Phosphoinositide 3-kinase is activated by MUC1 but not responsible for MUC1-induced suppression of Toll-like receptor 5 signaling

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Submitted 26 October 2006; accepted in final form 11 June 2007

Kato K, Lu W, Kai H, Kim KC. Phosphoinositide 3-kinase is activated by MUC1 but not responsible for MUC1-induced suppression of Toll-like receptor 5 signaling. Am J Physiol Lung Cell Mol Physiol 293: L686–L692, 2007. First published June 22, 2007; doi:10.1152/ajplung.00423.2006.—MUC1 is a membrane-tethered mucin-like glycoprotein expressed on the surface of various mucosal epithelial cells as well as hematopoietic cells. Recently, we showed that MUC1 suppresses flagellin-induced Toll-like receptor (TLR) 5 signaling both in vivo and in vitro through cross talk with TLR5. In this study, we determined whether phosphoinositide 3-kinase (PI3K), a negative regulator of TLR5 signaling, is involved in the cross talk between MUC1 and TLR5 using various genetically modified epithelial cell lines. Our results showed 1) activation of MUC1 induced recruitment of the PI3K regulatory subunit p85 to the MUC1 cytoplasmic tail (CT) as well as Akt phosphorylation, 2) MUC1-induced Akt phosphorylation required the presence of Tyr20 within the PI3K binding motif of the MUC1 CT, and 3) mutation of Tyr20 or pharmacological inhibition of PI3K activation failed to block MUC1-induced suppression of TLR5 signaling. We conclude that whereas PI3K is downstream of MUC1 activation and negatively regulates TLR5 signaling, it is not responsible for MUC1-induced suppression of TLR5 signaling.

MUC1 (MUC1 IN HUMAN AND Muc1 in nonhuman species) is a transmembrane (TM) mucin-like glycoprotein expressed on the surface of epithelial cells lining various mucosal organs including the respiratory, gastrointestinal, and reproductive tracts as well as by various hematopoietic cells (4, 7, 13). MUC1 also is overexpressed by most carcinomas, including more than 90% of mammary cancers (7). The deduced amino acid sequence of the MUC1/Muc1 gene indicates a three-domain structure of the protein, an NH2-terminal extracellular (EC) domain, a TM domain, and a COOH-terminal cytoplasmic tail (CT) domain (8, 18, 19). The EC domain contains variable numbers of 20 amino acid tandem repeats that can be repeated more than 120 times (12). The CT domain contains 72 amino acids, 7 of which are evolutionarily conserved tyrosines that are potential sites of phosphorylation. MUC1 CT tyrosine phosphorylation occurs within consensus sequence motifs for various signaling kinases and adaptor proteins including phosphoinositide 3-kinase (PI3K), Shc, PLCγ1, c-Src, and Grb-2 (37, 40). We have shown that Muc1 is a receptor for Pseudomonas aeruginosa flagella (21) and results in phosphorylation of its CT as well as activation of the MAPK or ERK (22).

Toll-like receptors (TLRs) belong to a family of type I TM proteins recognizing a wide variety of microbial components and play a crucial role in the innate immune system (14). Currently, 11 TLRs have been identified. TLR5 specifically recognizes flagellin, the major constituent of bacterial flagella and a virulence factor for gram-positive and gram-negative bacteria (9, 11). TLR5 engagement by flagellin leads to activation of the transcription factors NF-κB and activator protein-1 (AP-1) in a myeloid differentiation factor 88 (MyD88)/IL-1 receptor-associated kinase (IRAK)-dependent manner and induces proinflammatory cytokine production (5, 27, 30, 33). Because both MUC1/Muc1 and TLR5 are located on the airway epithelial cell surface and recognize bacterial flagellin as a common ligand, it is possible that these two flagellin receptors cross talk when they are exposed to flagellin during bacterial infection. In our recent publication, we (23) demonstrated that MUC1/Muc1 plays an anti-inflammatory role during airway bacterial infection based on the following results: 1) mice lacking Muc1 expression revealed significantly enhanced inflammatory responses when treated intranasally with either P. aeruginosa or its flagellin; 2) both primary airway epithelial cells and alveolar macrophages from Muc1 null mice exhibited significantly greater inflammatory responses to flagellin compared with those from their wild-type littermates; 3) knockdown of MUC1 in polarized nontransformed human bronchial epithelial cells (NHBE) resulted in an increase in flagellin-induced inflammatory responses; and 4) overexpression of MUC1 in a human epithelial cell line suppressed flagellin-induced TLR5 signaling. In this study, we sought to identify the mechanism of cross talk between MUC1/Muc1 and TLR5.

