST-GPR on chip was measured for profilin II (108 nM) compared with profilin I (500 nM). No mutational or structural analyses are available that might explain this preference in isoform binding, but many examples have been reported previously (Witke, 2004). SPR confirmed the binding to dynamin A of profilin II (Figure 2C and Table 1) and GST-Abp1 (Figures 2B and 3B). The measured binding affinity of immobilized GST-Abp1 to dynamin A is in the low nanomolar range (2.6 nM), one order of magnitude stronger than for MyoK. The interaction between profilin II and immobilized GST-Abp1 was also tested in SPR because of signals of variable intensity in blot overlays (unpublished data). The SPR signal for this interaction was clear and robust with a measured dissociation constant of 11 nM (Figure 3C). One additional interaction was tested in SPR that could not be easily tested in blot overlays, i.e., the ability of the GPR loop to dimerize or oligomerize. Low affinity (1.7 M) of the GPR loop for itself was measured indicating that, in vivo, MyoK might form homomers only at very high local concentrations (Supplemental Figure 2D). Long tail M1s have the potential to oligomerize because they contain complementary SH3 and TH2 domains. Nevertheless, as the GPR loop does not contain an SH3 domain, no precise sequence can be inferred as a potential homotypic binding site.
MyoK and Abp1 Are Enriched at the Constriction Point in the Phagocytic Furrow

MyoK, MyoB, Abp1, and the Arp2/3 complex are enriched in the phagocytic cup and especially at the lip formed by the tip of the advancing lamella (Figure 4A; [Schwarz et al., 2000; Insall et al., 2001; Clarke and Maddera, 2006]). Rapid fixation in ultracold methanol of cells ingesting dumbbell-shaped budded yeasts increased both temporal and spatial resolution, allowing us to capture all stages of phagocytic cup formation. Phagocytosis of this large particle is slow and the inversion of curvature at the bud neck induces a transient stalling of the uptake machinery, causing an accumulation of cytoskeletal proteins. A spatially well-defined collar structure is formed that we call the phagocytic furrow. This structure was well visualized by MyoK or Abp1 labeling (Figure 4, B–E). Sections through the collar revealed a ring of myosin I or Abp1 surrounded by another ring of cortical myosin II (Figure 4, D and E). MyoK together with Abp1 is

Figure 2. A network of direct protein-protein interactions linking the GPR loop of MyoK, Abp1, profilins, and dynamin A was revealed by blot overlays. GST-Abp1 directly interacts with immobilized GPR loop (A) and dynamin A (B). Purified profilin I and II were overlaid on membranes blotted in parallel (C). Both isoforms clearly interact with immobilized Abp1 and dynamin A. Weak interaction of profilin II with Abp1 and profilin I with WASp PRD are detected although the signal intensity of the control (GST) is similar. A Ponceau staining is shown (left) and corresponding overlays are shown (right). GST and the MyoD head domain were used as negative controls for GST fusion or Histagged proteins, respectively.

Figure 3. SPR confirms and quantifies the direct interactions detected by blot overlays. (A–C) The affinity of the GPR loop (A), dynamin A (B), and profilin II (C) to immobilized GST-Abp1 were determined by SPR. A GST-coupled chip was used in the reference chamber. Experimental curves are in color, and fitted curves are in black. The range of concentrations of analytes used is indicated in Table 1. (D) Schematic representation of the protein-protein interaction network unraveled in this study. Arrows indicate direct interactions. When determined, equilibrium constants are indicated. Interactions with actin have been shown previously (blue arrows). A dashed arrow indicates indirect interaction identified by anti-Abp1 immunoprecipitation.
largely more enriched in the furrow than in the cup (compare Figure 4A to C), not only confirming interaction studies but also suggesting a specific role of MyoK in membrane constriction. Therefore, further characteriza-
tion of this structure is complementary to the understanding of MyoK function.

In electron micrographs, actin delineated an organelle-free layer around the phagocytic cup (Figure 4F, closed arrowheads). The phagocytic furrow formed around budded yeasts was characterized by an actin-rich, organelle-free zone of triangular section (Figure 4, G and H, arrows). This zone was subdivided in a light actin rim, continuous with the layer surrounding the cup (Figure 4, G and H, closed arrowheads), and a denser, darker area, located just below or at the site of maximal concave curvature (Figure 4G, open arrowheads). The degree of continuity of the actin layer depends on the stage of closure. At early stages, actin entirely lined the cup (Figure 4H), and Abp1 and coronin patches were visible at the cup base (Figures 4C and 5D), probably hindering vesicle fusion during cup formation (Mercanti et al., 2006). At later stages, actin still filled the closing lips, but was absent from the cup base (Figure 4G), rendering it accessible for membrane remodeling. A schematic view of the phagocytic furrow is depicted in Figure 4I.

