Toll-like receptors (TLRs) are proteins involved in recognition of foreign pathogen-associated molecular patterns and activation of processes leading to innate immune recognition. We show that stimulation of fibroblasts with a TLR5 ligand, flagellin, can induce proliferation of serum-starved cells or prevent cell cycle exit upon serum withdrawal independently of autologous growth factor secretion. Other TLR ligands, such as poly(I:C) and lipopolysaccharide, can have a similar effect only if the action of type I interferons is blocked. Flagellin stimulation can prevent cell cycle arrest induced by overexpression of exogenous cyclin-dependent kinase inhibitor p27. Stimulation of TLR5 and overexpression of MyD88, but not TRIF, TIRAP, or TRAM, result in p27 degradation, which can be suppressed by dominant negative Akt and mutation of the p27 C-terminal Thr^{187} site. These data provide evidence for a non-immune and cell autonomous role of TLR signaling, whereby TLR stimulation provides a positive signal for cell division.

Members of the Toll-like receptor family are germ-line-encoded receptors that play an essential role in initiating the immune response against pathogens. Thirteen mammalian TLR^1 paralogues have now been identified (10 in human and 12 in mice), which recognize a wide variety of pathogen-associated molecular patterns from bacteria, viruses, and fungi, as well as certain host-derived molecules (1). TLRs are type I transmembrane glycoproteins with an extracellular domain composed of numerous leucine-rich repeats and an intracellular region containing a TIR homology domain (2, 3). The TIR domains interact with several TIR domain-containing adapter molecules (MyD88, TIRAP, TRIF, and TRAM), which activate a cascade of events resulting in transcription factor induction (4). The common signaling feature among all TLRs is the activation of the transcription factor NFκB, which has been implicated in the control of expression of inflammatory cytokines and maturation molecules. A subset of TLRs induces the production of type I interferons (IFNs) (5, 6), which mediate antiviral, growth-inhibitory, and immunomodulatory responses. Recently, Rakoff-Nahoum et al. (7) discovered a novel non-immune role of TLRs. They showed that TLR signaling can maintain epithelial homeostasis through proliferation and repair tissue after direct epithelial injury. In general, fibroblasts play an essential role in the latter process by inducing epithelial growth and differentiation not only in the intestinal epithelium but also in the skin and lungs by secreting keratinocyte growth factor, interleukin-6, interleukin-8, and transforming growth factor-β, factors required by epithelial cells in their differentiation process to repair tissue after injury (7). The discovery of the TLR-induced proliferation and the recent work describing a role in tissue repair prompted us to investigate the potential direct link between TLR signaling and cell cycle control.

Cell cycle progression of mammalian cells is controlled by a series of cyclin-dependent kinase (cdk) complexes that ensure that all the criteria for a faithful cell division have been met. Exposure of quiescent cells (G_0 phase of the cell cycle) to mitogens gives rise to activation of cyclin-cdk complexes, with concomitant dissociation and/or degradation of cdk inhibitors p16^{INK4a}, p21^{Cip1}, and p27^{Kip1}. Together with cyclin E-cdk2 complexes, cyclin D-cdk4 complexes phosphorylate proteins of the retinoblastoma family, which normally repress transcription of genes required for S-phase entry. After retinoblastoma phosphorylation, cells traverse S phase and following G_2 phase undergo cell division.

The tumor suppressor p27^{Kip1} (p27) plays a critical role in regulating progression through the G_1-S phases of the cell cycle (8). The activity of p27 protein is modulated by changes in its abundance principally by degradation, sequestration, and distribution between the nucleus and cytoplasm. Phosphorylation of p27 at Thr^{187} by cyclin-cdk complexes triggers its degradation mediated by SCF^{Skp2} ubiquitin ligase (9, 10). Increased levels of Skp2 or cyclin E result in increased degradation of p27 (11). Akt can phosphorylate p27 at Thr^{187} located in its nuclear localization signal and prevents nuclear localization of p27 (12, 13). Fujita et al. (14) found that Akt can phosphorylate p27 at Ser^{10}, Thr^{187}, and Thr^{188} and that 14-3-3 binds to phosphorylated p27 in the cytoplasm. Overexpression of cyclin D3 or activation of mitogen-activated protein kinase that up-regulates cyclin D1 can result in sequestration of p27 and prevents its association with its targets (15).

