Arrestin-2 and G Protein-coupled Receptor Kinase 5 Interact with NFκB1 p105 and Negatively Regulate Lipopolysaccharide-stimulated ERK1/2 Activation in Macrophages

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Toll-like receptors (TLRs) are a recently described receptor class involved in the regulation of innate and adaptive immunity. Here, we demonstrate that arrestin-2 and GRK5 (G protein-coupled receptor kinase 5), proteins that regulate G protein-coupled receptor signaling, play a negative role in TLR4 signaling in Raw264.7 macrophages. We find that lipopolysaccharide (LPS)-induced ERK1/2 phosphorylation is significantly enhanced in arrestin-2 and GRK5 knockdown cells. To elucidate the mechanisms involved, we tested the effect of arrestin-2 and GRK5 knockdown on LPS-stimulated signaling components that are upstream of ERK phosphorylation. Upon LPS stimulation, IκB kinase promotes phosphorylation and degradation of NFκB1 p105 (p105), which releases TPL2 (a MAP3K), which phosphorylates MEK1/2, which in turn phosphorylates ERK1/2. We demonstrate that knockdown of arrestin-2 leads to enhanced LPS-induced phosphorylation and degradation of p105, enhanced TPL2 release, and enhanced MEK1/2 phosphorylation. GRK5 knockdown also results in enhanced IκB kinase-mediated p105 phosphorylation and degradation, whereas GRK2 and GRK6 knockdown have no effect on this pathway. In vitro analysis demonstrates that arrestin-2 directly binds to the COOH-terminal domain of p105, whereas GRK5 binds to and phosphorylates p105. Taken together, these results suggest that p105 phosphorylation by GRK5 and binding of arrestin-2 negatively regulates LPS-stimulated ERK activation. These results reveal that arrestin-2 and GRK5 are important negative regulatory components in TLR4 signaling.

Pathogen-associated molecular patterns in microbes serve as ligands to activate a recently described class of pattern recognition receptors called Toll-like receptors (TLRs).13 TLRs have been identified to date, with TLR1, -2, -4, -5, -6, and -11 displayed on the cell surface and TLR3, -7, -8, and -9 localized intracellularly (1). Activation of TLR signaling constitutes one of the earliest responses of an organism to microbe invasion and understanding the signaling pathways stimulated by these TLRs is an area of intense interest. The TLRs as well as interleukin receptors have a conserved ~200-amino acid region in their cytoplasmic tails, known as the Toll/interleukin-1 receptor domain. Within the TIR domain are conserved regions that are crucial for signaling. After ligand binding, the TLRs dimerize and undergo conformational changes that are required for the recruitment of downstream signaling molecules (2). These include the adaptor molecule Myd88 (myeloid differentiation primary response protein 88), interleukin-1 receptor-associated kinases, TAK1 (transforming growth factor-β-activated kinase), TAK1-binding protein (TAB1 and -2), and TRAF-6 (tumor necrosis factor (TNF) receptor-associated factor 6) (3–6).

TLRs primarily activate two major signaling pathways, nuclear factor κ-B (NFκB) and mitogen-activated protein kinase (ERK, JNK, and p38) pathways. The NFκB pathway results in activation of transcription factors that are thought to act as a “master switch” for inflammation by regulating the transcription of genes involved in immunity and inflammation (7–9). The NFκB family of transcription factors includes homo- or heterodimers of p50 (a product of p105), p52 (a product of p100), p65 (RelA), c-Rel, and RelB. Activation of NFκB is controlled by an inhibitory subunit, inhibitor of NFκB (IκB), which retains the NFκB subunits in the cytosol. The IκB family of proteins includes IκBα, -β, and -ε, NFκB1 p105 (p105) (a precursor of p50), p100 (a precursor of p52), and Bcl3 (7). Upon activation by LPS and other agents, IκB is primarily phosphorylated by the IκB kinase (IKK) complex that results in the ubiquitination and degradation of the IκB proteins via the proteasomal pathway.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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5 The abbreviations used are: TLR, Toll-like receptor; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; pERK, phospho-ERK; JNK, c-Jun N-terminal kinase; IκB, inhibitor of NFκB; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; LPS, lipopolysaccharide; IKK, IκB kinase; HA, hemagglutinin; siRNA, small interfering RNA; GST, glutathione S-transferase; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase.
Degradation of these inhibitory proteins results in the release of the NFκB transcription factors, which then translocate into the nucleus to effect gene transcription (7).

