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# Amphiphysin IIm, a Novel Amphiphysin II Isoform, Is Required for Macrophage Phagocytosis

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## Summary

**Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive immune response. Amphiphysin II participates in receptor-mediated endocytosis, in part, by recruiting the GTPase dynamin to the nascent endosome. We demonstrate here that a novel isoform of amphiphysin II associates with early phagosomes in macrophages. We have ablated the dynamin-binding site of this protein and shown that this mutant form of amphiphysin II inhibits phagocytosis at the stage of membrane extension around the bound particles. We define a signaling cascade in which PI3K is required to recruit amphiphysin II to the phagosome, and amphiphysin II in turn recruits dynamin. Thus, amphiphysin II facilitates a critical initial step in host response to infection.**

## Introduction

Macrophages are essential to both the innate and acquired immune response because of their unique ability to phagocytose and degrade pathogens, to present antigens to T cells, and to stimulate an inflammatory response. Macrophages recognize infectious agents using pattern-recognition receptors that bind to conserved motifs on the surface of the pathogen, or through Fc and complement receptors after opsonization of the pathogen (Janeway, 1992). Phagocytic receptors stimulate the polymerization of actin at the point of particle contact, membrane extension around the particle, and subsequent particle internalization (Aderem and Underhill, 1999). Although it is clear that this process is tightly regulated, the molecular mechanisms that control actin polymerization and membrane recruitment to the forming phagosome remain largely unknown.

Cells have evolved a variety of strategies to internalize particles, including receptor-mediated endocytosis and phagocytosis (Aderem and Underhill, 1999). Receptor-mediated endocytosis, the process by which macromolecules and small particles enter cells, is mediated by clathrin while phagocytosis, the uptake of particles $>0.5$ $\mu$m, is actin dependent. Dynamin 2 is a GTPase that has a critical role in the scission of forming clathrin-coated endocytic vesicles from the plasma membrane (Schmid et al., 1998). Flies with a temperature-sensitive mutation in *shibire*, the *Drosophila* dynamin homolog, have a severe defect in receptor-mediated endocytosis that results in the accumulation of partially invaginated clathrin-coated pits at the nonpermissive temperature (Poodry et al., 1973; VanderBliek and Meyerowitz, 1991). In mammalian cells, dynamin localizes to the neck of forming endosomes, and inhibition of dynamin function prevents receptor-mediated endocytosis (Herskovits et al., 1993; VanderBliek et al., 1993; Damke et al., 1994; Takel et al., 1995). Dynamin is therefore thought to function either by generating the mechanical force necessary for vesicular fission from the plasma membrane or by acting as a classical GTPase switch that activates a downstream effector (Hinshaw and Schmid, 1995; Takel et al., 1995; McNiven, 1998; Sweitzer and Hinshaw, 1998; Sever et al., 1999).

Dynamin is targeted to forming endosomes through its interaction with amphiphysin (David et al., 1996; McMahon et al., 1997). Amphiphysin colocalizes with dynamin in nerve terminals and has been shown to assemble with dynamin into ring-like structures around membrane tubules (David et al., 1996; Takei et al., 1999). The C-terminal SH3 domain of amphiphysin interacts with the proline-rich domain of dynamin in a phosphorylation-dependent manner (David et al., 1996; Bauerfeind et al., 1997; Grabs et al., 1997; Slepnev et al., 1998). Amphiphysin also interacts with clathrin and the clathrin adaptor protein AP2 through sites in its N-terminal and central domains (David et al., 1996; McMahon et al., 1997; Slepnev et al., 1998; Owen et al., 1999); this interaction is also phosphorylation dependent (Slepnev et al., 1998). Overexpression of the SH3 domain of amphiphysin in cells inhibits receptor-mediated endocytosis (Shupliakov et al., 1997; Wigge et al., 1997a; Owen et al., 1998). Two major forms of amphiphysin have been identified: amphiphysin I, which is expressed primarily in the brain, and amphiphysin II, which is ubiquitously expressed (Butler et al., 1997).

We raised a panel of monoclonal antibodies directed against proteins that associate with phagosomes in macrophages (Morrissette et al., 1999). One of these antibodies, M8D10, recognizes a novel isoform of amphiphysin II, which we have called amphiphysin IIm. We report here that a mutant form of this amphiphysin lacking the SH3 domain, AmphIIm$^{SH3-}$, cannot bind to dynamin. Further, we show that expression of AmphIIm$^{SH3-}$ in macrophages significantly impairs phagocytosis at the stage of membrane extension.