The link between TLR signaling and cell cycle control has yet to be explored, and it is not clear whether certain (or all) TLR ligands can affect proliferation of cells independently of secreted factors. Using the immortalized Rat1 cell line as a model system (16), we sought to determine whether TLR signaling was capable of regulating cell cycle progression in fibroblasts with or without a contribution of autologous cytokines and how the signal from activated TLRs is transmitted to key cell cycle regulators. We have found that flagellin, a TLR5 agonist, can induce cell cycle entry by overcoming p27-induced cell cycle

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The abbreviations used are: TLR, Toll-like receptor; GFP, green fluorescent protein; LPS, lipopolysaccharide; siRNA, small interfering RNA; IR, Toll/interleukin receptor; IFN, interferon; NFκB, nuclear factor κB; FCS, fetal calf serum; BrdUrd, bromodeoxyuridine; cdk, cyclin-dependent kinase; MLB, mild lysis buffer; E3, ubiquitin-protein isopeptide ligase; IRAK, interleukin-1 receptor-associated kinase.

Received for publication, January 24, 2005, and in revised form, March 22, 2005
Published, JBC Papers in Press, March 23, 2005, DOI 10.1074/jbc.M500877200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 21, Issue of May 27, pp. 20620–20627, 2005
Printed in U.S.A.
arrest though a MyD88-dependent pathway involving Akt. Our findings also suggest that the differential capacity of TLR3 and TLR4 ligands to induce cell cycle progression is dependent on the ability of these ligands to produce IFN-β.

Experimental Procedures

**Cell Culture—**HEK293T and HEK293 cells were obtained from American Type Culture Collection, and Rat1 cells were obtained from the Imperial Cancer Research Fund (London, UK). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM l-glutamine (Invitrogen). Cells were cultured at 37 °C with 5% CO2. The p27-inducible Rat1 cell line (17) was cultured in the presence of 2 μg/ml tetracycline to repress p27 production. For proliferation studies, cells were treated with siRNA duplexes, arrested expression was analyzed by Western blotting. For gene knock down and then stimulated with flagellin (100 ng/ml) or FCS, and cyclin D1 expression was analyzed using FuGENE 6 (Roche Applied Science), with 500 ng of either synthetic phosphodiester oligodeoxynucleotides were synthesized by MWG and used at 10 μg/ml (1018: TGACTGTTAGCCTGCTGATGA). Poly(I:C) (Invivogen) was used at the concentrations indicated. Flagellin from Salmonella muenchen (Calbiochem) was used at indicated concentrations. Polyvinyl (10 μg/ml) was added, where indicated, to block the effects of potential endotoxin contaminants in the flagellin preparations. Rat IFN-β was purchased from PBL. Brefeldin A was supplied by Pharmingen, used at a 1:1000 dilution, and added at the same time as the ligand.

**Luciferase Assay—**Rat1 Cells were transiently transfected in triplicate using FuGENE 6 (Roche Applied Science), with 500 ng of either NFκB or IFN-β luciferase reporters (18) in 6-well tissue culture plates with cells at 50% confluence. Six h after transfection, cells from each of the 6 wells per plate were divided into 24 wells in 96-well plates. The following day, for each transfection, cells were stimulated in triplicate with TLR ligands for 6 h (making a total of 9 wells for each TLR ligand), harvested, and analyzed for luciferase activity using Promega Steady-Glo reporter assay reagents and the Fusion instrument from Packard. Each experiment, as described here, was repeated three times; results generally deviated by <10% of the mean value.

**Cell Cycle Analysis—**For flow cytometric analysis, stimulated or non-stimulated cells were detached with trypsin and fixed in 70% ethanol. Nuclei were stained with propidium iodide at 10 μg/ml to analyze total DNA content. To determine newly synthesized DNA, cells were labeled with 33 μM bromodeoxyuridine (BrdUrd) for 30 min. Total DNA content and BrdUrd incorporation were determined as described previously (16). The experiment was performed by using a FACSCalibur, and data were analyzed with CELLQUEST software.

**Small Interfering RNA Treatment of Cells—**Small interfering RNA duplexes targeting the coding region of MyD88 (GGAAGUGGACUCCAGACCTT) were synthesized by Dharmacon. To determine the efficacy of gene silencing, reporter assay and Western blot analysis were performed. Briefly, cells were transfected with siRNA duplexes (50 nm) according to the manufacturer’s recommendations. Forty-eight hours after siRNA treatment, cells were lysed for Western blot analysis or transfected with NFκB reporter as outlined above and stimulated with TLR ligands. Cells were also starved for 24 h after siRNA treatment and then stimulated with flagellin (100 ng/ml) or FCS, and cyclin D1 expression was analyzed by Western blotting. For gene knock down proliferation studies, cells were treated with siRNA duplexes, arrested in a serum-free medium, stimulated with the respective ligand, labeled with BrdUrd, and harvested for fluorescence-activated cell-sorting analysis.