The prototypical mitogen-activated protein kinase (MAPK) pathway involves a MAP3K that phosphorylates and activates a MAP2K that in turn phosphorylates and activates a MAPK. The most commonly studied MAPKs are ERK1/2, JNK1/2/3, and p38/MSK1/2 (10). These are activated in response to stimulation by extracellular signals, including ligands for TLRs, G protein-coupled receptors (GPCRs), tyrosine kinase receptors, and cytokine receptors. These kinases can regulate gene transcription by phosphorylating transcription factors in the nucleus as well as cytosolic components of the cell. In addition to the regulation of MAPKs by MAP3Ks, some of these MAP3Ks have been shown to play crucial roles in the activation of the NFκB pathway. The MAP3K transforming growth factor-β-activated kinase 1 (TAK1) has been shown to functionally interact with IKKs and thus regulate the NFκB pathway (11, 12). In addition, MKK6, ASK1, and members of the MLK3 (mixed lineage kinase-3) family have all been shown to regulate NFκB activation through interactions with upstream regulators of IκB phosphorylation (13–15). Thus, activation of MAP3Ks can result in stimulation of the NFκB pathway as well as the MAPK pathway.

Nonvisual arrestins (arrestin-2 and -3) are multifunctional adaptor/scaffolding proteins that regulate GPCR desensitization, trafficking, and signaling (16). Activation of GPCRs leads to a G protein-dependent as well as G protein-independent signaling (17, 18). This also results in the activation of a group of kinases called G protein-coupled receptor kinases (GRKs, GRK1–7) that phosphorylate residues in the intracellular domains of the GPCRs (19). This phosphorylation consequently results in arrestin binding that uncouples the receptor from G proteins (20, 21). Arrestin binding also recruits molecules, such as adaptins and clathrin, necessary for receptor internalization (22–24). In addition, arrestins can serve as a scaffold to regulate the RAF-MEK-ERK and ASK1-MKK-JNK pathways (16, 25, 26). More recent studies have shown that arrestins can also regulate NFκB pathways stimulated by TNF-α (27, 28) and LPS (29). Arrestins appear to interact with multiple components in the NFκB pathway, including TRAF6 (29), IKK (27), and IκBa (28).

To further explore the role of arrestins and GRKs in regulating LPS signaling, we focused on the ERK arm of the LPS sig-
Arrestin-2 and GRK5 Inhibit LPS-induced ERK Activation

Effect of LPS on ERK1/2 phosphorylation in Raw cells stably transfected with HA-vector or HA-Arrestin-2. Cells were treated for the indicated time periods. A representative blot is shown (top panel). Western blotting, quantitation, and analysis were performed as described in the legend to Fig. 1B. Only pERK2 quantitation is shown (bottom panel). Results were similar for pERK1 quantitation. n = 6, *p < 0.05.

Materials—LPS, phorbol 12-myristate 13-acetate, and tubulin antibody were from Sigma. Protease inhibitor mixture tablet was from Roche Applied Science. Arrestin constructs have been described previously (23). TPL2, ERK2, and p105/p50 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); phospho-ERK1/2 (pERK1/2), phospho-p105, and phospho-MEK antibodies were from Cell Signaling; and HA antibody was from Covance. Full-length HA-p105 was from Dr. Ghosh (33), whereas the various deletion constructs of HA-p105 have been described previously (34). HA-TPL2 constructs were from P. Tsichlis (35). HA-p105N antibody used for immunoprecipitating endogenous p105 has been described (36).

Cell Culture—Raw264.7 macrophage and HEK293 cells were from ATCC and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin at 37 °C and 5% CO2. For creating stable cell lines expressing HA-arrestin-2 or HA-p105, Raw264.7 cells were electroporated with 1 μg of pcDNA3-HA-arrestin-2 or HA-p105 and selected using neomycin (1 mg/ml).

siRNA—The siRNA sequence against mouse arrestin-2 has been described previously (37). Control siRNA and mouse arrestin-2, GRK2, GRK5, and GRK6 siRNAs were from Dharmacon. Transfection of siRNAs in Raw264.7 cells was performed using the Amaxa Nucleofector (program D-032). The cells were transfected and analyzed for knockdown 48 h after electroporation.

