Members of the guanine exchange factor (GEF) family of scaffold proteins are involved in the integration of signal flow downstream of many receptors in adaptive immunity. However, the full complement of GEFs that function downstream of Toll-like receptors (TLRs) requires further identification and functional understanding. By systematically integrating expression profiles from immune and epithelial cells with functional studies, we demonstrate that protein kinase A anchoring protein 13 (AKAP13), a scaffold protein with GEF activity, is an activator of NF-κB downstream of TLR2 signaling. Stimulation of the human macrophage cell line THP-1 and epithelial cells with a TLR2 ligand caused a significant up-regulation in AKAP13 mRNA, corresponding to an increase in protein expression. Analysis of TLR2 reporter cell lines deficient in AKAP13 expression revealed significantly reduced NF-κB activation and reduced secretion of interleukin-8 and MCP-1 in response to specific ligand stimulation. Furthermore, NF-κB activation was partially inhibited by a GEF-deficient AKAP13 mutant. AKAP13 was also involved in phosphorylation of JNK but not of extracellular signal-regulated kinase ERK1 and -2 following ligand stimulation. Together, our results suggest that AKAP13 plays a role in TLR2-mediated NF-κB activation and suggest that GEF-containing scaffold proteins may confer specificity to innate immune responses downstream of TLRs.

**AKAP13, a RhoA GTPase-specific Guanine Exchange Factor, Is a Novel Regulator of TLR2 Signaling**

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Oren Shibolet1, Cosmas Giallourakis1, Ian Rosenberg1, Tobias Mueller1, Ramnik J. Xavier1,2, and Daniel K. Podolsky1,2

From the 1Gastroenterology Unit, Center for the Study of Inflammatory Bowel Disease, and 2Center for Computational and Integrative Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Toll-like receptors are essential regulators of innate immunity, initiating a signaling cascade culminating in the activation of nuclear factor κB (NF-κB),3 mitogen-associated protein kinases (MAPK), and interferon response factors. Recent studies have demonstrated that the combinatorial recruitment of adaptor proteins provides an initial level of signaling specificity from the receptors. Uncontrolled activation of TLRs may be detrimental to the host and is therefore tightly regulated. Previous reports show that multiple inhibitory proteins are involved in down-regulating TLR signaling (1). However, knowledge of the full spectrum of mechanisms that “fine tune” specific TLR activation is incomplete.

TLRs can selectively activate the MyD88-dependent pathway (TLR1, -2, -5, -6, -7, -8, and -9), MyD88-independent pathway (TLR3), or both (TLR4) (2) and are localized on intracellular organelles and/or the cell membrane (3, 4). Another way of achieving TLR signaling specificity is through heterodimerization of receptors for optimal signal activation (e.g. with TLR2 and TLR1/6) (5).

Utilization of members of the GTPase family that function as molecular switches is another potential mechanism for regulating TLR signal induction (6). Rho family GTPases control multiple cellular processes and have been previously reported to regulate TLR signaling. GTPases in turn are controlled by GEFs and GTPase-activating proteins (GAPs) that activate and inactivate them, respectively (7). However, only limited data connect GEFs to TLR signaling (8, 9).

Here we show that AKAP13, a scaffold protein that is a member of the A-kinase anchoring protein family and also has GEF activity, is induced by TLR2 ligands and mediates NF-κB activation via TLR2. We also demonstrate that this activation depends on the GEF function of AKAP13 and that AKAP13 knockdown reduces TLR2-induced JNK phosphorylation but not extracellular signal-regulated kinase 1 and 2 (ERK1 and -2) or p38.

**EXPERIMENTAL PROCEDURES**

**Computational Analysis**—Utilizing the PFAM data base, we identified a nonredundant set of 76 genes, which encode for either the RhoGEF domain (PF00621) or dedicator of cytokinesis/DOCK (PF06920) domain in the human genome (supplemental Table 1). The DOCK domain was included because it has been shown to have GEF activity (10).

The cap analysis of gene expression (CAGE) site on the World Wide Web contains 70 clusters based on hierarchical clustering of 159,075 CAGE tag clusters across 23 tissue/cell type conditions (11). The cluster ID = 64 contains 979 cluster tag IDs associated with the 5’ ends of genes that are expressed in immune cells, such as macrophages both at base line and upon activation with agents such as LPS and CpG. These 979 clusters mapped to 746 mouse genes with unique Entrez gene identifiers.
AKAP13 Regulates TLR2 Signaling