**Plasmid Constructs—**MyD88, TIRAP (MAL), TRIF (TICAM-1), TRAM (TICAM-2), and IRAK were amplified from a human cDNA library and cloned into pCMV vectors. pCMVp27 and pBabePuro-p27 as well as pCMVp27 and pBabePuro-p27 were cloned into pCMVp27 and pBabePuro-p27 as described previously (19). The dominant active Thr308/Ser317 mutant of Akt (AcAkt) was obtained from Brian Hemmings (20), and the kinase-dead version of Akt (dnAkt) was a kind gift of Boudewijn Burgering (21).

**Transfection of HEK293T and HEK293 Cells—**Cells were transiently transfected using FuGENE 6 (Roche Applied Science), with 500 ng of the indicated plasmid together with 250 ng of pCMVGFp for normalization of expression levels. Twenty-four h after transfection, HEK293T cells were lysed for Western blot analysis. Transfected with TLRs were stimulated for 18 h with the respective TLR ligand and harvested for Western blot analysis.

**Biochemical Analysis—**Biochemical analysis of harvested cells was performed as described previously (16). Briefly, harvested cells were lysed in mild lysis buffer (MLB) containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 20 mM NaF, and 0.3 mM ortho-sodium vanadate. Total cellular protein (an amount between 10 and 40 μg, determined by Bradford assay; Bio-Rad) was used for SDS-PAGE and immuno blotting (Invitrogen). After incubation with primary antibodies, proteins were detected with peroxidase-conjugated goat anti-rabbit and mouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) and enhanced chemiluminescence (ECL; Amersham Biosciences). For kinase assays, cells were lysed in MLB, and 40–60 μg of total protein was immunoprecipitated with 2 μl of the respective antibody for 2 h at 4 °C in the presence of protein A-Sepharose. Beads were washed four times in MLB and once in kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM dithiothreitol). Beads were then resuspended in 40 μl of kinase reaction buffer supplemented with 0.1 mg/ml bovine serum albumin, 50 μM ATP, 1 μg of histone H1, and 7 μCi of [γ32P]ATP and incubated for 20 min at 30 °C. The reaction was stopped by the addition of 4× LDS loading buffer; radiolabeled histone H1 was resolved by SDS-PAGE and analyzed on PhosphorImager (Amersham Biosciences).

**Immunofluorescence Analysis—**HEK293 cells were transfected with p27 expression plasmid; 6 h later cells were divided into a 6-well plate containing polylysine-coated 22 × 22-mm coverslips. The following day, cells were stimulated with flagellin (100 ng/ml) for 0 and 8 h. Coverslips were washed in phosphate-buffered saline, fixed with 3.7% paraformaldehyde, and stained for p27. Briefly, p27 antibody was diluted 1:50 in 0.1% saponin/phosphate-buffered saline and incubated for 1 h at room temperature. Cells were washed in 0.1% saponin/phosphate-buffered saline, and secondary antibody-Alexa 594 conjugate (1:500 dilution) was added and incubated for 30 min in the dark at room temperature. Cells were washed, and coverslips were mounted onto slides using a 1:10 dilution of 4′,6-diamidino-2-phenylindole (nuclear stain; Invitrogen) in fluoromount (SouthernBiotech), and p27 protein was detected by direct fluorescence microscopy. Photographs were taken at ×4 magnification using Axioplan 2 imaging.

**Antibodies—**The following antibodies were used: anti-p27 C19 (sc-528; Santa Cruz Biotechnology), anti-ckd2 M2 (sc-163; Santa Cruz Biotechnology), anti-cyclin A H-432 (sc751; Santa Cruz Biotechnology), anti-cyclin E M20 (sc-481; Santa Cruz Biotechnology), anti-cyclin D1 (2926; Cell Signaling), anti-pRb (9058; Cell Signaling), anti-ckd6 (2136; Cell Signaling), anti-Akt (9272s; Cell Signaling), anti-IRAK (H273; Santa Cruz Biotechnology), anti-GFP (Roche Applied Science), and goat anti-rabbit Alexa 594 (Molecular Probes).

