The Serine/Threonine Kinase Akt Promotes Fcγ Receptor-mediated Phagocytosis in Murine Macrophages through the Activation of p70S6 Kinase*

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Fcγ receptor (FcγR) clustering by immune complexes activates multiple signaling pathways leading to phagocytosis. We and others have previously reported that Akt is phosphorylated in response to FcγR clustering. However, the functional consequence of Akt activation by FcγR is not known. Using Raw 264.7 macrophage cells transfected to overexpress either constitutively active myristoylated (Myr)-Akt or a dominant-negative CAAX-Akt and bone marrow macrophages (BMMs) from wild-type and transgenic mice expressing macrophage-specific Myr-Akt, we analyzed the function of Akt in phagocytosis. We report that overexpression of Myr-Akt resulted in significant increase in phagocytic efficiency, whereas CAAX-Akt down-regulated phagocytosis in Raw 264.7 cells. Likewise BMMs expressing Myr-Akt displayed enhanced phagocytic ability. Analyzing the downstream effectors of Akt, we demonstrate that p70S6 kinase is constitutively phosphorylated in Myr-Akt-expressing BMMs. p70S6 kinase is reported to influence actin cytoskeleton and cell migration, suggesting that Akt may influence phagocytosis through the activation of p70S6 kinase. Consistent with this, overexpression of either wild-type or constitutively active but not a kinase-inactive p70S6 kinase in Raw 264.7 cells significantly enhanced phagocytosis. Likewise suppression of p70S6 kinase with rapamycin down-regulated phagocytic efficiency conferred by the expression of constitutively active Akt. These findings demonstrate a novel role for Akt in phagocytosis through the activation of p70S6 kinase.

IgG immune complexes (ICs)1 are cleared by monocytes/macrophages via Fcγ receptors by a process termed phagocytosis (1). Murine macrophages express two classes of activating FcγRs, FcγRI and FcγRIIIa, which are associated with the low molecular weight y-subunit bearing a tyrosine-based activation motif (2). Upon encountering an immune complex FcγRs on macrophages are clustered and the immunoreceptor tyrosine-based activation motifs (ITAMs) are phosphorylated by the membrane-associated Src kinases (3, 4). The phosphorylated ITAMs recruit several signaling enzymes and adapter-enzyme complexes, activating a cascade of signaling events that culminates in phagocytosis and generation of inflammatory mediators. In addition these macrophages also express the inhibitory FcγRII(b) that contains a tyrosine-based inhibitory motif (ITIM), which when phosphorylated recruits inositol phosphates to down-regulate the phagocytic response (5–8).

PtdIns 3-kinase is a critical enzyme that is recruited by the phosphorylated ITAMs tifs of FcγR and generates 3' phosphoinositol lipids including PtdIns 3,4,5-trisphosphate (9–12). The generation of PtdIns 3,4,5-trisphosphate is necessary for the recruitment and activation of pleckstrin homology domain-containing enzymes such as Vav, the guanine nucleotide exchange factor for Rac that promotes cytoskeletal changes necessary for phagocytosis. Inhibition of PtdIns 3-kinase function, either directly or by blocking upstream molecules such as Gab2 that is necessary for membrane recruitment of PtdIns 3-kinase (13), has been shown to abrogate phagocytosis.

Protein kinase B/Akt is a pleckstrin homology domain-containing, serine/threonine kinase that is also activated by PtdIns 3-kinase products and is often used as an indicator of PtdIns 3-kinase activation. Kinase activity of Akt is turned on following membrane translocation of Akt, mediated by the binding of its pleckstrin homology domain with PtdIns 3,4,5-trisphosphate and phosphorylation of Akt on threonine and serine residues. It is generally accepted that threonine phosphorylation of Akt is the function of PDK-1 (14, 15). The mechanism by which serine 473 of Akt is phosphorylated is somewhat controversial. Thus, some studies suggest that Akt undergoes autophosphorylation at Ser-473, while others suggest a distinct kinase(s) (PDK-2) (16–20). Nevertheless forced expression of Akt using membrane-targeting signals results in constitutive activation of Akt. Two such membrane-targeted Akt constructs have been reported: one a viral gag fusion designated gag-protein kinase B and another containing the Src myristoylation signal designated myristoylated Akt (Myr-Akt) (21, 22). Both these membrane-targeted versions display ligand-independent serine and threonine phosphorylation and enzyme activity. It is thought that the upstream kinases that are required for phosphorylation and activation of Akt may have some basal activity that is sufficient to activate the membrane-targeted forms of Akt (14, 23, 24). Activated Akt has been shown to influence a variety of cellular functions including cell survival,
cell cycle regulation, NFκB-dependent gene transcription, and actin remodeling and cell migration through the activation of ribosomal p70S6 kinase (24–26).