A number of reports indicated that PI3K suppresses inflammation during the early stage of bacterial infection (see Ref. 6 for review). In support of this notion, Yu et al. (39) have recently demonstrated that TLR5-mediated PI3K activation negatively regulates flagellin-induced proinflammatory gene expression. In this report, we focused on the possible role of PI3K in mediating the suppressive effect of MUC1 on flagellin-induced proinflammatory response.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. The antibodies (Abs) and reagents and their sources were anti-CD8 Ab A (mouse monoclonal; Serotec, Raleigh, NC), anti-mouse IgG (Serotec), anti-CD8 Ab B (rabbit

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polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Akt (pAkt)/Akt Abs and anti-p85 Ab (rabbit polyclonal, Cell Signaling Technology), anti-β-tubulin Ab (mouse monoclonal), CT33 Ab (rabbit polyclonal recognizing MUC1 CT; Ref. 22), horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary Abs (KPL, Gaithersburg, MD), LY-294002 (Cell Signaling Technology), and wortmannin (Alexis Biochemicals, San Diego, CA). Anti-CD8 Ab A was used for activation of the chimera, whereas anti-CD8 Ab B was used for immunoprecipitation (IP) as originally described (24). Flagellin was isolated from P. aeruginosa strain K (PAK) as described previously (9).

**Construction of mutant plasmids and stable cell lines.** pCD8/MUC1 in which (9) (whereas the domain of CD8 covalently linked to the CD8 transmembrane domain of CD8 covalently linked to the CD8 transmembrane domain of CD8) was kindly provided by Dr. Andrew Gewirtz (Emory University, Atlanta, GA). This mutant lacks the intracellular Toll/IL-1R homology region of TLR5 and has been shown to fail to respond to flagellin (9), presumably by soaking up the ligand or preventing dimerization with wild-type proteins.

**Cell culture.** HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD). All the stably transfected HEK293 cell lines were maintained in the presence of 800 μg/ml G418 (GIBCO BRL) (38).

**CD8 Ab treatment and IP/immunoblotting.** In experiments designed to activate MUC1 CT, HEK293-CD8/MUC1, or HEK293-CD8/MUC1(Y20F) cells were starved in serum-free DMEM at 37°C in 5% CO2 for 16 h and treated at 37°C with 5.0 μg per 500 μl per well of anti-CD8 Ab A or isotype-matched normal mouse control IgG in serum-free medium. In experiments intended to inhibit dephosphorylation of phosphorylated tyrosine moieties, HEK293-CD8/MUC1 cells were starved in serum-free DMEM at 37°C in 5% CO2 for 4 h and pretreated with protein phosphatase inhibitor pervanadate (50 μM Na3VO4 and 0.6 mM H2O2; Ref. 25) for 30 min followed by stimulation with flagellin (200 ng/ml) in serum-free medium [24-well plates for pAkt immunoblotting (IB) and 6-well plates for IP]. For direct IB analysis, the cells were lysed on ice for 20 min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, protease inhibitor cocktail, and 1% Nonidet P-40. The lysates were centrifuged at 12,000 g for 10 min at 4°C, and protein concentrations were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). For IP, lysates containing equal amounts of protein were IPed with anti-CD8 Ab B and protein A agarose (Invitrogen) at 4°C for 16 h with continuous agitation. IPs were separated by SDS-PAGE and electroblotted to polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA). Positive control lysates for pAkt from HEK293 cells were treated with 0.6 mM Na3VO4 and 0.6 mM H2O2 for 15 min, which were mixed immediately before the treatment. Membranes were blocked in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (henceforth, TBS buffer) containing 0.05% Tween 20 and 5% nonfat dry milk for 30 min and incubated with primary Abs to Akt, pAkt, or p85. β-Tubulin Ab was used as a control to confirm equal gel loading of the samples. IBs were washed and incubated with appropriate secondary Abs and visualized using SuperSignal West Pico chemiluminescence reagents (Pierce).

**Transient transfection and luciferase reporter assay.** HEK293, HEK293-MUC1, or HEK293-control cells were plated in 24-well plates (4.8 × 104 cells per plate) and transfected with a NF-κB luciferase reporter, Renilla reporter, and human TLR5 expression plasmids (400 ng/well; Ref. 23) together with or without pCD8/MUC1 or pCD8/MUC1(Y20F) (200 ng/well) using Lipofectamine 2000 according to the manufacturer’s protocol. At 24 h posttransfection, the cells were pretreated with or without wortmannin (100 nM) for 30 min and treated with flagellin (100 ng/ml) for 6 h. HEK293-CD8/MUC1 and HEK293-CD8/MUC1(Y20F) cells were cotreated with anti-CD8 Ab A or negative control IgG. Relative luciferase activity was determined by normalizing with Renilla luciferase activities. The total amount of plasmid DNA was kept constant by adding the empty vector (pCDNA3.1) for each transfection. All assays were performed in triplicate, and single representative experiments are shown. Data are expressed as means ± SE.