**RESULTS**

**Poly(I:C), LPS, and Flagellin Activate NFkB in Rat1 Cells—**To transiently transfected cells with a construct containing the luciferase reporter gene under the control of NFkB response element and stimulated cells with various TLR ligands. Stimulation of cells with poly(I:C), LPS, and flagellin (Fig. 1A) had a significant effect on the activity of the NFkB reporter in Rat1 cells. As shown in Fig. 1A, the ability of the flagellin preparation from S. muenchen to activate the NFkB reporter was not blocked by polymixin and was unlikely to be due to endotoxin contamination. We also tested whether activation of NFkB was mediated through MyD88 using siRNA technology. We found that NFkB responses to flagellin were completely blocked; in response to LPS there was a partially drop in luciferase activity, and in response to poly(I:C) the activity remained constant in the absence of MyD88 (data not shown). These results suggest that TLR3, TLR4, and TLR5 are functional receptors in Rat1 cells, and in further experiments the function of these TLRs in cell cycle regulation was explored.
Control of Cell Cycle Entry and Exit by TLR Ligands—We next examined whether ligands for TLR3, TLR4, and TLR5 can induce cell cycle entry. Rat1 cells were starved in medium without serum for 24 h, and then varying amounts of poly(I:C), LPS, or flagellin were added to the culture medium for 20 h (Fig. 1B). Approximately 1% of control cells without any stimulation incorporated BrdUrd. Activation of TLR5 with flagellin (1 ng/ml) induced cell cycle entry in 10% of cells, and increasing the concentration of flagellin to 10 and 100 ng/ml resulted in enhanced incorporation of BrdUrd in 13% and 23% of cells, respectively. Stimulation of cells with poly(I:C) (5 μg/ml) or LPS (50 and 500 ng/ml) did not change BrdUrd incorporation, except for the highest concentration of poly(I:C) (50 μg/ml), which promoted cell proliferation. We also tested whether flagellin in the absence of serum can prevent cells from cell cycle exit and sustain proliferation of cells. Subconfluent growing Rat1 cells were washed in serum-free medium and replenished with fresh medium without serum (Starved cells), fresh medium with FCS (FCS), or fresh medium without serum but with 10 ng/ml flagellin. Cells were stimulated for 14 h and analyzed for cell cycle distribution by fluorescence-activated cell sorting. Data here are representative of one of four experiments performed.

Control of Cell Cycle Entry and Exit by TLR Ligands—We next examined whether ligands for TLR3, TLR4, and TLR5 can induce cell cycle entry. Rat1 cells were starved in medium without serum for 24 h, and then varying amounts of poly(I:C), LPS, or flagellin were added to the culture medium for 20 h (Fig. 1B). Approximately 1% of control cells without any stimulation incorporated BrdUrd. Activation of TLR5 with flagellin (1 ng/ml) induced cell cycle entry in 10% of cells, and increasing the concentration of flagellin to 10 and 100 ng/ml resulted in enhanced incorporation of BrdUrd in 13% and 23% of cells, respectively. Stimulation of cells with poly(I:C) (5 μg/ml) or LPS (50 and 500 ng/ml) did not change BrdUrd incorporation, except for the highest concentration of poly(I:C) (50 μg/ml), which promoted cell proliferation. We also tested whether flagellin in the absence of serum can prevent cells from cell cycle exit and sustain proliferation of cells. Subconfluent growing Rat1 cells were washed in serum-free medium and replenished with fresh medium containing either G1-G0 (bottom left quadrant), S phase (top left and right quadrants), or G2-M (bottom right quadrant). Data here are representative of one of four experiments performed. 

FIG. 1. TLR stimulation induces cell proliferation. A, Rat1 cells transfected with NFκB reporter plasmid were analyzed for luciferase activity induced by a panel of TLR ligands. Data are mean values ± S.E. from one experiment (as described under “Experimental Procedures”) representative of at least three independent experiments. B, cell cycle distribution of serum-starved Rat1 cells stimulated for 18 h with flagellin, LPS, or poly(I:C) at different concentrations, as assessed by BrdUrd incorporation and DNA content flow cytometry. BrdUrd incorporation was monitored with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (vertical axis), and DNA content was monitored by propidium iodide staining (horizontal axis). Controls are indicated at the bottom. The numbers in the quadrants represent the percentage of cells in either G0-G1 (bottom left quadrant), S phase (top left and right quadrants), or G2-M (bottom right quadrant). Data here are representative of one of four experiments performed. C, serum-containing medium was removed from growing Rat1 cells and replaced with fresh medium without serum (Starved cells), fresh medium with FCS (FCS), or fresh medium without serum but with 10 ng/ml flagellin. Cells were stimulated for 14 h and analyzed for cell cycle distribution by fluorescence-activated cell sorting. Data here are representative of one of four experiments performed.
mechanisms that lead to entry of the cells into the S phase of the cell cycle and that prevent cell cycle exit following growth factor deprivation.