## Results

### Identification and Characterization of the Antigen Recognized by M8D10

In order to identify novel components of the phagocytic machinery in macrophages, we generated a large panel of monoclonal antibodies directed against proteins that

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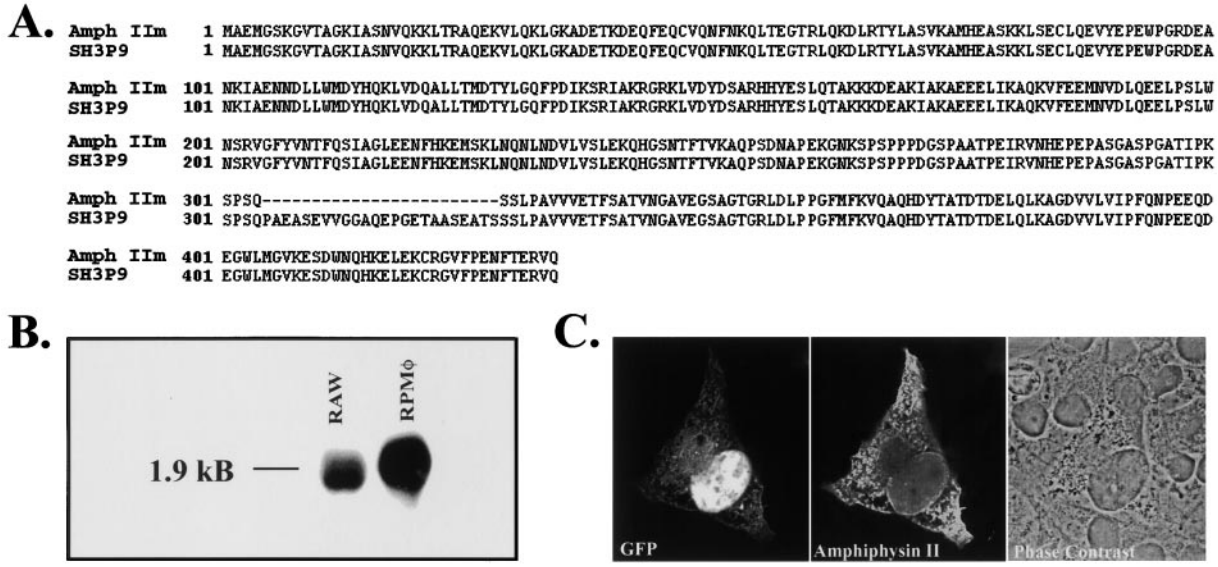


Figure 1. Cloning and Confirmation of Amphiphysin II as the Ligand for M8D10

(A) A macrophage expression library was probed with M8D10, and the resulting clone was sequenced. The sequence was aligned with amphiphysin II sequences in the GenBank database. The macrophage sequence is most similar to the murine embryonic amphiphysin II isoform, SH3P9, differing only in a 24–amino acid deletion.

(B) A Northern blot of mRNA from the RAW 264.7 macrophage cell line and plate-adhered resident peritoneal macrophages was probed with the amphiphysin IIm sequence (shown in [A]) and revealed a single transcript of 1.9 kb.

(C) COS7 cells were cotransfected with a plasmid expressing amphiphysin II and one expressing GFP. The cells were stained for immunofluorescence with the M8D10 antibody. Transfected cells were identified by GFP expression. Only transfected cells were positive for M8D10 staining.

associate with phagosomes (Morrissette et al., 1999). The cognate antigen for one of these antibodies, M8D10, was identified to be amphiphysin II by expression library screening. The sequence of amphiphysin II cloned from murine macrophages, which we have called amphiphysin IIm, was most closely related to SH3P9, a murine embryonic isoform (Sparks et al., 1996) that contained an additional insert of 24 amino acids (Figure 1A). Expression of amphiphysin IIm was confirmed by Northern blot analysis that revealed a single transcript of 1.9 kb in both murine resident peritoneal macrophages and the murine macrophage-like cell line RAW 264.7 (Figure 1B). We have been unable to identify any other amphiphysin II isoforms in macrophages either by Northern analysis or by RT–PCR (Figure 1B; data not shown). M8D10 does not recognize endogenous proteins in COS7 cells; therefore, to verify that M8D10 was specific for amphiphysin II, we cotransfected COS7 cells with a plasmid expressing full-length amphiphysin II (either amphiphysin IIm [Figure 1C] or SH3P9 [data not shown]) and a plasmid expressing GFP. Only the transfected COS7 cells, identified by GFP expression, expressed amphiphysin II (Figure 1C). M8D10 did not recognize amphiphysin I when it was expressed in COS7 cells (data not shown).

Macrophages engulf particles via a number of phagocytic receptors, including the Fc receptor (IgG-opsonized particles), the complement receptor (C3bi-opsonized particles), and a variety of receptors that recognize specific sugars (zymosan, a *Saccharomyces cerevisiae* cell wall particle). Phagocytosis initiated by these different receptors utilize some common and some unique cytoskeletal elements (Allen and Aderem, 1996). Amphiphysin IIm is recruited to early phagosomes stimulated by multiple phagocytic receptors, including phagosomes containing IgG-opsonized sheep red blood cells (SRBCs), C3bi-opsonized SRBCs, and zymosan (Figures 2A–F). This association is transient, and the protein is shed from maturing phagosomes with kinetics that precisely mirror that of actin (Figures 2G–L; data not shown).