We and others have previously demonstrated that Akt is phosphorylated in response to FcγR clustering in myeloid cells (7, 13). However, it is not known whether there is a functional consequence to Akt activation by FcγR clustering. In this study we have examined the details of Akt activation during FcγR-mediated activation of macrophages including the kinetics of Akt activation and the functional relevance of this activation. We report that serine and threonine phosphorylation and enzymatic activity of Akt are induced after FcγR clustering. In this study we have examined the details of Akt activation during FcγR-mediated activation of macrophages including the kinetics of Akt activation and the functional relevance of this activation. We report that serine and threonine phosphorylation and enzymatic activity of Akt are induced after FcγR clustering.

**Fig. 1.** FcγR clustering induces Akt activation in murine BMMs. Murine bone marrow macrophages were serum-starved overnight and subsequently stimulated for the time points indicated in the figure with heat-aggregated IgG (IC). A, WCLs were analyzed by Western blotting with phospho-specific Akt antibodies. The same membranes were reprobed with Akt antibody. The lower panel represents fold induction of phospho-Akt in the activated samples over the resting sample. Here phospho-Akt signals were first normalized to total Akt present in each lane. Values obtained from three independent experiments are represented as mean ± S.D. B, WCLs were analyzed with phosphothreonine Akt antibody (upper panel). The middle panel is a reprobe of the same membrane with Akt antibody. The lower panel is a quantitation of Akt threonine phosphorylation as described above. C, Akt immunoprecipitates were used in *in vitro* kinase assays with GSK-3 as the substrate. Phosphorylation of GSK-3 was detected using phospho-GSK-3 antibody (upper panel). The middle panel is a reprobe with Akt antibody. IgH and IgL stand for the heavy chain and light chain of the immunoprecipitating/activating antibodies, respectively. Phospho-GSK-3 signals were normalized to total Akt in each lane. The graph represents mean ± S.D. of values obtained from three independent experiments.

**MATERIALS AND METHODS**

**Cells, Antibodies, and Reagents**

Raw 264.7 murine macrophage cells were obtained from ATCC and maintained in RPMI 1640 medium containing 3.5% fetal bovine serum. Actin and Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All phosphospecific antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse CD16/32 (FcγRIII) was purchased from BD Pharmingen. p70S6 kinase plasmids were a generous gift from Dr. J. Blenis (Harvard Medical School, Boston, MA). Plasmids encoding Myr-Akt and CAAX-Akt were kindly provided by Dr. D. Stokoe (University of California, San Francisco, CA) and Dr. Burgerting (Utrecht University, Utrecht, Netherlands), respectively. Rabbit polyclonal antibodies specific for murine FcγRII were kindly provided by Dr. J. C. Cambier (National Jewish Medical and Research Center, Denver, CO).

**Generation of Myr-Akt Transgenic Mice**

Transgenic mice with macrophage-specific expression were generated by methods described previously (27). The Myr-Akt-HA plasmid used for this purpose was a kind gift from Dr. P. Tsichlis (Thomas Jefferson University, Philadelphia, PA). The fms-Myr-Akt plasmid was constructed such that the GFP cassette between Apal and NotI site of Δ6.7msGFP plasmid described previously (24) was replaced by Myr-Akt-HA. The fms-Myr-Akt was linearized with SalI and PvuI, and fms-Myr-Akt-HA plasmid used for this purpose was a kind gift from Dr. J. Blenis (Harvard Medical School, Boston, MA). p70S6 kinase plasmids were a generous gift from Dr. J. Blenis (Harvard Medical School, Boston, MA).

**Culture of Murine Bone Marrow Macrophages**

BMMs were derived as described previously (28). Briefly bone marrow cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and supplemented with 20 ng/ml colony-stimulating factor-1 for 7 days. BMMs were activated by clustering FcγRs with heat-aggregated IgG for varying time points.

**Preparation of Heat-aggregated IgG**

Heat aggregated IgG was prepared according to methods described previously (28). In brief Chromopure mouse IgG at a concentration of...
FIG. 2. Akt promotes FcγR-mediated phagocytosis. A, Raw 264.7 cells were transiently transfected with 5 μg of plasmid encoding EGFP. Transfectants were analyzed for the expression of EGFP by fluorescence microscopy. B, Raw 264.7 cells were transfected with the plasmids indicated in the figure (1 μg of EGFP along with 5 μg of either vector alone, Myr-Akt, or CAAX-Akt). The transfectants were analyzed for their ability to phagocytose IgG-coated SRBCs by counting the total number of SRBCs ingested by 100 GFP-positive cells. Values obtained from three independent experiments are presented as mean and S.D. C, protein-matched WCLs from the transfectants were analyzed by Western blotting with phospho-Akt antibody (upper panel) and with phosphoserine Akt antibody (second panel). Akt immunoprecipitates were assessed for their ability to phosphorylate GSK-3 in an in vitro kinase assay (third panel), and expression of total Akt was assessed by Western blotting with Akt antibody (lower panel). These results are representative of three independent experiments. Transf, transfection; IB, immunoblot; pGSK3, phospho-GSK-3; IVK, in vitro kinase assay; C, control immunoprecipitate.