Interferon Blocks the Proliferative Effect of TLR3 and TLR4 Ligands—Because TLR3 and TLR4 exhibit a different pattern of signal transduction than TLR5, we sought to investigate why poly(I:C) (except for high concentrations) and LPS do not promote proliferation of serum-starved Rat1 cells. We analyzed the responses of several promoter constructs in transfected cells, in line with reports using transfected HEK293T cells, whereas the response to flagellin was minimal (Fig. 2A and B). To analyze whether production of IFN following poly(I:C) or LPS stimulation had a negative effect on proliferation of starved Rat1 cells, we treated serum-starved Rat1 cells with poly(I:C) or LPS in the absence or presence of a type I IFN receptor-neutralizing antibody (Fig. 2C and D). Neutralization of type I IFN signaling unmasked the proliferative effect of poly(I:C) and LPS, whereas the antibody on its own did not promote cell cycle entry of serum-starved cells. Control antibody IgG2a did not have any effect (Fig. 2E). The IFN receptor-neutralizing antibody did not modify the activation of either NFκB or IFN-β promoters by TLR ligands (Fig. 2A), suggesting that IFN does not interfere with the TLR signaling pathway. We have also tested whether addition of rat IFN-β can reverse the proliferative effect of flagellin treatment. As shown in Fig. 2F, exogenous IFN prevents cell cycle entry induced by flagellin but not by FCS. Thus, TLR3, TLR4, and TLR5 promote cell cycle entry of G_{1}-G_{0}-arrested cells, but this effect is masked by the autocrine antiproliferative effect of type I IFN induced by TLR3 and TLR4.

TLR5 Signaling Overcomes p27-induced Growth Arrest—We analyzed expression levels of various proteins involved in G_{1}-S-phase transition upon TLR5 signaling (Fig. 3, A and B). Serum-starved Rat1 cells were incubated in the presence of either flagellin or FCS, and cells were harvested at different time points over a 24-h period. Flagellin (10 ng/ml) stimulation resulted in induction of cyclin D1 (4 h) and cyclin A (16 h), disappearance of the cdk inhibitor p27 (16 h), and the appearance of hyperphosphorylated retinoblastoma (16 h), suggesting that flagellin induced cell cycle entry accompanied by typical markers of proliferation, passage though the restriction point and entry into the S phase of the cell cycle. We also noticed that flagellin stimulation induces phosphorylation of Akt, GSK3β dephosphorylation on Tyr^{216} (22), and phosphorylation on Ser^{9}, 21, although with lower potency than FCS. None of the treatments induced apoptosis as judged from the absence of poly-(ADP-ribose) polymerase cleavage (Fig. 3A).

We also measured cdk2-associated kinase activity in starved cells and cells stimulated with flagellin and FCS (Fig. 3C). FCS induced cdk2 activity with faster kinetics than flagellin; a 14-h stimulation of cells with FCS resulted in high cdk2-associated kinase activity, whereas an 18-h stimulation with flagellin was needed to observe functional activation of cdk2.

To assess whether TLR5 signaling can antagonize inhibitory activity of exogenous p27, we used Rat1 cells infected with a retrovirus expressing p27 under the control of tetracycline-regulatable promoter. Cells in the presence of tetracycline (p27...
expression repressed) grew normally (Fig. 4B), whereas tetracycline removal induced high levels of p27 (Fig. 4D), and cells arrested in G0-G1 (Fig. 4A). In cells cultured in the absence of tetracycline, flagellin reduced the levels of p27 (Fig. 4D) and restored a normal growing profile (Fig. 4C). Thus, unlike growth factor stimulation that induced the cell cycle progression with faster kinetics, TLR5 signaling can antagonize the growth-arresting capacity of overexpressed p27.