### Amphiphysin IIm Lacking a Dynamin 2-Binding Site Inhibits Endocytosis and Phagocytosis

Amphiphysin II participates in receptor-mediated endocytosis, in part, by targeting dynamin to the forming endosome (Leprince et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997b; Volchuk et al., 1998). This implies that deletion of the dynamin-binding SH3 domain of amphiphysin will generate a dominant-negative inhibitor of amphiphysin II function. This mutant was generated (AmphIIm$^{SH3-}$), and, as predicted, V5 epitope-tagged full-length amphiphysin II coimmunoprecipitated with dynamin, while the AmphIIm$^{SH3-}$ mutant was not able to bind dynamin (Figure 3A). Mutant AmphIIm$^{SH3-}$ was expressed in a bicistronic vector with GFP (pTIGZ2 vector) in RAW-TT10 macrophages, allowing transiently transfected cells to be identified by their green fluorescence (Gold et al., 1999; Underhill et al., 1999). Cells expressing high levels of AmphIIm$^{SH3-}$ were severely inhibited in receptor-mediated endocytosis as determined by the uptake of DiI-labeled acetylated LDL in macrophages (Figure 3B), demonstrating that this mutant acts as a dominant-negative inhibitor of amphiphysin II function.

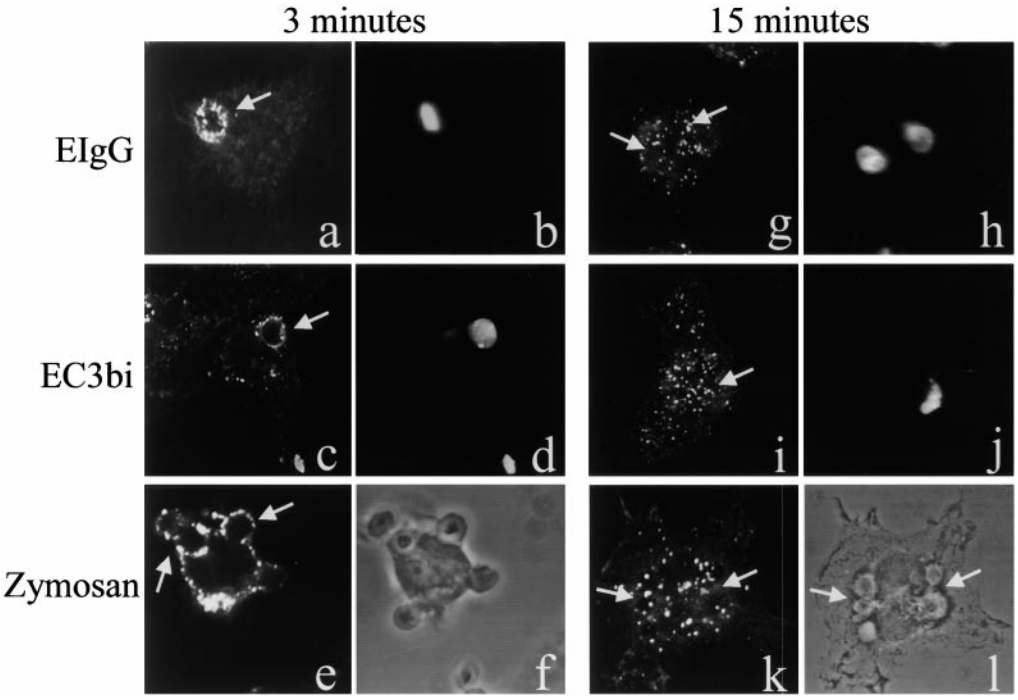


Figure 2. Amphiphysin II Localizes to Early Macrophage Phagosomes

Resident peritoneal macrophages were incubated with IgG-opsonized SRBCs (EIgG), complement-opsonized SRBCs (EC3bi), or zymosan for the indicated amounts of time. The cells were stained with M8D10 (A, C, E, G, I, and K) to identify amphiphysin II localization. SRBCs were identified with anti-SRBC antibody (B, D, H, and J), and zymosan were identified on phase images (F and L).

We used the AmphIIm$^{SH3-}$ mutant to investigate the role of amphiphysin II in phagocytosis. Both epitope-tagged full-length amphiphysin IIm and AmphIIm$^{SH3-}$ localized to phagosomes with the same kinetics as actin (Figure 3C). This demonstrated that the SH3 domain of amphiphysin II is not required for its localization to the site of particle binding and established that the mutant is correctly localized to exert an effect on phagocytosis. Further, as predicted by the biochemical data above, dynamin 2 was not enriched at the site of particle binding in cells expressing AmphIIm$^{SH3-}$ (data not shown). RAW-TT10 cells were transiently transfected with AmphIIm$^{SH3-}$, and phagocytosis of either tetramethylrhodamine-labeled zymosan, IgG-opsonized SRBCs, or C3bi-opsonized SRBCs was assessed as a function of the level of expression of the GFP/dominant-negative protein by two-color flow cytometry. AmphIIm$^{SH3-}$ inhibited Fc receptor-mediated phagocytosis by 74%, complement receptor-mediated phagocytosis by 73%, and zymosan phagocytosis by 82% (Figure 4).

The defect in phagocytosis was not due to an effect of AmphIIm$^{SH3-}$ on the level of phagocytic receptors, since transfected cells expressed normal cell surface levels of Fc receptors (CD16 and CD32) and C3bi receptors (Mac-1) (data not shown). In addition, particle binding at 4°C was also unimpaired (data not shown). Cell viability and other actin-based processes were unaffected by the expression of the mutant, since RAW-TT10 cells expressing AmphIIm$^{SH3-}$ polymerized actin at the site of particle binding (Figure 3C) and spread normally in response to phorbol esters (data not shown). In addition, mutant amphiphysin had no effect on interferon-γ (IFNγ)-induced upregulation of MHC class II, demonstrating that these cells are capable of both IFNγ-induced protein synthesis and trafficking of the newly synthesized protein to the membrane (data not shown).