750 μg/ml was heated at 62°C for 30 min and then cooled on ice immediately and used directly to stimulate the cells.

Cell Stimulation, Lysis, Immunoprecipitation, and Western Blotting

Raw 264.7 cells and BMMs were activated by clustering FcγRs with heat-aggregated IgG. Resting and activated cells were lysed in TN1 buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM Na₃P₂O₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, and 10 μg/ml each aprotinin and leupeptin), and postnuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Invitrogen). Immune complexes bound to beads were washed two times with lysis buffer and boiled in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the antibody of interest, and developed by enhanced chemiluminescence.

In other experiments Raw 264.7 cells were stimulated with IgG-coated SRBCs. Here the cells were mixed with IgG-coated SRBCs, centrifuged briefly to increase contact between the Raw 264.7 cells and the SRBCs, and placed at 37°C. Stimulation was terminated at varying time points by lysing in TN1 buffer. Cell lysates were then either subjected to immunoprecipitation with Akt antibodies for Akt kinase assays as described below or boiled in SDS sample buffer and analyzed by Western blotting.

Western Blot Data Quantitation

The enhanced chemiluminescence signal was quantitated using a scanner and a densitometry program (Scion Image). To quantitate the phosphospecific signal in the activated samples, we first subtracted background, normalized the signal to the amount of actin or total target protein in the lysate, and plotted the values as -fold increase over unstimulated samples as described previously (28).

Akt in Vitro Kinase Assay

Cells were incubated for 10 min in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). By centrifuging at 13,000 rpm for 10 min the lysates were cleared of nuclei. The amount of protein in the lysates was estimated. Equal amounts of the protein extracts were immunoprecipitated with Akt antibody. Immunoprecipitations with control antibodies were also done. The immune complexes bound to beads were washed two times with lysis buffer and two times with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 10 mM MgCl₂) and subsequently incubated with 40 μl of kinase buffer supplemented with 20 μM ATP and 1 μg of GSK-3 fusion protein as a substrate. The reaction was carried out for 30 min at 30°C. The reaction was stopped by boiling in SDS sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min, and proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose filters, probed with phospho-GSK-3 antibody, and developed by enhanced chemiluminescence.

Transfection

To study the influence of Akt and p70S6 kinase on phagocytosis, Raw 264.7 cells were transfected using the Nucleofector (Amazka Biosystems) according to the manufacturer’s recommendations. In brief, 3 × 10⁵ cells in 100 μl of Solution V (“Kit V,” Amazka Biosystems) at room temperature were mixed with 5 μg of the Akt constructs or the p70S6 kinase constructs along with 1 μg of EGFP-encoding plasmids. The cells were transfected...
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FIG. 3. Bone marrow macrophages expressing constitutively active Akt display enhanced phagocytic ability. A, wild-type and Myr-Akt-expressing BMMs were serum-starved overnight and then stimulated for the time points indicated in the figure with heat-aggregated IgG. Protein-matched WCLs were analyzed by Western blotting with phosphothreonine Akt antibody (upper panel). The same membrane was reprobed with actin antibody (lower panel). B, phagocytic efficiency of wild-type and Myr-Akt-expressing BMMs was assessed by counting the number of IgG-coated SRBCs ingested by 100 macrophages. The graph represents values obtained from three independent experiments. Statistical analysis was performed using Student’s t test (p value, 0.0007). C, wild-type and Myr-Akt-expressing BMMs were analyzed by flow cytometry for the expression of FcγRII/III using monoclonal antibody 2.4G2 (panels on the left) and for FcγRI expression using pooled human IgG (panels on the right). The dashed lines represent labeling with secondary antibody alone. Solid lines represent labeling with the primary antibodies followed by the secondary fluorescein isothiocyanate-conjugated antibody. D, 2.4G2 immunoprecipitates from wild-type and Myr-Akt-expressing BMMs were probed with anti-murine FcγRII (mFcγRII) antibody. IB, immunoblot; IP, immunoprecipitation; pThr, phosphothreonine; ′, minutes; R, resting.