In order to analyze the expression pattern of RhoGEF/DOCK genes in the human genome and assess which were enriched in the immune system, we analyzed a compendium of 79 human tissues/cells profiled by microarray technology. The public version of the GNF human expression atlas version 2 (12) was obtained from Novartis (available on the World Wide Web), including the primary .cel files, which used the U133a Affymetrix chip and a custom chip (GNF1H). The data set was filtered by requiring that a probe set have a threshold value of >20 in at least one sample and a maximum-minimum expression value of >100. A total of 28,852 reliable probes met the above filtering criteria. For each, the corresponding UniGene ID and LocusLink ID were identified based on the combined annotation tables provided at the Novartis and NetAffx sites on the World Wide Web. Of the 28,852 probe sets, 26,789 mapped to 16,811 UniGene IDs. To represent the human expression profiles of RhoGEF, hierarchical clustering with the centroid linkage method was performed using DCHIP using the Pearson correlation coefficient, with relative expression levels displayed.

For the 76 RhoGEF/DOCK genes that we identified based on their PFAM domains, at least one probe for 54 of the 76 genes was represented on the arrays and met our filtering criteria as well as the DCHIP default filtering criteria. Heat maps of hierarchical clustering of tissue expression and correlation values are based on a single probe set per gene chosen at random so as not to bias the visual presentation.

To ascertain if any RhoGEF genes were enriched in the immune system of the normal tissue/cell compendium, we used the Wilcoxon rank sum test. To calculate the Wilcoxon statistic, we divided the 79 tissue/cell types into those of immune system origin (n = 22) and those that are not part of the immune system (n = 57), making two sample classes. The p values from the Wilcoxon test for each RhoGEF/DOCK gene were then calculated with a significance threshold considered (p < 0.05). As shown in supplemental Fig. 1, RhoGEF denoted by a Wilcoxon score of p < 0.05 identified by a tick mark on the left of the heat map.

Cell Lines and Antibodies—HEK293 cells, stably transfected with TLR2, were a gift from Dr. Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10 μg/ml ciprofloxacin (Sigma), and 10 μg/ml G418 (Invitrogen). 3T3 RelA-deficient fibroblasts were a gift from Dr. Thomas D. Gilmore (Biology Department, Boston University, Boston MA) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin. Cells were grown at 37 °C in a 5% CO₂ atmosphere within a humidified incubator. Polyclonal rabbit antisera against AKAP13 was kindly provided by Dr. John Scott (Vollum Institute, Oregon Health and Science University, Portland, OR). Polyclonal rabbit antibodies against p65, GFP, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal rabbit antibodies against phospho-JNK, phospho-ERK1/2, phospho-p38, total JNK, ERK1/2, and p38 were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal mouse antibodies against Tollip were purchased from Alexis Biochemicals (San Diego, CA) (Axxora Platform). [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

Plasmids and Small Interfering RNA—FLAG-GFP-tagged and GFP-tagged AKAP13 were a gift from Dr. John Scott (13). All AKAP13 mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). For NF-κB activity determination, cells were transfected using Lipofectamine 2000 (Invitrogen) with 1 ng of Ig-κ pIV luciferase reporter plasmid and Renilla plasmid. Activity was measured using the Dual Luciferase reporter assay system (Promega, Madison, WI) in a BD moonlight 3010 luminometer (BD Biosciences) in accordance with the manufacturer’s instructions and normalized to Renilla activity. siRNA to AKAP13 were purchased from Ambion Inc. (Austin, TX): 1) (ID1475) forward sequence (5’-GGAGAAGGAGAGATTTTCTT) and reverse (5’-AGAATTTTCCTTCCTCCCTT); 2) (ID1569) forward (5’-GAGAAGGATTCTAAGAC) and reverse (5’-GCTTT-TAGAATTTTCC). Several negative control siRNAs were purchased from Ambion Inc. and assessed for their ability to knock down AKAP13 expression. Negative control 1 siRNA (catalog number AM4611) had the least effect and was used in all experiments.