### Amphiphysin IIm Acts at the Stage of Membrane Extension

To determine the point at which phagocytosis was arrested, AmphIIm$^{SH3-}$-expressing RAW-TT10 cells were studied by scanning electron microscopy (SEM). Ten minutes after contacting IgG-coated SRBCs, control cells (expressing pTIGZ2 alone) showed characteristic membrane ruffles extending around the particles (Figure 5A), while AmphIIm$^{SH3-}$-expressing cells only generated small pedestals beneath the bound SRBCs (Figure 5B). After unbound SRBCs were washed away and phagocytosis was allowed to proceed for an additional 50 min at 37°C, the pTIGZ2 control cells had internalized ~90% of the particles (Figure 5C; Table 1). In contrast, <30% of the particles associated with the AmphIIm$^{SH3-}$-expressing cells were internalized (Figure 5D; Table 1), and, most importantly, the small membrane pedestals never extended around the particles.

Phosphoinositide 3-kinase (PI3K) is an important regulator of macrophage phagocytosis, and inhibition of PI3K causes incomplete phagosome closure (Araki et al., 1996; Cox et al., 1999), a phenotype very similar to that observed in cells expressing mutant amphiphysin. Interestingly, treatment of macrophages with wortmannin, a PI3K inhibitor, prevents the recruitment of amphi-

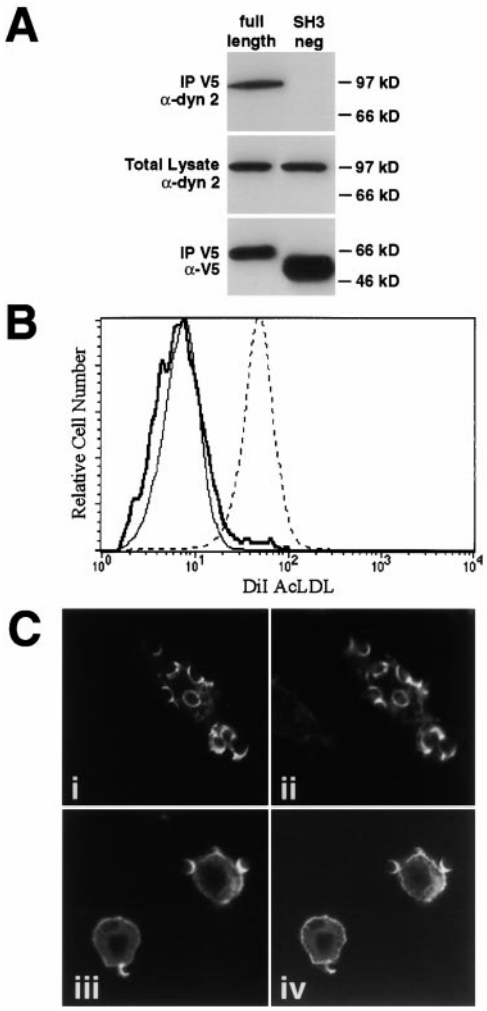


Figure 3. AmphIIm$^{SH3-}$ that Cannot Bind Dynamin Functions as a Dominant-Negative Inhibitor in Macrophages

(A) Eighteen hours after RAW-TT10 macrophages were transiently transfected with either full-length V5-tagged amphiphysin IIm or with V5-tagged AmphIIm$^{SH3-}$, total protein extract was prepared and immunoprecipitated with anti-V5 antibody. The upper panel shows the postimmunoprecipitation samples probed with the anti-dynamin 2 antibody. The middle panel shows total extract before immunoprecipitation probed with the anti-dynamin 2 antibody. The lower panel shows the postimmunoprecipitation samples probed with the anti-V5 antibody.

(B) RAW-TT10 cells were incubated with 10 μg/ml DiI AcLDL for 10 min and then analyzed by FACS. The dotted line represents the amount of DiI AcLDL endocytosed by untransfected cells, and the thick solid line represents the amount of DiI AcLDL incorporated by cells expressing high levels of AmphIIm$^{SH3-}$. The thin solid line is the amount of AcLDL taken up by untransfected cells at 4°C.

(C) RAW-TT10 macrophages were transiently transfected with full-length V5-tagged amphiphysin II (i and ii) or with V5-tgged AmphIIm$^{SH3-}$ (iii and iv), incubated with zymosan for 10 min, and stained with the anti-V5 antibody (i and iii). Actin was detected with phalloidin (ii and iv).

physin to the phagocytic cup (Figure 6). It is thus possible that PI3K acts upstream of amphiphysin during phagocytosis, and that it stimulates membrane extension around the particle through amphiphysin.