Flow Cytometry Analysis of FcγR Expression

Analysis of FcγRII/III Expression—BMMs were tested for expression of FcγR by incubating with anti-murine FcγRI/II/III monoclonal antibody 2.4G2 at a concentration of 10 μg/ml for 30 min at 4 °C. The cells were washed and incubated with fluorescein isothiocyanate-labeled mouse F(ab′)2 anti-rat Ig secondary antibody for 30 min at 4 °C.

Analysis of FcγRI Expression—BMMs were incubated with 100 μg/ml pooled human IgG for 30 min at 4 °C. Unbound antibody was washed off. Cells were incubated with F(ab′)2 fragments of anti-human IgG for 30 min at 4 °C. All cells were subsequently washed, fixed in 1% paraformaldehyde, and analyzed by flow cytometry on an Elite EPICS fluorescence-activated cell sorter (Coulter, Hialeah, FL). Data from 10,000 cells/condition were recorded to yield the percentage of cells expressing receptors.

Preparation of IgG-coated Sheep RBCs

Sheep RBCs (Colorado Serum, Denver, CO) were washed in phosphate-buffered saline and labeled with PKH26 Red. Labeled cells were then washed in phosphate-buffered saline and incubated with a subagglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37 °C for 1 h. Unbound IgG was removed by washing the cells with phosphate-buffered saline.

Phagocytosis Assays

The IgG-coated SRBCs described above were added to BMMs or transfected Raw 264.7 cells. The cells were pelleted by low speed centrifugation to increase contact between SRBCs and phagocytes. The samples were incubated for 1 h at 37 °C to study phagocytosis. Cells were then subjected to brief hypotonic lysis with water to get rid of externally bound RBCs prior to fixation in paraformaldehyde to be viewed under a fluorescence microscope. That the phagocytosis was via Fc receptors was confirmed by the lack of ingested particles observed in samples incubated with fluoresceinated RBCs that were not opsonized with IgG. Phagocytosis was measured by counting the total number of RBCs ingested by 100 transfectants (GFP-positive) or BMMs. In all experiments, the percentage of cells actually phagocytosing was between 35 and 45%.

All experiments were repeated at least three times. Statistical analysis was performed using the Student’s t test, setting a p value of ≥0.05 as significant.

RESULTS

FcγR Clustering Induces Akt Activation in Murine Bone Marrow-derived Macrophages—We have previously reported that FcγRIIa clustering in human monocytes cells and FcγRII/II clustering in murine macrophages induces serine phosphorylation of Akt (7, 29). To test whether clustering of FcγR by ICs would likewise induce serine phosphorylation of Akt as well as to determine whether Akt kinase activity is induced, murine BMMs were stimulated for varying time points with heat-aggregated IgG, and the following analyses were performed. First serine phosphorylation of Akt was assessed by Western blotting protein-matched whole cell lysates (WCLs), from resting and activated BMMs, with phospho-Ser Akt antibody (Fig. 1A, upper panel). Results indicated that serine phosphorylation
Akt Promotes FcγR-mediated Phagocytosis—Having established methods to efficiently transfect Raw 264.7 cells, we then examined the functional consequence of Akt activation on FcγR-mediated phagocytosis. To test the functional consequence of Akt activation on FcγR-mediated phagocytosis a novel transfection method was standardized to achieve efficient transfection in Raw 264.7 cells. Here Raw 264.7 cells were transfected with 5 μg of a plasmid encoding EGFP in Solution V using the Amaxa Biosystems Nucleofector program U-14. Transfectants were harvested 24 h later and analyzed by flow cytometry. Results indicated that 63% of the cells were GFP-positive. Shown in Fig. 2A is a fluorescence image of the transfected cells. This strategy represents an important breakthrough in our capacity to introduce plasmid DNA into myeloid cell lines.

Akt Promotes FcγR-mediated Phagocytosis—Having established methods to efficiently transfect Raw 264.7 cells, we then examined the functional consequence of Akt activation by FcγR clustering. Recent studies have indicated that Akt promotes cytoskeletal changes and cell migration through the activation of p70S6K (30). Since actin cytoskeletal changes are critical for phagocytosis, we asked whether Akt likewise influenced FcγR-mediated phagocytosis. In these experiments Raw 264.7 cells were transiently co-transfected with 5 μg of plasmids encoding Myr-Akt, CAAX-Akt, or vector alone along with 1 μg of a plasmid encoding EGFP. Myr-Akt is constitutively anchored in the plasma membrane and is active, whereas CAAX-Akt, which is also anchored in the membrane due to the membrane-targeting signal of Ki-Ras, the CAAX motif, functions as a dominant-negative (31). GFP-positive cells were assessed for their ability to phagocytose IgG-coated, PKH26 Red-labeled SRBCs. A total of 100 GFP-positive cells were analyzed in each experiment. Results from three independent experiments indicated that overexpression of Myr-Akt significantly enhanced phagocytic efficiency, while the overexpression of CAAX-Akt dampened the same (Fig. 2B).