**Figure 5.** Actin-binding proteins are enriched in distinct territories in the phagocytic furrow. (A–I) Sections (xy plane) and magnifications of the phagocytic furrow represent averaged projections over two or three confocal sections (0.3–0.5 μm depth). (A–F) Four to five distinct protein layers or territories are observed in sections through the furrow. A MyoK layer is surrounded by a myosin II (A) and plasma membrane (4C4) layer (B). Whereas there is a weak overlap with MyoK (C–F) and Abp1 (D), actin or coronin extensively overlap with Arp3 (E–F). For en face views (xz plane) through the phagocytic furrow, images were deconvoluted and reconstructed in 3D. The plane of the furrow at minimal ring diameter determined the midplane. A gallery of successive sections through the furrow, parallel to the midplane is also shown. (G–I) Confocal images illustrating the positioning in the phagocytic furrow of GFP-MyoB, YFP-MyoC, and GFP-dynamin A relative to the cytoskeletal proteins mentioned above. (J) Schematic representation of the different territories observed. For whole cells sections (A–E and I), bars are 2 μm. For magnified views of the furrow, bars are 1 μm.
Finally, myosin II built a more distal ring (Figure 5A). The plasma membrane marker 4C4 was depleted from the phagosomal membrane (Mercanti et al., 2006) and was found exclusively at the outer membrane (Figure 5D), with a sharp boundary at the cup lip. The gap between the proximal MyoK/Abp1 ring and the plasma membrane is expected to be filled with myosin II and coronin. Whereas coronin was strongly accumulated at the furrow (Figure 5, C–E), myosin II, although mainly cortical, was also moderately present at or just next to the site of constriction defined by MyoK and Abp1 (Figure 5, A and G). Arp3 was enriched at the cup lip and at the phagocytic furrow, only partially overlapped with coronin and displayed a more proximal localization that also overlapped with Abp1 (Figure 5E). Actin was localized relative to MyoK and Arp3 (Figure 5F), confirming that the bulk of actin was located similarly to coronin during phagocytosis (Maniak et al., 1995). Therefore, MyoK and Abp1 build the intermediate layer between the membrane and the Arp2/3 complex, whereas the Arp2/3 complex layer connects MyoK-Abp1 to the bulk of the actin network.

The M1 isoforms in Dictyostelium have different and overlapping functions in endocytosis and chemotaxis (Falk et al., 2003; Rivero, 2008). Whether this is reflected by subtle differences in their respective localizations in these processes has not been investigated rigorously. GFP-MyoB, YFP-MyoC, and MyoK were all localized in the proximal ring at the furrow (Figure 5, A, B, and G). MyoB overlapped with Abp1 and localized closer to the membrane than Arp3 (Figure 5H). However, M1s exhibited fine differences in their respective localizations. MyoK was present throughout the furrow, whereas MyoB and MyoC were often enriched in the upper part of the furrow, toward the cup lip. Only MyoB displayed, like Abp1, a similar enrichment at the furrow and the cup lip. Interestingly, M1s were enriched at the phagosomal membrane but were depleted from the plasma membrane cortex. Only a faint MyoB signal lined the cortical membrane in the continuity of the cup lip (Figure 5, G and H). In contrast, myosin II was enriched at the plasma membrane but only a faint signal lined the invaginated membrane of the cup lip, facing the particle. Thus, MyoB and myosin II built invected gradients crossing precisely at the tip of the cup lip (Figure 5G). MyoK, MyoB, and MyoC line the phagosomal membrane around the furrow, in contrast to cortical myosin II. Nevertheless, they might all be involved in aspects of membrane constriction. Thus, despite structural divergence, MyoK is representative of M1 function in phagocytosis and its interaction network might be overlapping with other M1s.

Abp1 is not only binding to MyoK but also to dynamin A. Therefore, GFP-dynamin A was visualized during phagocytosis of budded yeasts. Although coronin delineated the furrow and the progressing cup, dynamin A was localized on vesicles crowding around the cup base (Figure 5I), which are particularly visible in Figure 5I, bottom. In conclusion, detailed characterization of the phagocytic furrow indicates that the complex interaction network can be subdivided into two subnetworks, one subnetwork involving MyoK, Abp1, and actin and the other subnetwork involving Abp1 and Dynamin A.