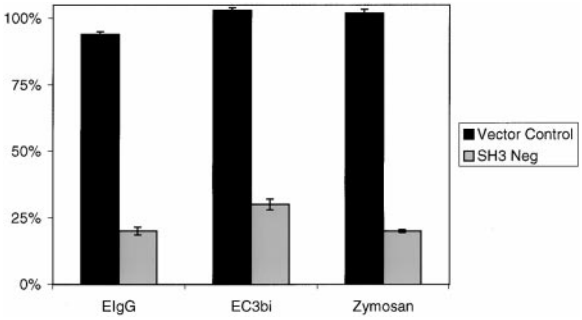


Figure 4. AmphIIm$^{SH3-}$ Inhibits Macrophage Phagocytosis

Transiently transfected RAW-TT10 macrophages internalized the indicated particles for 10 min. Uninternalized particles were removed, and the cells were analyzed by flow cytometry. Phagocytosis is expressed as the percent of highly expressing transfected cells internalizing particles relative to the percent of untransfected cells internalizing particles. Control and AmphIIm$^{SH3-}$ cells expressing the same level of GFP were compared. The data shown represent a minimum of three independent experiments, and error bars reflect standard error of the mean.

## Discussion

We have demonstrated that a novel isoform of amphiphysin II associates with early phagosomes and that a dominant-negative mutant of this protein blocks phagocytosis by preventing membrane extension around the particle. The fact that inhibition of amphiphysin IIm function prevents phagocytosis mediated by a range of receptors suggests that it has a general role in particle internalization.

The amphiphysins are a family of proteins that have a role in receptor-mediated endocytosis (Wigge and McMahon, 1998). To date, two genes have been defined that encode amphiphysin I and amphiphysin II. Amphiphysin I, which is found exclusively in the brain, contains distinct functional domains. Its N-terminal coiled-coil domain promotes dimerization of the molecule, a DPW/F domain that interacts with adaptor protein-2 (AP-2), a proline-rich linker domain that binds to clathrin, and a C-terminal SH3 domain that binds to dynamin and synaptojanin (David et al., 1996; McPherson et al., 1996; de Heuvel et al., 1997; Grabs et al., 1997; Micheva et al., 1997; Wigge et al., 1997a, 1997b; Owen et al., 1999). The association of amphiphysin with its binding partners is regulated by phosphorylation; phosphorylation of amphiphysin inhibits its binding to clathrin and AP-2, while the phosphorylation of dynamin and synaptojanin prevent their binding to amphiphysin (Bauerfeind et al., 1997; Slepnev et al., 1998).

Amphiphysin II is distributed ubiquitously and has a wider diversity of splice variants than amphiphysin I (Butler et al., 1997; Tsutsui et al., 1997; Wechsler-Reya et al., 1997; Ramjaun and McPherson, 1998). Some amphiphysin II splice variants have a very similar structure to amphiphysin I and appear to bind all the same proteins (Butler et al., 1997; Leprince et al., 1997; McMahon et al., 1997; Ramjaun et al., 1997, 1999; Ramjaun and McPherson, 1998; Volchuk et al., 1998). Other splice variants have deletions of the established binding sites for clathrin and AP-2, but it is not known whether they

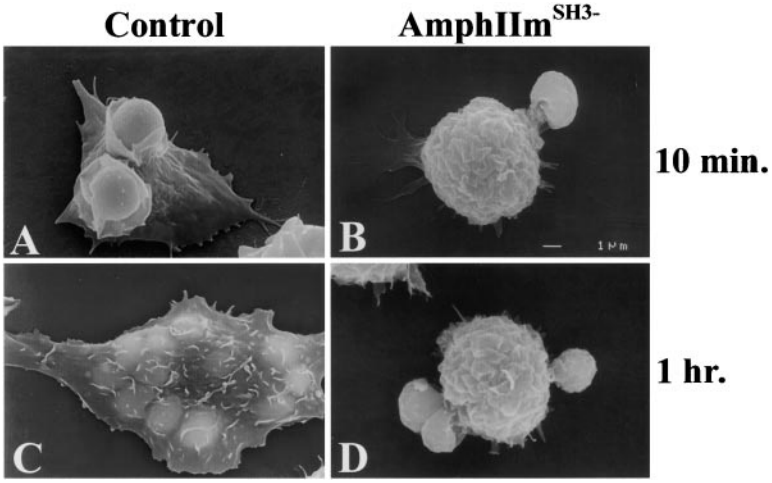


Figure 5. AmphIIm$^{SH3-}$ Inhibits Membrane Recruitment to the Forming Phagosome

Transiently transfected cells were sorted by FACS, and cells expressing the highest levels of GFP were studied by SEM. Sorted cells were incubated with IgG-coated SRBCs for 10 min (A and B) or for 1 hr (C and D). After a 10 min internalization, control pTIGZ2-transfected cells demonstrated membrane ruffling at the site of particle binding (A), and after 1 hr ~90% of the particles were internalized (C). By contrast, cells expressing AmphIIm$^{SH3-}$ only had small pedestals beneath the bound particles at both time points (B and D). The size bar shown in (B) applies to all SEMs.

can bind these proteins via alternative sites (Butler et al., 1997; Ramjaun and McPherson, 1998; Ramjaun et al., 1999). The amphiphysin II isoform that we have identified in macrophages, amphiphysin IIm, is most similar to SH3P9, a murine transcript identified in embryos (Sparks et al., 1996). Both of these transcripts have a large segment of the central domain spliced out of them, including the two defined clathrin binding sites. Further, neither of these transcripts have the defined AP-2 binding motif or the N-terminal insert domain previously defined to cause plasma membrane targeting. Despite the lack of these targeting domains, amphiphysin IIm localizes to phagosomes, and a dominant-negative form of the protein inhibits particle internalization and endocytosis. Although the dominant-negative mutant of amphiphysin IIm lacks the SH3 domain, it localizes to phagocytic cups, suggesting that the phagosomal targeting domain does not reside in this region of the protein. It is, however, possible that this mutant dimerizes with the endogenous protein and that targeting is mediated by the native SH3 domain.