Immunoprecipitation and Western Blotting—10-cm dishes of HEK293 cells were co-transfected with the respective constructs. At 48 h after transfection, cells were rinsed with ice-cold phosphate-buffered saline and harvested by the addition of 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and Roche protease inhibitor mixture). Cells were scraped, and lysates were centrifuged at 4 °C for 20 min at 48,000 g. For immunoprecipitation, 100 μg of supernatant protein was incubated with 25 μl of 25% protein A-agarose pre-equilibrated in lysis buffer and 10 μl of polyclonal antibodies for 2 h at 4 °C. Samples were then centrifuged for 10 s, and the pellets were washed three times with 0.5 ml of lysis buffer. Bound proteins were eluted by the addition of 15 μl of SDS sample buffer and boiling for 5 min and then analyzed by SDS-PAGE and immunoblotting. For Western blotting of ERK, p105, and TPL2, cells were quickly washed with cold phosphate-buffered saline before lysis with buffer containing 1% Triton X-100, protease, and phosphatase inhibitors. The lysates were clarified, and then protein concentration was determined, and equivalent amounts of protein were loaded on the gels for Western blot analysis. Immunoblotting was performed as described previously (23). For immunoprecipitation experiments and for immunoblotting TPL2, the secondary antibodies were horseradish peroxidase-conjugated and analyzed by chemiluminescence and densitometry. For arrestin-2/3, pERK/ERK, p105/p50, actin, and tubulin, immunoblotting was performed using fluorescent secondary antibodies and blots were quantitated using the Licor Odyssey scanner.

GST-p105 Expression and Purification—GST or GST-p105 fusion proteins were grown overnight at 37 °C, diluted 1:100 in LB-ampicillin, grown for 2 h at 37 °C, and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 3 h at 30 °C. Cells were pelleted (6000 g for 10 min) and resuspended in phosphate-buffered saline containing protease inhibitors (5 mM EDTA, 10 μg/ml leupeptin, 0.1 mg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride). Cells were treated for 10 min with 1 mg/ml lysozyme and then aliquoted and stored at −80 °C until needed. Aliquots were thawed on ice; 0.5% (final) Sarkosyl, 1% (final) Triton X-100 were added; and cells were frozen and thawed. DNase was added to each aliquot (4 μl of 1 mg/ml per 400–500 μl aliquot) and incubated at 4 °C for 30 min. The sample was centrifuged for 30 min (100,000 × g in a TLA-45 rotor), and the supernatant was then incubated with 50% glutathione-agarose bead slurry for 1 h at 4 °C and washed three times. Protein amounts were determined by Bradford assay.

Arrestin and GRK Binding to p105—GST or GST-p105 (497–968) fusion protein (0.63 μM each) immobilized on glutathione-agarose beads were incubated with 80 nM purified arres-
Arrestin-2 or arrestin-2-R169E in 100 μl of binding buffer (20 mM HEPES, pH 7.2, 120 mM potassium acetate, 0.1 mM dithiothreitol, 0.1% Triton X-100) at 4 °C for 1 h. Beads were centrifuged (1000 g) for 1 min and washed three times with binding buffer. Bound arrestin was eluted with SDS sample buffer, boiled for 1 min, electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblotting using a β-arrestin 1 monoclonal antibody (BD Transduction Laboratories) at 1:1000 dilution. For GRK binding studies, 0.4 μg of purified GRK2 or GRK5 was incubated with 10 μg of GST or GST-p105-(497–968) in interaction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 μg/ml leupeptin, 0.1 mg/ml benzamidine, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.02% Triton X-100, 1 mM dithiothreitol) at 4 °C for 1 h. Beads were then washed in interaction buffer, and bound GRKs were eluted and analyzed by Western blotting using anti-GRK2/3 (1:10,000) or anti-GRK4–6 (1:5000) monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY).