**Fine Localization of MyoK at The Phagocytic Furrow Depends on the GPR Loop and Farnesylation**

The GPR loop mediates protein–protein interactions, whereas the farnesylated tail targets a GFP construct to the plasma membrane. Thus, which domain is determinant for function and localization? To test for the impact of GPR loop deletion, a YFP fusion protein lacking the GPR loop (aa 122-261), MyoKΔloop, was overexpressed in myoK null cells. In contrast, in the MyoKΔfarnesyl construct, the farnesylation motif was masked by in-frame fusion of the C terminus to a myc tag (Schwarz et al., 2000). Although localizations of full-length MyoK, whether expressed at endogenous levels or overexpressed, were identical, localizations of the overexpressed MyoKΔloop or MyoKΔfarnesyl were aberrant (Figure 6A). YFP-MyoKΔloop was mostly cytosolic and not enriched at the furrow (Figure 6A). However, the GPR loop alone is not sufficient to confer furrow localization to a GFP fusion (Figure 6A). Moreover, YFP-MyoKΔloop diffusely colocalized with coronin but was clearly excluded from the proximal Abp1 ring (Figure 6B). In contrast, MyoKΔfarnesyl was diffusely present throughout the phagocytic furrow, where it localized with actin and coronin rather than Abp1. We conclude that localization of MyoK with actin in the phagocytic cup requires both the motor domain and the GPR loop, and farnesylation is required for proximal localization of the protein at the phagosomal membrane. The phagocytosis efficiency of the deletion mutants was quantified by flow cytometry. MyoK full-length complemented the myoK null uptake defect. In contrast, the MyoKΔloop construct did not complement (Figure 6D). The early uptake defect of MyoKΔfarnesyl-expressing cells was confirmed (Schwarz et al., 2000), even suggesting a slight dominant negative effect. Therefore, an intact MyoK protein is required for efficient uptake of big particles such as 4.5-μm beads or yeasts. Moreover, phagocytosis efficiency correlates with proper MyoK localization.

**MyoK and Abp1 Have Distinct Roles in Phagocytosis**

The phagocytic uptake of 4.5-μm fluorescent beads by abp1 and myoK null cells was quantitated by flow cytometry, revealing counterintuitive opposite differences between the two mutants (Figure 7C). The uptake defect observed in myoK null cells was mild (20% deficiency) but confirmed previous results obtained on adherent cells by counting internalized fluorescent yeast (Schwarz et al., 2000). Overexpressing MyoK complemented the myoK null defect and even increased uptake above wild-type levels, revealing a positive regulatory role for MyoK. In contrast, abp1 null mutants showed a markedly increased uptake that was strongly inhibited by overexpression of Abp1, revealing a negative regulatory role for Abp1. These observed differences were independent of the genetic background as abp1 null mutants in the AX-2 or DH1–10 backgrounds showed similar uptake phenotypes (unpublished data). To elucidate the mechanism of uptake, we examined the content of phagosomes purified at very short intervals (2–12 min) during ingestion. Detailed temporal profiling revealed a difference in the recruitment of MyoK and Abp1 that correlated with an early and late phase of actin dynamics (Supplemental Figure 3). MyoK, profilin, and myosin II were transiently enriched on 2- to 6-min phagosomes, whereas actin and Abp1 were also present early but dissociated later. Together, these data indicated that Abp1 and MyoK have distinct roles in phagocytosis.

The fact that MyoK and Abp1 interact but have antagonistic impacts on phagocytic uptake in vivo indicates that they might not depend on each other for localization to the phagocytic cup. MyoK localization was distinct from coronin and cortical myosin II in both wild-type cells and abp1 null mutants (Figure 7A). Its recruitment and precise localization at the phagocytic furrow were not dependent on the presence of Abp1. The reverse was also true. Abp1 localization at the furrow was indistinguishable between wild-type and myoK null cells and was similar to that of MyoK, relative to coronin and myosin II (Figure 7B). Although endogenous
MyoK and Abp1 interact and colocalize, they are not mutually required for their recruitment to the phagocytic furrow.

As a first step to investigate the antagonistic effects of Abp1 and MyoK, we unsuccessfully attempted to generate a double knockout strain. To design alternative strategies to dissect this antagonism we reasoned that, if Abp1 and MyoK are major players in regulating uptake efficiency and PakB regulates this, then expression of constitutively activated PakB (PakB\(^{H9004PL}\)) should have a strong effect on phagocytosis but this effect should remain MyoK dependent. Expressing PakB\(^{ΔPL}\) was difficult in wild-type cells and the tolerated level of expression was low (Figure 7E), whereas expression in myoK-null cells was easy and the level of expression very high (Figure 7E). Functionally, the impact was also extremely strong in wild-type cells, inhibiting uptake to \(\sim 80\%\), whereas it had little effect in the myoK-null background. Interestingly, this inhibition of uptake was similar to the one observed when overexpressing Abp1. We conclude that MyoK is likely the major physiological target of PakB phosphorylation for the uptake of large particles and that, together with Abp1, they form a complex circuit that acts as a switch to regulate phagocytosis (Figure 7, F and G).