The above transfectants were also tested in each experiment for the expression of the Akt constructs used. Western blotting of protein-matched WCLs from the transfectants indicated that indeed Myr-Akt was constitutively phosphorylated on serine/threonine residues and was kinase-active, whereas CAAX-Akt was not (Fig. 2C). Western blotting with total Akt antibody indicated that both Myr-Akt and CAAX-Akt were expressed at equivalent levels. Taken together these data suggest that Akt is an upstream regulator of FcγR-mediated phagocytosis. To examine whether the increase in FcγR function in Myr-Akt-expressing Raw 264.7 cells could be due to a change in FcγR expression flow cytometry and Western blot analyses were performed. Results indicated that overexpression of either Myr-Akt or CAAX-Akt had no influence on the expression levels of FcγR (data not shown).

Transgenic BMMs Expressing Constitutively Active Akt Display Enhanced FcγR-mediated Phagocytosis—The above experiments were performed in a transiently transfected macrophage cell line. Thus, to examine the effects of Akt more thoroughly in primary cells, we next used BMMs isolated from wild-type and transgenic mice with macrophage-specific expression of Myr-Akt. The expression of the Myr-Akt transgene was confirmed by Western blotting cell lysates with Ser(P) Akt antibody. The results shown in Fig. 3A demonstrate that IC stimulation of BMMs results in serine phosphorylation of endogenous Akt in both wild-type and Myr-Akt-expressing BMMs. In contrast, Myr-Akt was constitutively serine phosphorylated in the transgenic BMMs. These BMMs were then used in phagocytosis assays as described above. The results shown in Fig. 3B indicate that Myr-Akt-expressing BMMs display significantly enhanced phagocytic ability. Notably only 35–45% of the cells were ingesting the SRBCs in both wild-type and Myr-Akt-expressing BMMs. These results indicate that macrophages expressing constitutively active Akt were able to ingest a greater number of IgG-coated SRBCs/phagocyte.
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To test whether the enhanced phagocytic ability could be explained by a difference in FcγR in the two genotypes of macrophages, FcγR expression was measured. Flow cytometry analyses using the anti-murine FcγRIII/II monoclonal antibody 2.4G2 demonstrated that both wild-type and Myr-Akt-expressing BMMs express equivalent levels of Fcγ receptors (Fig. 3C). Since 2.4G2 recognizes both FcγRII and -III, Western blotting analysis was performed on 2.4G2 immunoprecipitates using antibodies specific for FcγRII. The results shown in Fig. 3D indicate that FcγRII expression is equivalent in both cell types. Likewise FcγRII expression levels measured by the binding of human IgG to these high affinity receptors indicated no gross differences in the expression levels between wild-type and Myr-Akt-expressing cells (Fig. 3C). Taken together these results indicate that Akt enhances the ability of macrophages to ingest IgG-coated particles.

Myr-Akt-expressing Cells Have Constitutively Phosphorylated p70S6 Kinase—p70S6 kinase is a downstream effector of Akt that has been shown previously to influence cytoskeletal changes and cell migration (30). To test the hypothesis that Akt promotes phagocytosis through the activation of p70S6 kinase, p70S6 kinase phosphorylation was assessed in wild-type and Myr-Akt-expressing BMMs. Thus whole cell lysates from resting and IC-stimulated BMMs were analyzed by Western blotting with phospho-p70S6 kinase antibody (Fig. 4A, upper panel). Results indicated that p70S6 kinase phosphorylation is induced by IC stimulation in wild-type BMMs. In contrast p70S6 kinase phosphorylation levels were constitutively elevated in Myr-Akt-expressing BMMs. The same membranes were reprobed with actin antibodies to ensure equal loading of protein in all lanes (Fig. 4A, middle panel). The lower panel is a quantitative measure of p70S6 kinase phosphorylation normalized to actin in each lane and expressed as fold increase over the phosphorylation in the resting, wild-type BMMs.

In parallel experiments phosphorylation of extracellular signal-regulated kinase in response to IC stimulation was measured in these cells. The results shown in Fig. 4B indicate that extracellular signal-regulated kinase phosphorylation is not constitutively elevated in Myr-Akt-expressing BMMs, thus establishing the specificity of Akt influence on p70S6 kinase.