Flagellin-induced Proliferation Involves Intracellular Mechanisms—To analyze whether treatment of Rat1 cells with flagellin or FCS results in secretion of soluble factors that might contribute in an autocrine fashion to the growth-promoting activity of TLR signaling, cells were treated for 2 h with flagellin or FCS, washed, and replenished with fresh medium without serum. Neither serum-treated nor flagellin-treated cell supernatants contained factors that promote cell cycle entry of starved cells (data not shown). We attempted to analyze the composition of cyclin-cdk-p27 complexes following flagellin stimulation in the presence of cycloheximide or actinomycin D to explore whether protein or RNA synthesis was required for changes in the complex composition, but both agents had an effect on the steady-state levels of individual molecules that prevented a reliable analysis of events leading to activation of cyclin-cdk complexes during TLR stimulation. However, blocking protein secretion with brefeldin A had no effect on induction of cyclin D1 levels following flagellin or FCS treatments of serum-starved Rat1 cells (Fig. 4E), suggesting that the effect of TLR5 signaling on cell cycle progression does not occur through an autocrine mechanism.

MyD88-dependent Pathway and NFκB Induction Are Required for the Proliferative Effect of Flagellin—To test whether the classical MyD88-dependent pathway is involved in the cell cycle effect observed in Rat1 cells treated with flagellin, we used a siRNA approach to knock down the levels of MyD88. Twenty-four h later, cells were serum-starved and stimulated for 20 h with flagellin or FCS. A, BrdUrd incorporation (vertical axis) and DNA content by propidium iodide staining (horizontal axis) were plotted. B, siRNA- and scrambled siRNA-treated cells were harvested 48 h after transfection and analyzed using an antibody against MyD88. C, cyclin D levels were assessed by Western blotting in starved cells treated and not treated with siRNA in the presence or absence of 100 ng/ml flagellin at the time points indicated. Data here are representative of one of five experiments performed.

**FIG. 3.** Protein expression in serum-starved Rat1 cells stimulated with flagellin (10 ng/ml) or FCS. A, expression of cell cycle proteins examined by Western blots. B, expression of cdk2 and p27. C, cdk2 kinase activity in cells stimulated with FCS or flagellin using histone H1 as a substrate.

**FIG. 4.** Flagellin antagonizes p27-induced growth arrest. Histograms show propidium iodide-stained cells prepared under the following conditions: A, in the absence of tetracycline; B, in the presence of tetracycline; and C, addition of flagellin (100 ng/ml) for 22 h in the absence of tetracycline as indicated. Cell extracts were analyzed for expression of p27. Equal loading was assessed by immunoblotting for β-tubulin. The last lane (ctrl) contains extract from HEK293T cells transfected with p27 expression plasmid. E, blocking protein secretion with brefeldin A does not inhibit cyclin D1 induction. Starved Rat1 cells were stimulated in the presence or absence of brefeldin A for 8 h, and cyclin D1 levels were analyzed by Western blotting. β-Tubulin levels were determined to control for equal loading.

**FIG. 5.** Small interfering RNA for MyD88 blocks flagellin-induced cell proliferation. Rat1 cells were treated with siRNA for MyD88. Twenty-four h later, cells were serum-starved and stimulated for 20 h with flagellin or FCS. A, BrdUrd incorporation (vertical axis) and DNA content by propidium iodide staining (horizontal axis) were plotted. B, siRNA- and scrambled siRNA-treated cells were harvested 48 h after transfection and analyzed using an antibody against MyD88. C, cyclin D levels were assessed by Western blotting in starved cells treated and not treated with siRNA in the presence or absence of 100 ng/ml flagellin at the time points indicated. Data here are representative of one of five experiments performed.
mediated by flagellin or FCS, siRNA specific for MyD88 prevented cell cycle entry induced by flagellin, but not by FCS. We controlled siRNA efficiency by performing a Western blot on MyD88 siRNA-treated Rat1 cells 48 h after transfection (Fig. 5B). In addition, treatment of starved cells with MyD88 siRNA abolished induction of cyclin D1 expression following flagellin treatment (Fig. 5C). These results suggest that the effect of flagellin on cell cycle regulation is mediated by the classical TLR signaling pathway involving the adapter MyD88.