Electrophoretic Mobility Shift Assay—HEK-TLR2 cells were grown in 6-cm dishes and transfected with AKAP13 siRNA or control siRNA. After 60 h, cells were stimulated with 1 μg/ml of palmitoyl-3-cysteine-serine-lysine 4 (pam3CSK4) (Invivogen, San Diego, CA) for 15, 30, or 60 min or with tumor necrosis factor-α for 30 min (R & D Systems, Inc., Minneapolis, MN) as a positive control. Nuclear extracts were prepared according to a protocol previously reported (14). Briefly, cells were washed in 1 ml of ice-cold phosphate-buffered saline, collected by centrifugation (3000 rpm for 5 min at 4 °C), and incubated in buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.3 mM Na₃VO₄, protease inhibitor (complete Mini®, Roche Applied Science)) for 10 min. Cells were then lysed in 0.8% Nonidet P-40 (13,000 rpm for 30 s at 4 °C). The pellet was resuspended in buffer C (20 mM HEPES, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.3 mM Na₃VO₄, 0.4 mM NaCl, and 200 mM Pefabloc SC (Roche Applied Science)) rocked for 15 min at 4 °C, and then centrifuged (15,000 rpm for 5 min at 4 °C). Protein concentration in the supernatant was determined by the Bradford method. competition assays, 1 μl of labeled oligonucleotide was purchased from Santa Cruz Biotechnology. Double strand oligonucleotides were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega Biosciences, San Luis Obispo, CA). The reaction was conducted in a total volume of 20 μl using 10 μg of nuclear protein extract, 1 μl of labeled oligonucleotide, 5 μl of EMSA buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 50 mM NaCl, 50 mM Tris (pH 7.4), 0.4% poly(dI-dC) (0.25 μg/μl), and 4 μl of water. For competition assays, 1 μl of cold or mutant oligonucleotide was added to the reaction. The samples were loaded onto a 4% nondenaturating polyacrylamide/bisacrylamide gel and run in 0.5× TBE buffer. The gel was dried, and the resultant DNA-protein complexes were detected by autoradiography.
**AKAP13 Regulates TLR2 Signaling**

Western Blot Analysis—For the detection of AKAP13, p38 JNK/SAPK, ERK1 and -2, actin, and Tollip, cells were seeded into 12-well dishes and transfected with either AKAP13 siRNA or control siRNA. After 60 h, cells were stimulated with pam3CSK4 as described above and lysed in Nonidet P-40 lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 10 mM Na2P2O7, and protease inhibitor), centrifuged (15,000 rpm for 20 min at 4 °C), and the supernatant was assayed for protein concentration using the Bradford method. Extraction of protein from human intestinal tissue samples was performed following homogenization using the same lysis buffer described above. Equal amounts of protein were solubilized in Tris-glycine SDS sample buffer (Invitrogen) and electrophoresed on 4–12% gradient Novex® Tris-glycine gels (Invitrogen). Following electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes that were incubated with AKAP13- or phospho-MAPK-specific antibodies. Detection using horseradish peroxidase-linked antibody (Amer sham Biosciences) was performed according to the manufacturer’s instructions. Membranes were stripped and reprobed with anti-total MAPK, anti-actin, or anti-Tollip to confirm equal loading.

**Enzyme-linked Immunosorbent Assay (ELISA)**—ELISA kits for the detection of IL-8 and MCP-1 were purchased from BD Biosciences. Cytokine concentrations in cell culture supernatants were determined in accordance with the manufacturer’s instructions.

**Reverse Transcription-PCR**—THP-1 cells were seeded into 12-well plates at a density of 0.8 × 10^6 cells/well and stimulated with 160 nM phorbol 12-myristate 13-acetate (Sigma) for 24 h followed by stimulation with 1 μg/ml pam3CSK4 or 100 ng/ml LPS (Invivogen) for varying times. For reverse transcription, 2 μg of total RNA were reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad). Mouse tissues were obtained from naive C57/B6 mice. All animal experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital and Harvard Medical School. Human intestinal biopsies were obtained from noninflamed tissue from patients with inflammatory bowel disease undergoing routine screening for cancer surveillance. Informed consent was obtained from all patients prior to the procedure, and the protocol was approved by the Human Studies Committee of the Massachusetts General Hospital. Extraction of RNA from mouse and human tissues was performed using Trizol (Invitrogen) according to the manufacturer’s instructions. AKAP13 was PCR-amplified from 2 μl of cDNA using the following PCR program: 95 °C for 120 s, followed by 35 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences for mouse AKAP13 (XM_907140) were 5′-ATGGGGCGGATAGGAGGAGG-3′ (forward) and 3′-GCAAGGAGAAAGCAGCAGGCGGT-5′ (reverse) (161-base pair amplicon), and sequences for mouse GAPDH were 5′-GGAGCCAACGGGTACATCTC-3′ (forward) and 3′-GAGGGGCATCCACAGTCTCTTCT-5′ (reverse) (211-base pair amplicon).

Real time quantitative PCR was performed in the DNA Engine Opticon® 2 system (MJ Research, Boston, MA) using the iQ™SYBR®Green super mix (Bio-Rad). Briefly, 100 ng of reverse transcribed cDNA were used for each PCR with 250 nM forward and reverse primers. The thermal cycling conditions were composed of 4 min at 95 °C, followed by 35 cycles at 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 45 s. The threshold cycle (Ct) values were obtained for the reactions, reflecting the quantity of the template in the sample. AKAP13 ΔCt was calculated by subtracting the calibrator gene GAPDH Ct value from the AKAP13 Ct value and thus represented the relative quantity of the target mRNA normalized to GAPDH mRNA. The mRNA content of AKAP13 in untreated cells was defined as 1 arbitrary unit.