Phosphorylation Assays—2 μM purified GST-p105-(497–968) or GST was incubated with 20 nM GRK2 or GRK5 in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM [γ-32P]ATP, 5 mM MgCl2 for 30 min at 30 °C. Reactions were stopped with SDS buffer, electrophoresed on a 10% polyacrylamide gel, and the gel was dried and subjected to autoradiography. Phosphorylated p105 bands were excised and counted by liquid scintillation to obtain quantitative results.

RESULTS

Role of Arrestin-2 on LPS-induced ERK Phosphorylation—In order to test whether arrestin-2 is involved in LPS signaling in macrophages, we used electroporation to transfec Arrestin-2-specific siRNAs into Raw264.7 cells. Control siRNA-transfected cells were used as controls. As shown in Fig. 1A, we were able to knock down arrestin-2 levels by 70%, whereas arrestin-3 levels were unaffected by arrestin-2 siRNA transfection. LPS treatment of Raw264.7 cells resulted in transient activation of ERK1/2 that peaked at 15–30 min (Fig. 1B). Interestingly, ERK1/2 activation was 30–50% higher in the arrestin-2 knockdown cells compared with control cells (p < 0.01), suggesting that endogenous arrestin-2 mediates an inhibitory role in LPS-promoted ERK activation in macrophages. This effect appears to be specific for LPS, since phorbol 12-myristate 13-acetate-induced ERK1/2 phosphorylation was comparable in control and arrestin-2 knockdown cells (Fig. 1C). Because the knockdown of arrestin-2 caused a significant increase in LPS-induced ERK activation, we hypothesized that overexpression of arrestin-2 would result in inhibition of LPS-induced ERK activation.
Indeed, LPS treatment of Raw264.7 cells stably expressing HA-arrestin-2 (at levels ~2-fold higher than endogenous arrestin-2 levels) (data not shown) resulted in significantly attenuated ERK1/2 activation compared with control cells (Fig. 2).

Role of Arrestin-2 in LPS Signaling Upstream of ERK—Multiple laboratories have shown that LPS-induced ERK activation in macrophages, including Raw264.7 cells, requires the phosphorylation and degradation of the IκB protein p105 (30, 32, 35, 38). Under basal conditions, p105 is stoichiometrically associated with the MAP3K TPL2 and maintains TPL2 in an inactive state. Upon stimulation with LPS, p105 undergoes IKK-dependent phosphorylation and subsequent ubiquitination and degradation. This process releases TPL2 from p105, and the free TPL2 phosphorylates MEK1/2, which then phosphorylates and activates ERK1/2 (30, 32, 35, 38). To identify the step in the pathway where arrestin regulates LPS-induced ERK activation, we tested the effect of LPS on p105 phosphorylation in control and arrestin-2 knockdown cells. This was done by Western blotting using an antibody specific for phosphoserine 932, one of the sites in p105 phosphorylated by IKK upon LPS stimulation. As shown in Fig. 3A, arrestin-2 knockdown resulted in an ~2-fold increase in p105 phosphorylation after 15 min of LPS stimulation compared with control cells. Arrestin-2 knockdown also promoted a significant increase in the rate of p105 degradation (~2-fold increase in p105 degradation at 60 min after LPS stimulation in knockdown cells compared with control cells) (p < 0.01) (Fig. 3B). In contrast, total p50 levels remained constant suggesting that the decrease in p105/p50 ratio is because of p105 degradation (Fig. 3B). Additional experiments were conducted using arrestin-3 knockdown cells to test the specificity of LPS-induced p105 phosphorylation and degradation. Unlike arrestin-2, arrestin-3 knockdown did not affect LPS-induced p105 phosphorylation or degradation (supplementary Fig. 1). Taken together, these results suggest that the effect of arrestin-2 knockdown on LPS-induced ERK activation is at or above the level of p105 phosphorylation by IKK.