Amphiphysin IIm localizes to phagosomes with kinetics that precisely mirror that of actin. Amphiphysins have been associated with actin-based events in a variety of systems. Two human isoforms of amphiphysin II that are predominantly expressed in muscle colocalize with the cytoskeletal protein ankyrin (Butler et al., 1997); ankyrin has also been shown to associate with phagosomes (Moffat et al., 1996). Amphiphysin I colocalizes with actin patches at the leading edge of growth cones, and expression of an antisense construct of amphiphysin I inhibits neurite outgrowth (Mundigl et al., 1998). Disruption of the two yeast homologs of amphiphysin, *rvs161* and *rvs167*, disrupts endocytosis as well as normal actin dynamics (Munn et al., 1995; Sivadon et al., 1995; Balguerie et al., 1999), and the SH3 domain of Rvs167p was identified in a two-hybrid screen for actin binding proteins (Amberg et al., 1995).

Macrophages expressing AmphIIm$^{SH3-}$ bind particles normally and polymerize actin at the point of contact; however, the cells fail to extend membrane around the particles. This inhibition suggests that there is a defect in membrane insertion into the forming phagosome; since AmphIIm$^{SH3-}$ has no effect on IFN$\gamma$-induced upregulation of surface MHC class II molecules, the inhibition appears to be selective. Amphiphysin II binds to dynamin and synaptojanin, both molecules that regulate membrane traffic (Leprince et al., 1997; Ramjaun et al., 1997). We have recently shown that a dominant negative form of dynamin 2, dynK44A, also inhibits phagocytosis by preventing membrane extension (Gold et al., 1999). We show here that AmphIIm$^{SH3-}$ fails to bind dynamin and that, consequently, dynamin is not recruited to the phagosome. This suggests that amphiphysin IIm functions to recruit dynamin to the phagosome and that

Table 1. Bound, Incompletely Internalized Particles

| | Bound Particles | |
|---|---|---|
| | 10 min | 1 hr |
| Control | 435 ± 17* | 45 ± 5* |
| AmphIIm$^{SH3-}$ | 509 ± 32* | 375 ± 73* |

* Represents number of bound, incompletely internalized IgG SRBC per 100 macrophages examined by scanning electron microscopy. Error is standard error of the mean from three samples. Examination of parallel samples by fluorescence confirmed that after 1 hr ~90% of the particles on the control cells were internalized, while <30% of the particle associated with the AmphIIm$^{SH3-}$ expressing cells were internalized.

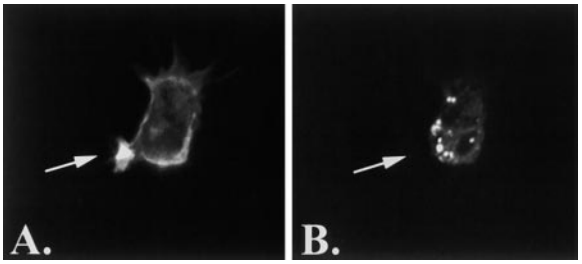


Figure 6. PI3K Is Necessary to Recruit Amphiphysin IIm to the Site of Particle Binding

Resident murine peritoneal macrophages were treated with wortmannin for 1 hr and incubated with zymosan for 10 min. Arrows indicate the site of zymosan binding. Actin polymerized under the bound particles (A), but amphiphysin IIm was not enriched in the actin pedestals (B).

dynamin facilitates membrane extension around the particle, although the precise mechanism by which this happens is unknown. It still remains possible that the inositol 5′-phosphatase synaptojanin also participates in phagocytosis.

Inositol lipid turnover occurs during phagocytosis and inhibition of PI-3 kinase prevents particle internalization at the stage of membrane extension around the particle (Araki et al., 1996; Cox et al., 1999). Indeed, inhibition of PI-3 kinase blocks the recruitment of amphiphysin IIm and dynamin to the phagosome. Thus, we have established a signaling pathway whereby engagement of a phagocytic receptor triggers rapid actin polymerization beneath the particle. Activation of PI-3 kinase is then required to recruit amphiphysin IIm to the phagosome, which in turn is necessary for the recruitment of dynamin. This cascade of events is essential for membrane extension around the particle and subsequent particle internalization. The uptake of bacterial pathogens is a critical step in initiating both the innate and acquired immune response; we have now demonstrated that amphiphysin IIm is important in this process.