**Immunohistochemistry**—HEK-TLR2 cells were seeded onto Lab Tek® chamber slides (Nalge Nunc International, Rochester, NY) and transfected with AKAP13 siRNA or control siRNA or left untransfected. Sixty hours later, cells were either stimulated with specific ligands or left unstimulated and then washed and fixed in ice-cold 100% methanol. Following fixation, cells were blocked with PBS containing 5% serum of the secondary antibody host and incubated with primary antibody followed by washing and incubating with fluorescein isothiocyanate-secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Following three further washes, the slides were mounted using 4',6-diamidino-2-phenylindole containing mounting media (Vectorshield, Vector Laboratories, Burling ton, CA) and examined in an inverted microscope under a ×40 objective Olympus model AX70 (Olympus America Inc., Center Valley, PA) and a laser confocal microscope model Radiance 2000 (Bio-Rad) using multitracking (line switching) for two-color imaging (×40). Image acquisition was performed with LaserSharp Scanning software (Bio-Rad).

**Rho-binding Domain Pull-down Assay**—HEK-TLR2 cells transfected with the indicated plasmids were grown for 24 h in the presence of 10% serum and were serum-starved for an additional 24 h. Cells were then incubated for 2 and 5 min with 1 μg/ml pam3CSK4. Cells were lysed, and Rho-binding domain pull-down was performed using the EZ-Detect™ Rho activation kit (Pierce) in accordance with the manufacturer’s instructions.

**Statistical Analysis**—Statistical significance between groups was assessed by Student’s t test. Data are expressed as means ± S.D. Triplicate determinations were performed in the IL-8 ELISA experiments, and all experiments were repeated at least three times. A p value equal to or less than 0.05 was considered to be statistically significant. Two-way analysis of variance was used to compare multiple time groups.

**RESULTS**

* A Combined Computational and Experimental Approach Suggests AKAP13 Is a Candidate TLR2 Signaling Cascade
Regulator—A combined computational and experimental approach outlined in Fig. 1 was developed in order to identify GEFs in the human genome that might act as modulators of TLR signaling. In step 1, we utilized the PFAM protein domain data base to generate a compendium of genes that are capable of encoding proteins with known or potential RhoGEF/DOCK activity, ultimately identifying 76 genes in the human genome encoding RhoGEF domains (Fig. 1 and supplemental Table 1; see “Experimental Procedures”). Next, we reasoned that, since a large proportion of TLR signaling components are enriched or selectively expressed in cells of the immune system or mucosal colonic epithelial cells, a bona fide TLR RhoGEF would share these characteristics (15). Accordingly, in step 2, we systematically analyzed the expression profiles of the identified RhoGEF genes on a genome-wide scale utilizing two complementary data sources: microarrays (left) and CAGE technology (right) (11, 12). Analysis of the expression patterns of 54 of 76 RhoGEF genes across a compendium of 79 human tissues/cells, revealed a subset of RhoGEFs (n = 24) co-expressed and selectively enriched in the immune system based on hierarchical clustering and Wilcoxon rank sum testing (p < 0.05) (supplemental Fig. 1). In addition, clustering of CAGE data representing promoter activity across 23 different mouse tissues/cells under various conditions revealed a list of 746 gene signatures enriched for immune/macrophage expression. Among the 746 genes, there were eight RhoGEFs selectively expressed in immune/macrophages as compared with other tissues profiled in this data set. In step 3, we integrated these two data sources via an AND logic rule that candidate TLR RhoGEFs must be enriched in expression in the immune system based on the CAGE data AND microarray profiles. This resulted in the identification of a consensus set of five candidate TLR RhoGEFs. These included AKAP13, which functions as a protein kinase A (PKA)-anchoring protein, and a RhoA-specific GEF. In step 4, we analyzed the expression profile of these five RhoGEFs in an independent data set, since the above two data sets had not included colonic epithelium, identifying AKAP13 as not only enriched in immune cells but also expressed in colonic epithe-
lial cells (step 4.1) (16). We validated our search results by looking at AKAP13 expression in human and mouse colon and ileum. AKAP13 mRNA was expressed in human and murine intestinal tissue. AKAP13 protein was detected by Western blot analysis in human tissue. Currently, available AKAP13 antibodies do not reliably recognize murine AKAP13 (Fig. 1, 4.2).