Arrestin-2 Knockdown Promotes TPL2 Release from p105 and MEK1/2 Phosphorylation—p105 degradation promotes the release of TPL2 and subsequent phosphorylation of MEK1/2. To test if TPL2 release from p105 is enhanced in arrestin-2 knockdown cells, endogenous p105 was immuno-

![FIGURE 4.](image-url)
precipitated from Raw264.7 cells following 0, 15, or 30 min of LPS treatment. There are two forms of TPL2 in macrophages, M1 and M30, that result from the use of alternative translational start sites (32) (Fig. 4A). TPL2 was completely associated with p105 before treatment with LPS, as assessed by p105 immunoprecipitation, whereas a significant amount of TPL2 was released from p105 after LPS treatment (Fig. 4B). TPL2 release peaked at 15 min after LPS stimulation and was reduced by the 30 min time point, probably due to degradation. The release of TPL2 at 15 min was significantly enhanced in the arrestin-2 knockdown cells compared with control cells (Fig. 4B). Moreover, this enhanced release of TPL2 at 15 min correlated well with the enhanced LPS-stimulated MEK1/2 phosphorylation that was observed in arrestin-2 knockdown cells compared with control cells (Fig. 4C).

Interaction of Arrestin-2 with p105—LPS-induced p105 phosphorylation is enhanced in arrestin-2 knockdown macrophages, suggesting that IKK-mediated phosphorylation of p105 is negatively regulated by arrestin-2. One could predict two possible ways that this could occur. Arrestin-2 binding to IKK or its upstream regulators might inhibit IKK phosphorylation of p105 or arrestin-2 binding directly to p105 might inhibit its ability to be phosphorylated by IKK. Our initial studies demonstrated that arrestin-2 knockdown had no effect on LPS-promoted IKKα/β phosphorylation (supplemental Fig. 2). Thus, we hypothesized that p105 might bind directly to arrestin-2, similar to the ability of arrestin-3 to directly bind IkBα (32). We initially tested this by overexpressing HA-tagged p105 and arrestin-2 in HEK293 cells followed by immunoprecipitation using arrestin-2 and HA antibodies. Immunoprecipitation of arrestin-2 co-immunoprecipitated HA-p105 (Fig. 5A, top panel), whereas immunoprecipitation of HA-p105 also co-immunoprecipitated arrestin-2 (Fig. 5A, third panel). These results suggest that arrestin-2 can interact with HA-p105. Interestingly, when we co-expressed arrestin-2-R169E (an activated form of arrestin-2 that binds to GPCRs in a phosphorylation-independent manner) (39) with p105, its interaction with p105 was significantly increased compared with wild type arrestin-2 (Fig. 5A). These results suggest that arrestin-2 probably binds to p105 in a conformationally sensitive and/or phosphorylation-dependent manner in vivo. To test if endogenous arrestin-2 interacts with p105 in macrophages, we treated Raw264.7 cells (stably expressing HA-tagged p105) with LPS for 0, 5, or 15 min. Immunoprecipitation of endogenous arrestin-2 co-immunoprecipitated HA-p105 in untreated cells, whereas LPS treatment appeared to decrease arrestin-2/p105 interaction, particularly at the 15-min time point (Fig. 5B). There was no co-immunoprecipitation of HA-p105 when the lysates were immunoprecipitated with preimmune serum (data not shown). These results suggest that endogenous arrestin-2 interacts with p105 and that this interaction may be regulated by LPS treatment. Arrestin-2 interaction with p105 probably attenuates LPS-promoted p105 phosphorylation/degradation.
To identify the regions of p105 that interact with arrestin-2, we used various deletion constructs of HA-tagged p105, including regions that encompass the NH₂-terminal region (p50) as well as the COOH-terminal region (also called IκBa). These deletion constructs were co-expressed with arrestin-2 in HEK293 cells, arrestin-2 was immunoprecipitated, and the eluates were then immunoblotted using HA-monoclonal antibody. These results reveal that arrestin-2 binds to the COOH-terminal region of p105 (Fig. 6A) and that this binding may involve the DD and PEST domains of p105 (Fig. 6A). Arrestin-2 did not bind to the NH₂-terminal region of p105 (or to HA-p50), suggesting that the interaction with p105 may be similar to the previously reported arrestin-3 interaction with the COOH-terminal PEST domain of IκBa (28).

To test whether arrestin-2 directly binds to p105, we performed binding assays using purified GST-p105-(497–968), arrestin-2, and arrestin-2-R169E. In line with the HEK293 cell results described above, these studies demonstrate that the COOH-terminal portion of p105 (residues 497–968) can directly interact with arrestin-2 (Fig. 6B). This interaction was enhanced ~2-fold when arrestin-2-R169E was used, again suggesting that p105/arrestin-2 interaction may be conformationally and/or phosphorylation-dependent. Taken together, these results demonstrate that arrestin-2 can directly bind to the COOH-terminal region of p105.