## Experimental Procedures

### Expression Cloning of Antigens

Ascites from the hybridoma line M8D10 was used to screen a day 16 mouse embryonic expression library (Novagen). Filters were probed with a biotinylated secondary antibody followed by a streptavidin-conjugated peroxidase and positive plaques were detected with a DAB color reaction (all reagents from Vector). Plaque-purified positives were isolated and the DNA insert was purified and sequenced with vector-specific primers on an ABI automated sequencer. GCG (Genetics Computer Group) and Sequencher (Gene Codes Corporation) software packages were used to align the gene sequences.

### Northern Blot Analysis

Total RNA was isolated (Trizol, GIBCO–BRL) from RAW 264.7 cells (American Type Culture Collection [ATCC]) and from plate-adhered resident peritoneal macrophages from ICR mice (Charles River). mRNA was generated from total RNA by passage over oligo dT columns (Message Maker, Pharmacia). mRNA (2 μg) for each sample was electrophoresed on a 1% agarose/formaldehyde gel and transferred to Hybond (Amersham). This Northern blot was hybridized with a randomly primed probe generated from the full-length amphiphysin IIm cDNA (Prime-It II, Stratagene) using standard techniques and exposed to Biomax MR film (Kodak) for 2 days.

### DNA Expression Vectors

Amphiphysin IIm was amplified from a murine thioglycolate–elicited peritoneal macrophage library (Stratagene) using the following primers: 5′-CAGGATGGCAGAGATGGG and 3′-CGTCACTGTACCC GCTCTG. The resulting 1.2 kb product was gel purified (Qiagen gel purification kit) and cloned into pcDNA3.1 zeo (Invitrogen).

AmphIIm$^{SH3-}$ was generated by amplifying base pairs 1–939 of the macrophage-specific amphiphysin isoform (5′ primer, GGCGGA TCCATGGCAGAGATGGGGAGCAAG; 3′ primer, GCGAATTCTTAC TCCACCACCACAGCCGGAAG). The product was cloned into the BamHI–EcoRI site in the pTIGZ2 vector (Gold et al., 1999; Underhill et al., 1999). In this vector, expression of AmphIIm$^{SH3-}$ is under the control of a tetracycline-repressible promoter. Removal of tetracycline from the media results in a bicistronic mRNA that concomitantly directs translation of the dominant-negative amphiphysin protein and GFP.

V5/His epitope–tagged amphiphysin II and AmphIIm$^{SH3-}$ were constructed by amplifying the appropriate region of the protein and TA cloning the cDNAs into the pcDNA3.1/V5/HisTOPO vector (Invitrogen).

The vector pSFFV-eGFP expresses enhanced green fluorescent protein under the direction of the spleen focus-forming virus (SFFV) LTR. The plasmid was constructed by first deleting the CMV promoter of pcDNA3 (Invitrogen) by excising the 0.9 kb BglII–BamHI fragment containing the promoter and ligating the vector closed. A 1.2 kb EcoRI–SalI fragment of p651 (kindly provided by Michel Nussenzweig) containing the SFFV LTR and a 0.8 kb SalI–NotI fragment of peGFP-N1 (Clonetech) containing the coding region for enhanced green fluorescent protein were cloned together into the EcoRI–Not site of the vector.

### Immunofluorescence Characterization

Murine resident peritoneal (RP) macrophages were isolated and cultured as previously described (Allen and Aderem, 1996). Synchronized phagosomes were created by centrifuging particles onto the cells at 1600 rpm and 4°C for 1 min. (Prior to exposure to C3bi-opsonized particles, cells were treated with 200 nM PMA for 30 min.) After washing with PBS, the cells were incubated in media at 37°C for the times indicated in the text. The cells were fixed in formalin (10 min, room temperature [RT]), permeabilized in 0.25% Triton X-100 in PBS (10 min, RT), washed twice in PBS, and incubated with primary antibody (M8D10 hybridoma supernatant and anti-sheep red blood cell [RBC] antibody purchased from Intercell) for 1 hr at RT. The coverslips were washed in PBS and incubated with the appropriate secondary antibodies (all FITC- and TxR-conjugated antibodies were from Cappel; Cy5 conjugates from Jackson). Actin was stained with rhodamine-phalloidin (Molecular Probes). After a 1 hr incubation, the slides were washed in PBS, rinsed briefly in distilled water, and mounted in a polyvinyl alcohol-based mounting media (Harlow and Lane). All confocal images were obtained on a Zeiss Axiophot microscope equipped with Biorad Confocal optics.

IgG-coated SRBCs were prepared by incubating SRBCs (ICN/Cappel) diluted in PBS with anti-sheep RBC IgG (Intercell) at RT for 60 min. Complement-coated SRBCs were prepared by incubating sheep red cells with anti-sheep RBC IgM (Intercell) at RT for 30 min, and the cells were washed and resuspended in RPMI with 10% C5-depleted human serum (Sigma) and incubated at 37°C for 1 hr. Zymosan (Molecular Probes) was prepared as previously described (Aderem et al., 1984).

PI3K was inhibited by incubating RP macrophages with 100 nM wortmannin (Sigma) for 1 hr. These cells were incubated with zymosan for 10 min, then prepared and examined as above.