**AKAP13 Is Expressed in Intestinal Epithelial and Immune Cell Lines**—AKAP13 is a PKA-anchoring protein that also functions as a RhoA-specific GEF. Basal expression of AKAP13 in epithelial and immune cell lines was assessed by reverse transcription-PCR. mRNA encoding AKAP13 was detected in all cell lines examined (SW480, HEK293, and HeLaS3) (Fig. 2A). Protein expression in these cell lines was shown using an AKAP13-specific rabbit antiserum (Fig. 2B). Protein expression in these cell lines was shown using an AKAP13-specific rabbit antiserum (Fig. 2B). To further validate our computational mining and integration strategy, we sought to determine whether AKAP13 and other RhoGEF candidates (ARHGEF2 and DOCK11) were selectively modulated by TLR ligands, such as pam3CSK4 (TLR2) and LPS (TLR4) in a time-dependent manner. Importantly, stimulation of either THP-1 or SW480 cells with TLR2 but not TLR4 ligands resulted in an increase of AKAP13 mRNA expression starting as early as 1 h after stimulation, reaching a peak of 8.5 ± 1.0-fold increase at 3 h and returning to base-line levels at 5 h (Fig. 2C).

**AKAP13 Regulates TLR2 Signaling**

Similar results were obtained when AKAP13 expression was assessed following TLR2 ligand stimulation in HEK293 cells stably transfected with TLR2 (HEK- TLR2) (data not shown). The induction of AKAP13 mRNA following TLR2 stimulation was also found to correlate over time with increasing AKAP13 protein in THP-1 (Fig. 2D). In contrast, ARHGEF2 mRNA peaked at only 2.5 ± 0.3-fold over base line after pam3CSK4 (supplemental Fig. 2A). In order to confirm that the cell lines we used were responsive to TLR ligand stimulation, interleukin-8 mRNA response, which is known to be strongly up-regulated in response to TLR stimulation, was used as a positive control (supplemental Fig. 2B). Thus, by integrating protein domain analysis and gene expression profiles on a genome-wide scale for RhoGEFs, we hypothesized that AKAP13 may be involved in TLR2 signaling, and we focused on elucidating its potential function.

To assess subcellular localization of AKAP13, HeLa cells were transfected with an AKAP13-GFP plasmid and stained with a lipophilic dye (Dil) specific for lipid bilayers in cell membranes and organelles. AKAP13 was localized to the cytoplasm and excluded from the nucleus and cell membrane (Fig. 2E). AKAP13 Is an Activator of NF-κB—To determine whether AKAP13 activates NF-κB, we transfected increasing concen-
trations of AKAP13 expression plasmid into HEK293 and SW480 cell lines and assessed NF-κB activation using a luciferase reporter assay. AKAP13 caused a concentration-dependent activation of NF-κB in HEK293 (Fig. 3A) and SW480 epithelial cells (data not shown). In order to elucidate whether AKAP13 activates NF-κB via the canonical or alternative pathways, AKAP13 expression plasmid was transfected into RelA-deficient 3T3 fibroblasts or control 3T3 cells. AKAP13 did not activate NF-κB in the RelA-deficient cell line as compared with controls, suggesting that AKAP13 activates NF-κB via the canonical pathway (Fig. 3B).

AKAP13 Mediates NF-κB Activation via TLR2 Signaling—The observation that AKAP13 expression is induced by TLR2 ligand stimulation and that AKAP13 activates NF-κB in HEK293 (Fig. 3A) and SW480 epithelial cells (data not shown). In order to elucidate whether AKAP13 activates NF-κB via the canonical or alternative pathways, AKAP13 expression plasmid was transfected into RelA-deficient 3T3 fibroblasts or control 3T3 cells. AKAP13 did not activate NF-κB in the RelA-deficient cell line as compared with controls, suggesting that AKAP13 activates NF-κB via the canonical pathway (Fig. 3B).

AKAP13 Mediates NF-κB Activation via TLR2 Signaling—The observation that AKAP13 expression is induced by TLR2 ligand stimulation and that AKAP13 activates NF-κB suggested that AKAP13 might function as a regulator of TLR signaling pathways, specifically TLR2. We used a siRNA based approach to determine whether AKAP13 is involved in TLR2-induced NF-κB activation in epithelial cells.

siRNA treatment knocked down AKAP13 mRNA and protein by 90 and 80%, respectively (Fig. 4A and B) in HeLaS3 cells. Since an association of AKAP13 with cytoskeletal actin has been previously described, we examined if AKAP13 silencing might disrupt the actin cytoskeleton and interfere with intra-cellular signaling. AKAP13 siRNA was cloned into a pSUPER expression plasmid expressing GFP. HeLaS3 cells were transfected and stained for actin and tubulin. Neither actin stress fibers nor tubulin were disrupted by AKAP13 knockdown (Fig. 4C; data not shown).