MyD88-induced Degradation of p27 Is Blocked by Dominant Negative Akt and Mutation of the p27Thr187 Site—To gain more insight into the mechanism of p27 regulation by TLR signaling, we transfected HEK293T cells with a p27 expression vector in combination with another vector expressing TLR3 or TLR5 (Fig. 6A). After overnight stimulation of cells with flagellin, the levels of p27 decreased significantly in cells transfected with TLR5, whereas poly(I:C) treatment did not have any effect. It is likely that the inability of TLR3 signaling to decrease p27 levels is due to the fact that HEK293T cells are capable of producing type I IFN in response to TLR3 signaling. Stimulation of HEK293 cells, which, in contrast to HEK293T cells, express endogenous TLR3 and TLR5, with flagellin in the presence of the proteasome inhibitor N\textsuperscript{G}, N\textsuperscript{G}-di-CBZ-l-arginine restored the expression of p27 to the levels observed in cells without flagellin treatment (Fig. 6B), indicating that TLR5 signaling can modulate p27 levels through inducing its proteasome-mediated degradation. These results confirm that TLR5 expression is required for the observed effect of flagellin on the regulation of p27 levels and that flagellin treatment exerts its effect on cell cycle control by inducing degradation of p27.

Following ligand stimulation, TLRs associate with different TIR adapters, and in the next experiment, we attempted to determine whether overexpression of TRIF, TIRAP, MyD88, or TRAM in HEK293T cells has any effect on expression of exogenous p27. We have found that among all the TIR adapters tested, only expression of MyD88 is capable of reducing the levels of p27 (Fig. 6C). Co-transfection of IRAK-1 also suppressed p27 expression (Fig. 6C). However, we did not detect p27 in complexes with IRAK or MyD88 (data not shown).

The levels of p27 can be regulated by the ubiquitin/proteasome pathway (23, 24), and it was demonstrated that cyclin E-cdk2 complex can phosphorylate p27 on a C-terminal cdk target site (19, 25). A kinase contact mutant of p27, which still binds cyclins, does not inhibit cyclin E-cdk2 complexes but becomes phosphorylated and rapidly degraded. A cyclin/cdk contact mutant of p27 that does not interact with kinase complexes is not phosphorylated and is stable. HEK293T cells were transfected with wild-type p27, cyclin/cdk contact mutant, or cdk target site mutant of p27 in the presence or absence of TRIF and MyD88, and the levels of p27 were measured (Fig. 6D). TRIF did not have any effect on the levels of p27, whereas MyD88 expression resulted in the accumulation of lower levels of wild-type p27. Mutation of the cdk target site TPKK to VPKK stabilized the total levels of p27, indicating that this site is involved in regulation of p27 levels following TLR5 and MyD88-mediated signaling. Surprisingly, the levels of the double cyclin/cdk contact mutant decreased, similarly to wild-type p27, in the presence of MyD88. This result implies that the degradation pathway induced by TLR signaling might include an action of a kinase distinct from cdk2. This kinase is not targeted by highly overexpressed p27 and possibly phosphorylates the TPKK motif in p27, thus generating a signal for proteasome-dependent degradation of p27. Although the identity of this kinase remains to be established, we can speculate that Akt might be involved in the process of p27 phosphorylation and subsequent degradation.

Akt has been shown to regulate p27 levels by increasing its degradation and/or inducing its cytoplasmic localization (see introduction). Akt was phosphorylated following treatment of
Rat1 cells with flagellin (Fig. 3), and the expression of dominant active Akt results in decreased levels of p27 (Fig. 6E). Moreover, degradation of p27 induced by overexpression of MyD88 can be blocked by a dominant negative version of Akt (Fig. 6E). We have also observed that flagellin treatment of HEK293 cells overexpressing p27 resulted in the accumulation of p27 in the cytoplasm (Fig. 7). Taken together, these data suggest that the proliferative pathway through TLR activation involves the degradation of p27 through the action of Akt.