Akt and p70S6 Kinase Are Activated during Phagocytosis—Our experiments demonstrate that FcγR clustering using heat-aggregated IgG results in the activation of Akt and p70S6 kinase. To test whether Akt and p70S6 kinase are activated in macrophages actively phagocytosing IgG-coated SRBCs, Raw 264.7 cells were allowed to phagocytose IgG-coated SRBCs for varying time points. Reactions were terminated by adding lysis buffer. Serine phosphorylation of Akt was analyzed by Western blotting protein-matched lysates with phosphoserine Akt antibody (Fig. 5A, upper panel). The same membranes were reprobed with Akt antibody (lower panel) to ensure equal loading in all lanes. Likewise Akt kinase activity was assessed in an in vitro kinase assay as described above (Fig. 5B), and phosphorylation of p70S6 kinase was assessed by Western blotting protein-matched lysates with phospho-p70S6 kinase antibody (Fig. 5C). Results from three independent experiments indicated that both Akt and p70S6 kinase are activated very early during the phagocytic process. Activation of these kinases is maximal at 5 min postincubation with IgG-coated SRBCs.

p70S6 Kinase Promotes FcγR-mediated Phagocytosis—The above findings suggest that Akt may influence FcγR-mediated phagocytosis through the activation of p70S6 kinase. Akt is reported to promote the activation of p70S6 kinase through the activation of mTOR, which phosphorylates p70S6 kinase (32). However, a role for p70S6 kinase in phagocytosis has not been established. Thus, to formally test whether p70S6 kinase could influence phagocytosis, Raw 264.7 cells were transiently transfected with 5 μg of empty vector, wild-type p70S6 kinase, kinase-inactive p70S6 kinase (100R p70S6K), or constitutively active p70S6 kinase (CA p70S6K) along with 1 μg of a plasmid encoding EGFP. All p70S6 kinase constructs were HA-tagged. CA p70S6K is a truncation mutant that lacks the C-terminal region (33), whereas 100R is kinase-inactive due to mutation of lysine to arginine in the catalytic domain (34). Phagocytic abil-
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Akt Influence on Phagocytosis Is through the Activation of p70S6 Kinase—The above experiments demonstrate that both Akt and p70S6 kinase promote FcγR-mediated phagocytosis. To test whether the influence of Akt on phagocytosis is mediated through the activation of p70S6 kinase, the influence of rapamycin on the ability of Myr-Akt to promote phagocytosis was next examined. Thus, Raw 264.7 cells were co-transfected with 5 μg of vector alone or plasmids encoding Myr-Akt or CA-Akt along with 1 μg of EGFP. The transfectants were harvested 24 h later, pretreated for 1 h with Me2SO or 20 nM rapamycin, and subsequently analyzed for their ability to phagocytose IgG-coated SRBCs. Results from three independent experiments are shown in Fig. 7C. Rapamycin treatment significantly down-regulated phagocytic efficiency of cells transfected with vector alone or with wild-type p70S6K. CA p70S6K is not sensitive to rapamycin as has been previously reported (33). In parallel experiments, expression of the p70S6K constructs and the effect of rapamycin on their phosphorylation were assessed by Western blotting protein-matched lysates from the transfectants with antibodies for phospho-p70S6K and p70S6K (Fig. 7D). The expression of CA p70S6K was confirmed by Western blotting anti-HA immunoprecipitates with anti-HA antibody as described in Fig. 5C (data not shown). Taken together, these findings establish for the first time a role for p70S6 kinase in FcγR-mediated phagocytosis.

As an additional approach to test the influence of p70S6 kinase on FcγR-mediated phagocytosis the following experiments were performed with cells treated with the mTOR inhibitor rapamycin. First the specificity of rapamycin was tested. Here Raw 264.7 cells were treated for 1 h with 20 nM rapamycin prior to stimulation with heat-aggregated IgG. Control cells were treated with Me2SO. Protein-matched lysates from resting and activated cells were analyzed with antibodies specific for phosphorylated p70S6 kinase (Fig. 7A). As seen in the figure, p70S6 kinase phosphorylation was detected in the Me2SO-treated, stimulated cells, whereas no p70S6 kinase phosphorylation was seen in cells treated with rapamycin. To ensure that the inhibitory effect of rapamycin was specific to p70S6 kinase, upstream signaling events such as Akt phosphorylation were assessed in parallel samples. The results shown in Fig. 7B indicate that rapamycin treatment did not influence Akt phosphorylation at the dose used to inhibit p70S6 kinase phosphorylation.

Second the influence of rapamycin on phagocytosis was examined. Here Raw 264.7 cells were transfected with 5 μg of vector alone or plasmids encoding wild-type, kinase-inactive, or CA p70S6K along with 1 μg of EGFP. Transfectants were harvested 24 h later, pretreated for 1 h with Me2SO or 20 nM rapamycin, and subsequently analyzed for their ability to phagocytose IgG-coated SRBCs. Results from three independent experiments are shown in Fig. 6A. In contrast, overexpression of kinase-inactive p70S6 kinase led to enhanced phagocytic efficiency (Fig. 6A). In contrast, overexpression of kinase-inactive p70S6 kinase led to down-regulation of phagocytosis. In all cases the total number of macrophages ingesting the SRBCs was not significantly different.