We next assessed the effect of AKAP13 knockdown on TLR2-mediated NF-κB activation. Cells were transfected with a NF-κB-luciferase reporter and either siRNA to AKAP13 or control siRNA and stimulated with specific ligand. In HEK-TLR2 cells stimulated with pam3CSK4, AKAP13 knockdown but not control siRNA caused a 65 ± 3.3% reduction in NF-κB activation (Fig. 5A). We performed Western blot analysis that confirmed that AKAP13 but not pathway-associated proteins (Tollip) and actin were knocked down (Fig. 5B). An additional AKAP13-specific siRNA 2 (see "Experimental Procedures") yielded similar results (data not shown), suggesting that the observed effects on NF-κB were indeed AKAP13-specific.

The effect of AKAP13 knockdown on cytokine secretion was evaluated. IL-8 and MCP-1 are proinflammatory cytokines that are secreted following NF-κB activation. AKAP13 knockdown inhibited TLR2-dependent secretion of IL-8 and MCP-1 as assessed by ELISA (Fig. 5C and D).
assay. AKAP13 knockdown caused a reduction in the nuclear content of the NF-κB consensus sequence binding oligonucleotides following stimulation of HEK-TLR2 cells with pam3CSK4 (Fig. 6A). NF-κB binding activity increased progressively over the 60-min time period (Fig. 6B). Specificity of the binding was confirmed by incubation with excess cold oligonucleotides. NF-κB binding was inhibited by cold competitor consensus sequence but not by mutant cold competitor (data not shown). Western blot analysis confirmed AKAP13 knockdown (data not shown).

We next assessed NF-κB p65 subunit nuclear translocalization following stimulation by immunofluorescent staining using anti-NF-κB p65 subunit antibody. AKAP13 siRNA but not control siRNA decreased p65 nuclear translocalization following stimulation with pam3CSK4 in HEK-TLR2 cells (Fig. 6C).

**AKAP13 GEF Function Modulates TLR2 Signaling**—TLR2 activation of NF-κB is reportedly associated with RhoA activation (17). We hypothesized that the RhoA-specific GEF function of AKAP13 might play a role in TLR2 signaling. To identify the specific domain involved in NF-κB activation via TLR2 signaling, several AKAP13 mutants were generated containing point mutations previously reported to alter specific AKAP13 functions (Fig. 7A). Only the mutation at tyrosine 2153 of AKAP13, which reduces interaction with RhoA and significantly interferes with GEF function, affected TLR2 signaling (13). Thus, transfection of the GEF-inactive (Y2153F) mutant but not the PKA binding domain-deficient mutant (A1251P/I1260P), the 14-3-3 binding domain-deficient mutant (S1565A), or the protein kinase D binding site-deficient mutant (S2737A), caused a reduction in NF-κB activation following stimulation with pam3CSK4 in the HEK-TLR2 cell line (Fig. 7B). The pleckstrin homolog-deficient mutant (W2324L) caused a small decrease in NF-κB activation that did not reach statistical significance. Western blots of total cell lysates confirmed that similar amounts of the mutants were expressed (Fig. 7C).

TLR2 ligand stimulation with heat-killed *Staphylococcus aureus* was previously shown to rapidly activate RhoA, with maximal activation ~5 min following stimulation (17). We therefore assessed RhoA activation following TLR2 stimulation in cells transfected with the AKAP13 GEF-deficient mutant using a Rho-binding domain pull-down assay. As previously shown, stimulation of HEK-TLR2 cells with a TLR2 ligand caused activated RhoA (data not shown). Transfection of the AKAP13 GEF-deficient mutant but not empty vector caused a significant inhibition of TLR2-induced RhoA activation (Fig. 7D and E). Together, these studies suggest that ectopic expression of the GEF-inactive mutant attenuates TLR2-mediated NF-κB activation via a RhoA-dependent pathway.
AKAP13 Regulates TLR2 Signaling

**DISCUSSION**

Toll-like receptors initiate signaling pathways that shape innate immunity. Previous studies have demonstrated that GTPases and phosphatidylinositol 3-kinase function as molecular switches in TLR activation (6, 20). GTPase signaling activity is regulated at multiple levels by GEFs, GAPs, and GDP dissociation inhibitors. In this report, we have focused on identifying GEFs that regulate responses induced by TLR2 ligands in epithelial cells. Using computational approaches and functional studies, we demonstrate that AKAP13, a RhoA-specific GEF and scaffold protein, regulates TLR2 signaling. Previously, limited data connecting GEFs to TLR signaling has been available. Inhibition of Vav1, a GEF for Rho family GTPases, by a specific Src-related tyrosine kinase inhibitor, blocked tumor necrosis factor secretion and nitric-oxide synthase accumulation in response to TLR9 stimulation (9). CpG-DNA-mediated up-regulation of NF-κB activity (but not MAPK activation) was also blocked by Vav1 inhibition in human macrophages (9). It was further shown using Vav1,2,3 null macrophages that Vav proteins are essential for transducing inflammatory signals emanating from MyD88, a key regulator of the TLR pathway (21). Further, the GEF for ARF-GTPase, cytohesin-1, is involved in CD14/TLR2-mediated phagocytosis of mycobacteria by human macrophages. Knockdown of cytohesin-1 resulted in a marked attenuation of phagocytosis of mycobacteria (22).