**Interaction of p105 with GRK5—Immunoprecipitation as well as in vitro binding analysis of arrestin-2-R169E and p105 suggest that arrestin interaction with p105 might be phosphorylation-dependent. Because arrestin interaction with GPCRs is dependent on phosphorylation of the receptor by GRKs, we hypothesized that GRKs might also be involved in the phosphorylation of p105 and potentially regulate arrestin-2 interaction. To test this, we examined whether purified GRK2 or GRK5 phosphorylates GST-p105-(497–968). As shown in Fig. 7A, p105 was effectively phosphorylated by GRK5 with a stoichiometry of ~1.4 mol of phosphate/mol of p105 after a 30-min incubation. In contrast, p105 was a poor substrate for GRK2. Note that GST was not phosphorylated by GRK2 or GRK5 (data not shown). We next examined if GRK2 or GRK5 directly interact with p105 and found that both kinases directly bind to GST-p105-(497–968) (Fig. 7B). To test the possible regions of GRK interaction with p105, we incubated lysates (from HEK293 cells overexpressing HA-p105) with the GST-RH (regulator of G-protein signaling homology) domains of GRK2, GRK5, and GRK6. We chose this region based on yeast two-hybrid studies that...
identified interaction between the RH domain of GRK2 (residues 3–190) and p105 (Alliance for Cell Signaling; available on the World Wide Web at http://www.signalinggateway.org/data/Y2H/cgi-bin/y2h.cgi). Incubation of HA-p105 lysates with the RH domain of the three kinases revealed binding of HA-p105 with the GST-GRK-RH domains. This binding was significantly higher for GRK5 than for either GRK2 or GRK6 (Fig. 7C).

Regulation of LPS-stimulated p105 Degradation and ERK Phosphorylation by GRK5—Based on our in vitro analysis, we hypothesized that GRK5 might regulate LPS-induced p105 degradation and ERK activation. To test this, we transfected Raw cells with control siRNA or GRK2 siRNA for 48 h. Lysates were then extracted, and Western blotting was performed using GRK2 monoclonal antibody (1:3000). Blots were stripped and reprobed with actin polyclonal antibody to check for loading. A representative blot is shown. n = 5. B–D, effect of LPS on p105 phosphorylation and degradation in control siRNA and GRK2 siRNA transfected cells. Raw264.7 cells transfected with control or GRK2 siRNA were treated with LPS for 0, 15, 30, and 60 min. Western blotting against the respective proteins was done as described in Fig. 3. Representative blots are shown in B. Quantitation is shown in C (p105 phosphorylation) and D (p105/50 ratio). n = 5. Quantitation was performed as described in Fig. 3.

GRK5 knockdown enhanced LPS-stimulated p105 phosphorylation and degradation ~2-fold (Fig. 9, B–D). These results suggest that endogenous GRK5 (but not GRK2 or -6) regulates LPS-stimulated (IKK-mediated) p105 phosphorylation and degradation in macrophages, possibly via GRK5 phosphorylation of p105.

We next tested the effect of GRK5 knockdown on LPS-induced ERK1/2 phosphorylation. Similar to the effect on p105 degradation, GRK2 or GRK6 knockdown did not affect LPS-stimulated ERK1/2 phosphorylation (Fig. 10A and data not shown, respectively). In contrast, the enhanced LPS-induced p105 degradation observed with GRK5 knockdown correlated well with an enhanced LPS-stimulated ERK1/2 phosphorylation (~2-fold at 15 min after LPS treatment) (Fig. 10B). Although we speculate that GRK5 phosphorylation of p105 might regulate arrestin-2 binding to p105, our initial studies suggest that GRK5 knockdown has no effect on arrestin-2 co-immunoprecipitation with HA-p105 in Raw cells (data not shown). Whether this means that GRK5 does not regulate arrestin-2 binding or that the knockdown was not effective enough to see a difference in p105/arrestin-2 binding is unclear. Nevertheless, these studies reveal that arrestin-2 and GRK5 directly interact with p105 and play a negative role in regulating p105 function, probably via regulation of IKK-mediated phosphorylation of p105. Future studies will test how these two proteins coordinately regulate the ERK pathway stimulated by LPS.