In parallel, overexpression of transfected p70S6 kinase proteins was assessed by Western blotting (Fig. 6B). Note that constitutively active p70S6K is not detected by the p70S6K antibody used. This is due to the fact that the antibody used was raised against the C terminus of p70S6K, which is deleted in the CA construct. Therefore, as an alternate method to ensure that CA p70S6K was indeed expressed anti-HA immunoprecipitates were probed with anti-HA antibody (Fig. 6C). These results indicate that p70S6 kinase promotes FcγR-mediated phagocytosis.

Finally, to test whether the effects of Akt on phagocytosis are...
A. DMSO Rapamycin

|   | 5' | 15' | 30' |   | 5' | 15' | 30' |
|---|----|-----|-----|---|----|-----|-----|
| R | p70S6K |  |
| WCL | IB: Anti-phospho p70S6K |  |
| IB: Anti-p70S6K |  |

B. DMSO Rapamycin

|   | 5' | 15' | 30' |   | 5' | 15' | 30' |
|---|----|-----|-----|---|----|-----|-----|
| R | Akt |  |
| WCL | IB: Anti-pSer Akt |  |
| IB: Anti-Akt |  |

**FIG. 7. Rapamycin reverses the enhancing effect of p70S6K on FcγR-mediated phagocytosis.** Raw 264.7 cells were treated with MeSO (DMSO) or 20 nM rapamycin for 1 h at 37 °C prior to stimulation with heat-aggregated IgG. A, protein-matched lysates were analyzed by Western blotting with phospho-p70S6K antibody (upper panel). The lower panel is a reprobe of the same membrane with anti-p70S6K antibody. B, parallel samples were probed with anti-Ser(P) Akt antibody (upper panel), and the membrane was reprobed with anti-Akt antibody (lower panel). C, Raw 264.7 cells were transiently co-transfected with 5 μg of vector alone, wild-type p70S6 kinase, kinase-inactive p70S6K (100R), or constitutively active p70S6K (CA) along with 1 μg of plasmid encoding EGFP. Transfectants were harvested 24 h later, pretreated for 1 h with 20 nM rapamycin or MeSO. Transfectants were assessed for their ability to phagocytose IgG-coated SRBCs. The graph represents mean and S.D. of values obtained from three independent experiments. D, protein-matched lysates from the transfectants were analyzed by Western blotting with phospho-p70S6K antibody (upper panel). The membranes were reprobed with p70S6K antibody. D, MeSO; R, rapamycin. Note that the CA p70S6K is a C-terminal truncation mutant that lacks the phosphorylation site. CA p70S6K is not recognized by either phospho-p70S6K antibody or the p70S6K antibody. Expression of this construct was verified by anti-HA Western blots of anti-HA immunoprecipitates as shown in Fig. 5C. Wt, wild type; IB, immunoblot; ′, minutes; R, resting; pSer, phosphoserine.

primarily mediated via p70S6K, Raw 264.7 cells were co-transfected to express both CAAX-Akt and CA p70S6K. The transfectants shown in Fig. 8E were analyzed for their phagocytic ability. The results shown indicate that cells expressing CA p70S6K have significantly enhanced phagocytic ability in comparison to vector-transfected cells (p value, 0.02). The presence of CAAX-Akt did not diminish the enhanced phagocytic ability due to CA p70S6K (p value, 0.38), suggesting that Akt is indeed upstream of p70S6K.

**DISCUSSION**

In recent work we and others have reported that Akt is phosphorylated on serine 473 in response to FcγR clustering in myeloid cells (7, 13, 35). In these studies Akt serine phosphorylation was merely used as an indicator of PtdIns 3-kinase activation by FcγR. It was not determined whether threonine phosphorylation of Akt and Akt kinase activity were likewise induced under these conditions. More importantly, there have been no studies examining whether phosphorylation of Akt in response to FcγR clustering has any functional consequence. Thus, in this study we examined in detail the kinetics of Akt activation in response to FcγR clustering and the influence of Akt on FcγR-mediated functional outcomes. We report that IC stimulation of macrophages induces serine/threonine phosphorylation of Akt along with its kinase activity. Our studies also demonstrate that Akt promotes FcγR-mediated phagocytosis working through the downstream effector p70S6 kinase.