DISCUSSION

Protein and Membrane Binding Domains of MyoK Are Essential for Efficient Particle Uptake

The M1 tails usually contain a membrane-targeting polybasic stretch and TH2 and SH3 protein–protein interactions domains. MyoK is a naturally occurring M1 variant lacking typical tail domains. In this protein, a TH2-like domain is inserted in the surface loop1 of the motor domain and interacts directly with F-actin, Abp1, and profilins. Despite this insertion, the motor domain of MyoK is able to bind F-actin in an ATP-dependent manner (Schwarz et al., 2000).
and is probably regulated by phosphorylation at the TEDS site. Farnesylation of the C terminus functionally replaces the TH1 polybasic stretch and directs a GFP fusion protein to the membrane. Both the GPR loop and farnesylation are needed for fine localization of MyoK at the phagocytic cup and for efficient phagocytosis. Interestingly, these domains alone are not sufficient for correct localization of GFP fusions to the distinct MyoK/Abp1 territory at the phagocytic furrow. Neither GFP-GPR nor GFP-MyoK tail constructs are enriched in the phagocytic cup. MyoK is the first myosin shown to bind directly profilin-actin and to contain a functional farnesylation motif.

The MyoK–Abp1 Interaction Network Is Well Conserved and Redundant

To understand the function and regulation of MyoK in phagocytosis, we identified its interaction network (Figure 3D). This network is highly redundant. Abp1, MyoK, and dynamin A bound to profilin, whereas Abp1 bound to MyoK and dynamin A and PakB bound to MyoK and Abp1. Although MyoK displays a divergent M1 domain organization, its interaction network is well conserved. Similar M1 interactions are found in other organisms and play a role in endocytosis. Profilin binding to dynamin was already shown in mouse brain (Gareus et al., 2006), and the SH3 domain of Abp1 binds the PRD of dynamin II in rat brain and human cell lines (Kessels et al., 2001; Onabajo et al., 2008) and Myo5p in yeast (Fazi et al., 2002). Therefore, Abp1 and profilins might be additional bona fide binding partners of TH2-like domains.

The measure of binding affinities between native full length proteins extends our understanding of protein organization in regions of actin dynamics and allows at least one prediction. Abp1 might displace profilin from MyoK and dynamin A. Indeed, Abp1 displays high binding affinities and profilin binding site overlap with Abp1 binding site. In addition, the affinity of Abp1 for the GPR loop was higher than for both isoforms of profilins and Abp1 affinity for...
cies are affected by these mutations. Therefore, MyoK and protein territories in the furrow, although phagocytosis efficiency to the juxtamembranous area. An outer layer of coronin would then restrict the actin action of the Arp2/3 complex next to the phagocytic membrane. As detailed above, MyoB and myosin II built the innermost proximal ring next to the phagosomal membrane. This ring was surrounded by an intermediate Arp3 ring and a distinct coronin ring (Figure 6, A–E). The thick coronin ring overlapped with the bulk of actin and was surrounded by a cortical myosin II layer, following an order strikingly reminiscent of the overall organization of the lamellipodium of migrating cells (Le Clainche and Carlier, 2008).

The accumulation of M1s at the site of constriction of the phagocytic furrow, is highly reminiscent of the accumulation of myosin IE around shared erythrocytes in macrophages (Swanson et al., 1999). Although myosin IE accumulates at sites of constriction, surrounded by an actin ring, myosin II localized at the cup base. In both systems, myosin I and myosin II have distinct localizations. Myosin II accumulates at the cup base or in the cortex, whereas M1s are enriched at sites of constriction, bound to the phagosomal membrane. As detailed above, MyoB and myosin II built inverted gradients at the cup lip crossing precisely at the tip of the lip (Figure 5G). Resting cortical tension depends on the expression levels of MyoB, MyoC, MyoK, and myosin II in an additive manner, but only myosin II is required for rapid increase in cortical tension upon stimulation (Dai et al., 1999). Therefore, we speculate that, during phagocytosis, myosin II provides tension to pull the convex cortical plasma membrane, dragging it inward like in the cytokinetic furrow. M1s provide tension to push on the concave phagosomal membrane and compress it around the particle.