### Immunoprecipitation and Immunoblotting

RAW-TT10 cells, a clone of the RAW 264.7 (ATCC) cell line that stably expressed the tetracycline transactivator (Gold et al., 1999; Underhill et al., 1999), were transfected with either V5 epitope-tagged full-length amphiphysin IIm or V5 epitope-tagged AmphIIm$^{SH3-}$. Eighteen hours after transfection, the cells were lysed on ice into lysis buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 1% Triton X-100) with protease inhibitor cocktail (Sigma, P8340) by rocking at 4°C for 15 min, followed by scraping. Lysates were spun at 18,000 × g at 4°C for 15 min. An aliquot of the supernatant was set aside, and the rest was incubated with 1 μl of anti-V5 antibody (Invitrogen) for 1 hr at 4°C with rotation, then with 50 μl of protein A Sepharose (Sigma) for 1 hr at 4°C with rotation. The beads were washed three times in lysis buffer, and the protein was eluted in sample buffer by boiling. The samples were run on a 10% SDS-PAGE acrylamide gel. Proteins were transferred to PVDF membrane (Millipore) and blocked overnight at 4°C in 10% nonfat dried milk in PBS. Membranes were incubated for 1 hr at RT with either the anti-dynamin 2 antibody Dyn2 (Henley et al., 1998) or the anti-V5 antibody as indicated in the manuscript, washed three times for 15 min in TBS/Tween, and incubated in a 1/10,000 dilution of peroxidase-conjugated secondary antibody (Cappel). After washing three times for 15 min in TBS/Tween, specific binding was detected using chemiluminescence (Amersham).

### Cell Surface Staining

Cells to be stained for FcγRII and FcγRIII were resuspended into FACS buffer (PBS, 2% FCS, 0.5 mM azide), while cells to be stained for Mac-1 were resuspended into 2.4G2 supernatant (ATCC) and incubated 15 min on ice. Primary antibody (biotinylated 2.4G2 for

FcR staining or biotinylated anti-mouse CD11b antibody, both from PharMingen) was added, and the cells were incubated on ice for 20 min. Cells were washed in FACS buffer, resuspended in diluted streptavidin-PE (Caltag), and incubated on ice for 15 min. The cells were washed, resuspended in FACS buffer with 1% paraformaldehyde, and analyzed on a FACScan (Becton Dickinson).

### Transfections

COS7 cells (ATCC) were cotransfected with pSFFVeGFP and the amphiphysin I, IIm, or SH3P9 in pcDNA3.1 at a 1:10 ratio using the calcium phosphate method. Forty-eight hours after transfection, the cells were prepared and stained (as described above) and analyzed by confocal microscopy.

RAW-TT10 cells were transiently transfected by electroporation. DNA (10 μg) was added to $5 \times 10^6$ RAW-TT10 cells in 250 μl of RPMI (JRH Biosciences) with 10% heat-inactivated FCS (Hyclone). The cells were electroporated at 280 volts, capacitance 960 μFd, and immediately washed in 5 ml RPMI with 10% FCS. The cells were plated and analyzed 18–24 hr later by FACS or confocal microscopy. In the experiments reported here, tetracycline was always absent from the media, resulting in strong activity of the tetracycline-regulated promoter.

### Phagocytosis Assay

TRITC-zymosan was purchased from Molecular Probes. TRITC-labeled SRBC "ghosts" were prepared by incubating SRBCs (ICN/Cappel) in hypotonic lysis buffer (1 mM $MgCl_2$, 100 mM EGTA in 0.02× PBS) with TRITC-BSA (Molecular Probes) on ice for 1 hr. Isotonicity was restored to the cells with 5× PBS, and the ghosts were resealed at 37°C for 1 hr. Unincorporated TRITC-BSA was removed by washing in PBS, and the ghosts were opsonized as described above.

The specified particles were centrifuged onto the transiently transfected RAW-TT10 cells at 1600 rpm and 4°C for 1 min. (Prior to exposure to C3bi-opsonized particles cells, were treated with 200 nM PMA for 30 min.) The cells were incubated at 37°C for 10 min. The extracellular particles were removed; ghosts were lysed with a 20 s water wash, TRITC-zymosan was digested for 10 min with 100 μ/ml lyticase (Sigma). The cells were resuspended in PBS/EDTA, fixed with 1% formalin, and analyzed by FACS.

### Scanning Electron Microscopy

Eighteen hours after transfection, cells expressing high levels of the indicated proteins were sorted onto thermonox coverslips (Nunc) using a FACStar plus (Becton Dickinson). Cells were adhered for 3 hr at 37°C, then incubated with IgG-opsonized SRBCs for the indicated amount of time. Cells were fixed in 3% glutaraldehyde in EM buffer (0.1 M cacodylate, 0.1 M sucrose) at RT for 1 hr, then washed with EM buffer. The cells were post-fixed in 1% $OsO_4$ in 0.1 M cacodylate, 4 mM CaCl [pH 7.3] for 30 min at RT. The cells were dehydrated through serial changes in ethanol (35%, 50%) for 5 min each, en bloc stained in 3% uranyl acetate 7and 0% ethanol for 30 min, and ethanol dehydration was completed (80%, 90%, 95%, 100%, 100%, each for 5 min). The coverslips were critical point dried, mounted onto scanning stubs, and air dried overnight. Cells were sputter coated with 30 nm gold/palladium and examined on a Jeol JSM-6300F SEM.

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