**DISCUSSION**

GPCRs are a major class of receptor molecules that are regulated by protein kinases (GRKs) and adaptor/scaffolding molecules (arrestins). While GRKs phosphorylate the agonist-occupied receptor, arrestins bind to the phosphorylated receptor and uncouple the receptor from G proteins. Recent studies have suggested that GRKs and arrestins could play much broader roles in cell signaling (16, 40). Although non-GPCR targets for GRKs have been identified (41–46), arrestins have recently been shown to be involved in receptor signaling pathways that are not considered within the typical GPCR paradigm (27, 29, 37, 47). These include receptor signaling from a variety of ligands ranging from insulin to cytokines. Although the major focus of this study is on the role of arrestin-2 in the LPS-induced ERK pathway, the rationale for testing the role of GRKs was...
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2-fold: 1) arrestin-2-R169E ("activated" form of arrestin) interaction with p105 was significantly greater compared with wild type arrestin, and 2) the Alliance for Cellular Signaling identified p105 as the only prey in a yeast two-hybrid screen using the GRK2 RH domain as the bait. Arrestins bind to GPCRs in a phosphorylation-dependent manner (phosphorylated by GRKs). The arrestin-2-R169E mutant binds to GPCRs independent of phosphorylation (23, 39, 48). Because this mutant bound to p105 better than the wild type, we hypothesized that arrestin-2 probably binds to p105 in a phosphorylation-dependent manner in vivo. Thus, we rationalized that one of the GRKs probably phosphorylates p105, which then increases arrestin-2 binding to p105. In vitro analysis reveals that the RH domains of GRK2 and GRK5 interact with the COOH-terminal portion of p105, although only GRK5 is able to effectively phosphorylate p105 in vitro. This suggests that p105 may be a substrate for GRK5 in vivo, although this was not tested in the present study. These results correlate with our findings on the role of endogenous GRK5 on p105 degradation (i.e. knockdown of GRK5 in macrophages significantly enhances IKK-mediated p105 phosphorylation and degradation). This is reflected downstream in the ERK pathway, which also shows an enhanced ERK phosphorylation in the GRK5 knockdown cells compared with controls. This suggests that arrestin-2 and GRK5 coordinately regulate the same pathway. Further studies are necessary to test if phosphorylation of p105 by GRK5 or other kinases regulates arrestin-2 binding to p105. In this regard, in addition to IKK, p105 has been shown to be phosphorylated by GSK3β (glycogen-synthase kinase 3β) (49) and more recently by TPL2 (50). Even if GRK5 phosphorylation of p105 does not regulate arrestin-2 binding to p105, our RNA interference data clearly demonstrate that GRK5 regulates LPS-stimulated IKK-mediated phosphorylation and degradation. Thus, it is also plausible that GRK5 phosphorylation of p105 or direct binding to p105 might regulate IKK phosphorylation of p105 independent of arrestin-2.

Stimulation of IKK by activated TLR4 is mediated via several protein-protein interactions. Of particular note is the role of TRAF6. Gao et al. (28) demonstrated that arrestin-3 interacts directly with IκBα and that this interaction regulates the TNF-α-induced phosphorylation and degradation of IκBα.

While this manuscript was in preparation, the same group provided evidence that LPS-induces interaction of arrestins with TRAF6, and they proposed that this was essential for the regulation by arrestin of IκBα phosphorylation via the TRAF6-IKK pathway in arrestin 2/3 double knock-out mouse embryo fibroblasts (29). Using TRAF6 knock-out mice, Hacker et al. (51) showed that although TRAF6 is essential for LPS-induced IκBα degradation, ERK activation is only partially dependent on TRAF6. Since regulation of p105 is important in ERK activation by LPS in macrophages, it is possible that TRAF6-independent pathways could regulate the p105-TPL2-ERK pathway. Furthermore, although TPL2-ERK activation is necessary for LPS-induced TNF-α mRNA expression in macrophages, bone marrow-derived macrophages from TRAF6 knock-out mice did not show any significant difference from wild type bone marrow-derived macrophages in terms of LPS-induced TNF-α mRNA expression (51, 52). Taken together, these results suggest that although TRAF6 is essential for the IKK-IκBα pathway,
the IKK-p105-ERK pathway may be regulated at least in part by TRAF6-independent pathways.