Organization of adjacent territories in the phagocytic furrow shows that the Arp2/3 complex layer is partially overlapping with both the MyoK–Abp1 layer and the coronin–actin layers. Indeed, Arp3 is pulled down together with an Abp1-GPR loop complex. Considering that Abp1/M1s recruit and activate the nucleation machinery in yeast, whereas coronin terminates nucleation by displacing the Arp2/3 complex from the actin filament in mammals (Kasonen et al., 2006; Sun et al., 2006; Cai et al., 2008), we suggest that MyoK-Abp1 is contributing to positioning and activation of the Arp2/3 complex next to the phagocytic membrane. An outer layer of coronin would then restrict the actin nucleation activity to the juxtamembranous area.

Finally, absence of MyoK and Abp1 or mislocalization of MyoK does not dramatically affect the distribution of protein territories in the furrow, although phagocytosis efficiencies are affected by these mutations. Therefore, MyoK and Abp1 are regulatory proteins that might affect the dynamic of cup progression rather than modify the stability of protein distributions.

MyoK and Abp1 Play a Regulatory Role in Phagocytosis
MyoK and Abp1 have distinct roles in phagocytosis. Despite direct binding of Abp1 to the GPR loop of MyoK and colocalization in the furrow, both proteins did not depend on each other for proper localization. Abp1 but not MyoK was enriched in patches at the bottom of the cup and at the cup lip (Figure 4C). Both proteins also displayed distinct temporal profiles during generation and maturation of early phagosomes and during their respective absence had an opposite impact on uptake efficiency. Because Abp1 is more abundant than MyoK (Figure 6C) and contains a ubiquitous SH3 protein-binding domain, it might perform tasks independent of MyoK, whereas both proteins interact and cooperate in the phagocytic furrow.

Because both proteins localized independently in the furrow (Figure 7, A and B), their recruitment to the cup probably results from redundant protein interactions. Many proteins involved in endocytosis interact with the SH3 domain of Abp1 in yeast and mammals (Kessels et al., 2001; Fazi et al., 2002; Pinyol et al., 2007). In Dictostelium, PakB potentially recruits Abp1 to the phagocytic cup. Indeed, PakB localizes to the phagocytic cup (de la Roche et al., 2005) and coimmunoprecipitated with Abp1 (Figure 1E). Other M1s might also be substrate for PakB (Figure 7G) and interact with Abp1. MyoB and MyoC bind CARMIL through their SH3 domain (Jung et al., 2001), but binding partners to their TH2 domain have not been investigated. MyoB was present together with Abp1 not only at the furrow but also at the lip and the base of the cup (Figure 5H). Therefore, the TH2 domain of MyoB might also bind Abp1 and target it to the phagocytic cup. In contrast, CAP and F-actin might localize MyoK to the furrow in absence of Abp1. CAP localizes to the cup (Sultana et al., 2009) and was identified by GST-GPR pull down (Figure 1D).

The MyoK–Abp1 Interaction Acts as a Switch in the Regulation of Phagocytosis
MyoK and Abp1 play positive and negative regulatory roles in phagocytosis, respectively. We propose that activation of MyoK by PakB-mediated phosphorylation and binding of profilin–actin to its GPR loop are the two major and coincidental events that impact positively on phagocytosis (Figure 7F). Conversely, absence of Abp1 relieves a negative regulation and increases uptake. This might seem counterintuitive, however, knockout mutants for dynamin A, the actin cross-linking protein Abp34, and both profilins I and II also show increased phagocytosis (Wienke et al., 1999; Rivero, 2008). Remarkably, both dynamin A and profilin II interacted directly with Abp1, further supporting the negative regulatory role of Abp1. Vice versa, overexpression of Abp1 inhibits uptake (Figure 7C) and also has a dominant-negative effect on cell motility (Wang and O’Halloran, 2006). This suggests a general mechanism by which dissociation of the Abp1 dimer and binding of the monomer negatively regulates M1s, including MyoK (Figure 7F).

Our observations can now be integrated into a robust mechanistic model presented in Figure 7G, as well as in Supplemental Figure S4, that explicitly describes the impact of all the knockout and complementation experiments presented here. We propose that MyoK is a central positive regulator of uptake, which integrates signals from PakB (TEDS site phosphorylation) and profilin (in the form of recruitment of profilin–actin complex, fuel for actin polymerization). In absence of MyoK, other M1s dependent on
A MyoK-Abp1-PakB Circuit Regulates Uptake