By using a computational screen, we identified the 76 Rho-GEF-encoding genes in the human genome. We reasoned that given that the majority of TLR signaling components exhibit high immune specificity, those RhoGEF genes that also exhibited preferential expression in the immune system represented likely RhoGEF candidates for TLR signaling modulation. We further asked, of the top five candidate TLR RhoGEFs, which exhibited transcriptional regulation themselves by TLR stimuli, given that the majority of TLR signaling components exhibit high immune specificity, those RhoGEF genes that also exhibited preferential expression in the immune system represented likely RhoGEF candidates for TLR signaling modulation. We further asked, of the top five candidate TLR RhoGEFs, which exhibited transcriptional regulation themselves by TLR stimuli, since it has been observed that TLR signaling components can be involved in feedforward or feedback circuits (23, 24). In this regard, we observed that AKAP13 specifically exhibited robust up-regulation of mRNA and protein expression following TLR2 ligand, but not TLR4 ligand, stimulation. Of note, AKAP13 has two conserved NF-κB transcription factor binding sites, the first −137 bases and the second +317 bases from the transcriptional start site, suggesting that it may be an NF-κB target. Thus, integrating multiple forms of data led us to focus on the functional role of AKAP13 in TLR2 signaling.

AKAP13 has been shown to function as a GEF for RhoA-GTPase and a scaffold anchor for PKA regulating the secondary pathway playing a role in many aspects of the immune response (18). Although the ERK1/2 pathway can be activated by Ras family GTPases (19), p38 and JNK are activated by Rho family GTPases. Following TLR engagement by ligand, signal transduction by the MyD88-dependent pathway leads to MAPK activation. We therefore assessed the role of AKAP13 in MAPK activation. HEK-TLR2 cells were transfected with AKAP13 siRNA and stimulated with specific ligand, and MAPK phosphorylation was determined using phosphospecific antibodies. AKAP13 knockdown diminished JNK phosphorylation 15 and 30 min following stimulation (Fig. 8A). JNK phosphorylation returned to base line 1 h after stimulation (data not shown). In contrast, phosphorylation of ERK1/2 and p38 was unaffected by AKAP13 knockdown (Fig. 8, B and C, respectively).

**FIGURE 7.** GEF function of AKAP13 was involved in NF-κB activation following TLR2 stimulation. A, the structure of AKAP13 is depicted. The mutations used in B are shown. HEK293 cells stably transfected with TLR2 were transiently transfected with NF-κB luciferase reporter/Renilla and with either GEF-deficient (Y2153F), pleckstrin homology-domain-deficient (W2324L), PKA binding site-deficient (A1251P/I11560P), protein kinase C binding site-deficient (A1251P/I11560P), protein kinase C binding site-deficient (S1565A) mutants of AKAP13 or not transfected (NT). B, cells were either stimulated with pam3CSK4 or left untreated (NS). After 6 h, luciferase activity was measured. Results represent mean ± S.D. of three independent experiments. *, p < 0.05 for Y2153F versus nontransfected (two-way measures of analysis of variance for group). C, Western blot analysis with anti-GFP antibodies confirmed equal expression of the various mutants. HEK293 cells stably transfected with TLR2 were transfected with either the GEF-deficient mutant or empty vector (GFP-C) for 24 h in the presence of 10% serum. After an additional 24 h of serum starvation, cells were stimulated with 1 μg/ml pam3CSK4 for 5 min or left untreated. Cell lysate was incubated with glutathione S-transferase-Rho-binding domain beads, and the bound activated RhoA (top) or total RhoA (bottom) was detected with monoclonal anti-RhoA antibody. D, blot from a representative experiment. E, densitometric quantitation of the ratio between activated RhoA and total RhoA from three experiments.
AKAP13 is involved in protein kinase D activation via recruitment of protein kinase C and phosphorylation of PKA in a complex that constrains the action of these broad-spectrum kinases (25). AKAP13 is also involved in actin stress fiber formation via a trimeric complex composed of AKAP13, RhoA, and PKA (13), in activation of serum response elements via G-coupled receptors (26), and, as recently shown, in mediating α1 adrenergic receptor-induced cardiomyocyte hypertrophy (27).