Arrestins interact with p105 in macrophages (this study) and IκBα in HEK293 cells (28) even under unstimulated conditions. This suggests that arrestin binding can occur through mechanisms independent of LPS signaling. Interestingly, treatment with LPS resulted in a decrease in arrestin-p105 interaction in macrophages stably expressing HA-p105. Although the mechanisms causing this decreased interaction is not clear, we speculate that a component of the LPS signaling pathway probably causes the decrease in arrestin-p105 interaction, thus facilitating phosphorylation by IKK. Gao et al. (28) showed that cross-talk between β-adrenergic receptor and TNF-α receptor can regulate arrestin binding to IκBα. Future studies will test if signals from GPCRs could regulate arrestin-2 binding to p105 or GRK5 phosphorylation of p105 in macrophages. We found that the effect of arrestin-2 knockdown on p105 phosphorylation and degradation was specific for this isoform of arrestin, since arrestin-3 knockdown did not affect p105 phosphorylation or degradation. Gao et al. (28) found that arrestin-3 was important in preventing IκBα phosphorylation and degradation. It is not clear if arrestin-2 and -3 affect different members of the IκB family in a specific manner or if this is a cell/tissue-specific effect. Whereas our studies were performed in macrophages, arrestin-3-IκBα interactions were studied in HEK293 cells. Furthermore, because p105 and IκBα share similar binding regions for arrestin interaction, and since GRK5 binds to a similar region in p105 as arrestin-2, it would be of interest to test if GRK5 could also regulate IκBα function.

Similar to GPCRs, TLR signaling also involves multiple adapter proteins and kinases that primarily lead to the activation of MAPK and NFκB pathways. Data presented here demonstrate that endogenous arrestin-2 and GRK5 affect the ERK1/2 arm of the LPS signaling pathway. Although arrestin-3 has been shown to modulate NFκB activation via IκBα degradation, the role of arrestin-2 and GRK5 in LPS-induced NFκB activation in macrophages will require further studies. Arrestins have been shown to affect all members of the MAPK pathways in response to GPCR stimulation (16). Although LPS activates all members of the
MAPK family including JNK and p38 MAPK (9), the role of arrestins and GRKs with regard to LPS-stimulated JNK and p38 MAPK pathways remains to be tested. Recently Wang et al. (29) provided evidence that arrestin-3 also plays a major role in cytokine production in response to TLR3 and TLR9 in bone marrow-derived macrophages from arrestin-3 knock-out mice. Whether arrestin-2 and GRK5 or the other GRKs play a role in TLR3 and TLR9 signaling is not known. It was also recently shown that p105 was necessary for ERK activation in response to most of the TLR ligands in macrophages derived from p105 knock-out mice (53). Thus, one could predict that arrestins and GRKs might play either specific or universal roles in various TLR signaling with regard to p105-mediated activation of ERK. In this study, we examined the role of GRK2, -5, and -6 in LPS stimulated ERK activation. Although both GRK5 and GRK2 were able to directly interact with p105, p105 was a better substrate for GRK5 than for GRK2. Moreover, RNA interference analysis reveals that endogenous GRK5 but not GRK2 or -6 affects LPS-induced p105 degradation as well as ERK activation in macrophages. Nevertheless, we cannot rule out a role for p105 interaction with additional members of the GRK family.

In conclusion, using an RNA interference approach, we present evidence that endogenous arrestin-2 and GRK5 play a significant role in the IKK-mediated phosphorylation and degradation of p105 in macrophages (Fig. 11). We provide further evidence that both arrestin-2 and GRK5 can directly interact with p105 and that GRK5 can phosphorylate p105 in vitro. The effect of these proteins on p105 is reflected downstream of the pathway, particularly on LPS-induced ERK activation in macrophages. Finally, data presented in this study demonstrate a role for arrestin-2 and GRK5 in linking the IKK-NFκB and the ERK pathway stimulated by LPS.

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