**DISCUSSION**

We identified in this work a role for TLR signaling in the control of cell proliferation. We found that stimulation of TLR5 expressed in Rat1 cells with flagellin induces cell cycle entry of serum-starved cells and prevents cell cycle exit upon serum withdrawal. Increased proliferation of fibroblasts induced by TLR ligands can have a significant role in several biologically relevant situations. In response to the presence of pathogens, proliferating fibroblasts will be a more efficient source of cytokines and growth factors necessary for adequate disposal of pathogens (26). During the preparation of this work, Rakoff-Nahoum et al. (7) demonstrated that TLRs also play a crucial role in the maintenance of intestinal homeostasis. Induction of intestinal injury in mice revealed a TLR-dependent response to commensal microflora components residing in the lumen of the intestine promoting the secretion of tissue-protective factors and consequently mediated the differentiation and proliferation of epithelial cells required for repair of the intestinal epithelium (7). In addition, TLR5 is highly expressed on intestinal epithelial cells (27), which supports a specialized role for TLR5 potentially in tissue repair. Whereas there is much evidence describing how epithelial cells depend on fibroblast soluble secreting factors to proliferate (28, 29), little is known about the activating components required to induce the proliferation and secretion of cytokines by fibroblasts. Chakravortty and Kumar (30) noted that induction of cell proliferation and collagen synthesis in intestinal subepithelial myofibroblasts was mediated by LPS, whereas Maas-Szabowski et al. (31) have reported that interleukin-1 promoted the growth of human dermal fibroblasts. Therefore, TLR activation permits the clearance of infection through cytokine induction and promotes cell growth in scenarios of tissue repair. We support the consensus that TLR signaling is involved in the control of cellular proliferation and that this effect is mediated through the MyD88-dependent signaling pathway. Interestingly, ligand binding to the endogenous TLR4 and TLR3 induced a similar effect on the cell cycle only when the antiproliferative effect of endogenous IFN was prevented by neutralizing the type I IFN receptor. The type of TLR ligands expressed by bacteria and viruses may have a discriminating effect on fibroblast proliferation through the inability to induce IFNs that mediate growth-inhibitory and immunomodulatory responses (32). Antagonizing the proliferative effects of TLR signaling through the action of interferons is biologically relevant in the case of viral infections as a means to limit the viral spread. Proliferation of cells induced by flagellin was not mediated by soluble factors, and blocking protein secretion during flagellin stimulation of cells did not prevent activation of cyclin D1 production. Therefore, TLR5 signaling can initiate an intracellular program of cdk activation that leads to cell cycle progression.

Importantly, TLR stimulation through TLR5 can overcome cell cycle arrest induced by exogenous expression of cdk inhibitor p27. Similarly to c-myc and E1A (16, 17), TLR5 signaling can rescue cells from growth arrest induced by p27 in Rat1 tet-p27 cells. Furthermore, stimulation of exogenously expressed TLR5 with flagellin, as well as overexpression of MyD88, results in degradation of p27 independently of cyclin E-cdk2. Several reports describe the role of Akt in regulation of p27 function. Akt can phosphorylate p27, preventing its nuclear localization (12, 13). Furthermore, the role of Akt in TLR signaling has been shown previously to be involved in NFκB induction (33–35) via a MyD88 complex with phosphatidylinositol 3-kinase, an upstream modulator of Akt function (33). We have shown that TLR5 signaling induces phosphorylation of Akt, that blocking Akt function restores the levels of p27 following expression of MyD88, and that flagellin stimulation induces accumulation of p27. Because nuclear p27 and Akt might not necessarily meet in the same cellular compartment, one possible hypothesis is that Akt that becomes activated in a complex with MyD88 and IRAK and phosphorylates and induces a sequestration of de novo synthesized p27. The levels of p27 present in the nucleus are then degraded following an SCF\(^{Skp2}\)-dependent ubiquitylation (36) and do not get replenished with newly synthesized p27 in the cytoplasm retained through Akt. One obvious question that needs to be addressed is how p27 becomes degraded in the cytoplasm. It needs to be established whether cyclin-cdk complexes contribute to the phosphorylation of p27 in this process or whether Akt directly phosphorylates and degrades p27 following TLR stimulation. Neither may be the case; Kamura et al. (37) recently described an E3 complex Kip1 ubiquitination-promoting complex (KPC), consisting of KPC1 and KPC2, which interacts with and ubiquitinates p27\(^{Skp2}\) localized in the cytoplasm (37). In conjunction with our work, this supports the hypothesis of a cyclin/cdk-independent degradation of p27 through TLR activation.

We have described a novel association of TLR signaling with cell cycle control, two fields that continue to evolve and now merge. Additional work will need to be performed to further elucidate the biological significance of these findings.

**Acknowledgments**—We thank Drs. Christophe Caux, Bruno Amati, Ruslan Medzhitov, and Massimo Tommasino for critical reading of the manuscript. We also acknowledge the gift of plasmids from Drs. Brian Hemmings and Boudewijn Burgersing.

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J. Biol. Chem. 2005, 280:20620-20627.
doi: 10.1074/jbc.M500877200 originally published online March 23, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500877200

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