Since multiple TLR signaling components are capable of activating NF-κB upon ectopic expression, we initially tested the ability of AKAP13 to promote NF-κB activation. Consistent with a previous report, expression of AKAP13 enhanced NF-κB activation (28). AKAP13 failed to activate NF-κB in RelA-deficient fibroblasts as compared with control NIH-3T3 fibroblasts, allowing us to surmise that AKAP13 activates NF-κB via the canonical pathway.

To elucidate a potential functional role in TLR2-induced NF-κB activation, we used an siRNA approach. However, prior to focusing on a regulatory role for AKAP13 in TLR2 signaling, we established that transient AKAP13 knockdown did not cause cell death or cytoskeletal alterations in HeLaS3 epithelial cells. Functional studies demonstrated that stimulation with a TLR2 ligand following AKAP13 knockdown in HEK293 cells stably transfected with TLR2 resulted in a reduction in NF-κB activation and p65 nuclear translocation, which was corroborated by a corresponding decrease in proinflammatory cytokine secretion. Furthermore, preliminary findings suggest that AKAP13 siRNA reduced TRAF6-mediated NF-κB activation (data not shown).

Following siRNA experiments, we analyzed the structure-function relationships of the various domains of AKAP13 in TLR2-mediated signal transduction. The GEF-deficient mutant Y2153L was found to exhibit decreased activation of NF-κB following TLR2 ligand stimulation, suggesting that AKAP13 GEF function is involved in mediating TLR2 signaling. This result is consistent with the function of AKAP13 as a GEF-specific for RhoA and a previous report showing RhoA-dependent TLR2 signaling regulation of the canonical IκB-kinase-mediated pathway induction of NF-κB (17). Interestingly, a mutation affecting the pleckstrin homology domain in AKAP13 had little effect on NF-κB activation. This finding is in agreement with previous reports suggesting that the Dbl and pleckstrin homology domains of AKAP13 have distinct and specific roles (29–31).

In addition to mediating NF-κB, our study revealed that AKAP13 also moderates TLR2 activation via JNK but not on ERK1/2 or p38. Two previous reports have connected Brx and Lbc, two fragments of AKAP13, to MAPK activation in other cellular contexts. Lbc induced activation of ERK2 in a RhoA-associated integrin-mediated activation of MAPK (32), whereas Brx activated estrogen receptor β by a p38- but not ERK-dependent pathway (33). Other AKAP family members and RhoA-GEFs are also associated with MAPK activation. The muscle-specific AKAP contains components of the ERK signaling pathway (34), and the Rho-GEFs Net1 and p115RhoGEF interact with CNK1, MLK2, and MKK7, components of the JNK signaling cascade (35), protein kinase C isoform α, which

**FIGURE 8.** AKAP13 modulated phosphorylation of JNK but not of p38 and ERK1/2 following TLR2 stimulation. HEK293 cells stably transfected with TLR2 were transiently transfected with siRNA against AKAP13, negative control siRNA oligonucleotides or not transfected. Cells were stimulated with pam3CSK4 for 0, 15, and 30 min. The expression of phospho-JNK (top) phospho-ERK1/2 (middle), and phospho-p38 (bottom) were analyzed by Western blotting using anti-phospho-specific antibodies (top panel for each blot). Blots were stripped and reprobed with anti-total-specific antibody (lower panel for each blot). The graphs represent mean ± S.D. of three independent experiments (two-way measures analysis of variance; *, p < 0.05 for time and group). Blots are from one representative experiment.
AKAP13 Regulates TLR2 Signaling

AKAP13 binds AKAP13 (25), modulates TLR2-mediated JNK activation (36), although no change in protein kinase C phosphorylation was observed following AKAP13 knockout in our study (data not shown).

Oda and Kitano (24) recently suggested that GTPases play a major role in mediating TLR signaling and operate as separate and distinct modules incorporating signals downstream of MyD88. In this regard, GEF modules may be predicted to impact TLR signaling. In the present studies, AKAP13, which contains both GEF and scaffolding domains, was found to be induced by TLR2 stimulation and regulate TLR2 signaling, suggesting a possible positive feedback loop effect.

In summary, this study identifies AKAP13 as a novel regulator of TLR2 signaling and demonstrates a role for the GEF domain in modulating NF-κB activation. Furthermore, AKAP13 knockdown was shown to regulate JNK phosphorylation. Additional work is necessary to identify AKAP13 binding partners in TLR2 signaling and the relative importance of its scaffolding versus catalytic function. Importantly, our data demonstrate the power of bioinformatics data mining in identifying functionally important TLR regulators highlighting the role of GEF proteins as important regulators of innate immunity.

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