Akt is a serine/threonine kinase that has been well investigated by several groups. It has been shown to play a role in a multitude of cellular functions such as cell survival, gene transcription, and more recently in actin remodeling (24). Thus, several studies have demonstrated that Akt is sufficient for the induction of NFκB-dependent gene transcription in response to platelet-derived growth factor and tumor necrosis factor (36–38). In other studies, Kane et al. (39) demonstrated that Akt enhances the degradation of IκBα to promote NFκB activation. Akt has also been proposed to phosphorylate and activate IKKα (37). Recent studies have revealed that Akt is an upstream regulator of the mTOR/p70S6 kinase pathway (32). Clearly Akt is a multifaceted enzyme and influences cellular events in a variety of ways that are not yet fully understood.

Our observations that activated Akt promotes phagocytosis are novel and unexpected. Therefore, we first analyzed whether the increased phagocytic efficiency of Myr-Akt-expressing macrophages could simply be explained by an influence on the expression levels of FcγR, mediated secondarily by potential cytokine induction by activated Akt. This latter, however, is an unlikely possibility since a 1-h rapamycin pretreatment reverses the effects of Myr-Akt. Consistent with this notion, flow cytometry and Western blot analyses revealed that the presence of Myr-Akt does not influence FcγR expression. Cox et al. (11) previously reported that a PtdIns 3-kinase-dependent event causes increased cell surface membrane area, which allows for enhanced pseudopod extension and phagocytosis of larger opsonized particles. An unidentified pleckstrin homology protein-matched lysates from the transfectants were analyzed by Western blotting with phospho-p70S6K antibody (upper panel). The membranes were reprobed with p70S6K antibody. D, MeSO; R, rapamycin. Note that the CA p70S6K is a C-terminal truncation mutant that lacks the phosphorylation site. CA p70S6K is not recognized by either phospho-p70S6K antibody or the p70S6K antibody. Expression of this construct was verified by anti-HA Western blots of anti-HA immunoprecipitates as shown in Fig. 5C. Wt, wild type; IB, immunoblot; ′, minutes; R, resting; pSer, phosphoserine.
domain-containing, PtdIns 3,4,5-trisphosphate-dependent molecule was proposed to be responsible for the latter effect. We speculate that Akt may be the unidentified molecule proposed by these investigators.

In recent studies a novel role for Akt has been proposed in actin remodeling and cell migration through the activation of the ribosomal kinase p70S6K (21, 30, 40–42). The 70-kDa ribosomal S6 kinase has been implicated in a number of cellular functions such as protein synthesis, cell cycle regulation, and neuronal cell differentiation (for a review, see Ref. 42). In addition, p70S6K appears to associate with Rac1 and Cdc42 and may thereby influence actin cytoskeleton (40, 42, 43). Consistent with these previous reports the data presented here indicate that Myr-Akt-expressing BMMs have constitutively high p70S6 kinase phosphorylation. In addition, overexpression of active p70S6 kinase, but not kinase-inactive p70S6 kinase, resulted in a significant enhancement of phagocytic efficiency. In further support of the Akt-p70S6 kinase influence on phagocytosis, rapamycin treatment of BMMs and Raw 264.7 cells resulted in suppression of phagocytosis even in the presence of overexpressed, constitutively active Akt. Taken together these results strongly suggest that Akt promotes phagocytosis through the activation of p70S6 kinase. Of note, inhibition of Akt using a dominant-negative Akt construct or inhibition of p70S6K activation using rapamycin resulted in reduced phagocytic efficiency but did not block phagocytosis. We propose that the Akt-p70S6K pathway is necessary only for efficient phagocytosis and that there may be other PtdIns 3-kinase-activated signaling pathways that play a role in phagocytosis.

The influence of PtdIns 3-kinase on FcγR-mediated phagocytosis has been examined by several groups. Most of these reports concur that PtdIns 3-kinase promotes phagocytosis at least through the activation of the low molecular weight GTP-binding protein Rac (44–46). Interestingly Rac is reported to be an upstream regulator of p70S6 kinase (30, 47). These previous observations combined with our current data suggest that the three downstream effectors of PtdIns 3-kinase, Akt, Rac, and p70S6 kinase, are all involved in the phagocytic process. It is not clear, however, whether these three molecules are on a linear pathway (Akt → Rac → p70S6 kinase) or whether Akt and Rac exert distinct influence on p70S6 kinase. We are currently investigating the relationship between Akt, p70S6 kinase, and Rac and the relative contribution of these molecules to FcγR-mediated phagocytosis. In summary, these data establish, for the first time, a novel role for Akt in enhancing FcγR-mediated phagocytosis through the activation of p70S6 kinase.

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The Serine/Threonine Kinase Akt Promotes Fc$\gamma$ Receptor-mediated Phagocytosis in Murine Macrophages through the Activation of p70S6 Kinase
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