**RESULTS**

**Stimulation of HEK293-CD8/MUC1 cells with anti-CD8 Ab activates the PI3K/Akt signaling pathway.** We previously showed that treatment of HEK293-CD8/MUC1 cells with anti-CD8 Ab increased phosphorylation at four of the seven tyrosine residues in the MUC1 CT (Y20, Y29, Y46, and Y60; Ref. 37). Y20, once phosphorylated, forms the canonical sequence motif (pTyr-X-X-Met) for binding to the Src homology 2 (SH2) domain on the p85 subunit of PI3K. Because it is well-known that activation of PI3K by various cell surface receptors results in phosphorylation of Akt, its downstream kinase (3), we determined whether activation of MUC1 also results in PI3K activation as well as Akt phosphorylation. HEK293-CD8/MUC1 cells were treated with anti-CD8 Ab or control IgG for various time periods before harvesting the cell lysates and subjecting them to IB using anti-pAkt Ab specifically recognizing pSer473, the site that is specifically phosphorylated following activation of PI3K (2). Figure 1A shows that...
the levels of pAkt increased significantly even at 15 min following treatment with anti-CD8 Ab but not with control IgG. In light of our (37) previous data showing the phosphorylation of Y20 following stimulation of these cells with anti-CD8 Ab, these results suggested that anti-CD8 Ab induced the activation of PI3K and subsequent phosphorylation of Akt in HEK293-CD8/MUC1 cells. To confirm the involvement of PI3K in anti-CD8 Ab-induced Akt phosphorylation, we pretreated the cells with two specific inhibitors for PI3K before stimulation with anti-CD8 Ab. As seen in Fig. 1B, pretreatment of the cells with either wortmannin or LY-294002 blocked anti-CD8 Ab-induced Akt phosphorylation. Together, these results indicated that phosphorylation of the MUC1 CT at Y20 induced Akt phosphorylation via activation of PI3K.

Stimulation of HEK293-CD8/MUC1 cells with anti-CD8 Ab recruits PI3K to MUC1 CT. Having shown that stimulation of CD8/MUC1 activated the PI3K-Akt signaling pathway, we next examined whether stimulation of CD8/MUC1 resulted in physical interaction between the MUC1 CT and PI3K as predicted from both phosphorylation of Y20 following CD8/MUC1 stimulation (37) and the presence of Y20 within the consensus motif sequence for PI3K binding. Because PI3K consists of two subunits, the regulatory subunit p85, which binds to its consensus motif sequence via a SH2 domain, and the catalytic subunit p110, which is activated upon p85 binding to its consensus motif sequence (3), we focused on the interaction between MUC1 CT and the p85 subunit. Cells were stimulated with anti-CD8 Ab, and cell lysates were IPed using anti-CD8 Ab followed by IB using anti-p85 Ab. As shown in Fig. 2A, cells stimulated with anti-CD8 Ab contained CD8/MUC1 associated with the p85 subunit, and this physical association was clearly detectable even at 2 min following anti-CD8 Ab treatment. In contrast, cells expressing a mutant CD8/MUC1 in which Y20 of MUC1 CT was replaced with phenylalanine failed to associate CD8/MUC1(Y20F) with the p85 subunit (Fig. 2B) indicating the phosphorylation of Y20 of MUC1 CT followed by the recruitment of PI3K upon activation of CD8/MUC1. Next, we determined whether Y20 on the MUC1 CT was involved in Akt phosphorylation after stimulation with anti-CD8. Figure 3 shows that whereas CD8/MUC1 cells phosphorylated Akt at all the time points tested (10, 15, and 20 min) following anti-CD8 Ab treatment, CD8/MUC1(Y20F) cells could not, even up to 20 min following the treatment, indicating that the presence of Y20 is necessary for anti-CD8 Ab-induced Akt activation. Collectively, these results indicated that stimulation of CD8/MUC1 cells with anti-CD8 Ab recruited PI3K to MUC1 CT through physical interaction between Y20 of MUC1 CT and p85 of PI3K, which, in turn, phosphorylated Akt. Thus these data indicate the functional relevance of p-Y20 of MUC1 CT in direct activation of PI3K/Akt pathway. Indirect association of MUC1 with PI3K/p85 through contact with c-Src was also demonstrated by Al Masri and Gendler (1).

Suppression of TLR5 signaling by MUC1 does not require PI3K binding to MUC1 CT. PI3K has been shown to play an anti-inflammatory role in a number of systems (6). This notion has recently been supported by Yu et al. (39), in which flagellin-induced proinflammatory gene expression was enhanced in the presence of PI3K inhibitors. Given the anti-inflammatory role of MUC1 during P. aeruginosa infection as well as intranasal instillation of flagellin (23), the interaction of PI3K with MUC1 CT, and the anti-inflammatory role of PI3K, we hypothesized that the anti-inflammatory activity of MUC1 exhibited during P. aeruginosa infection was mediated through the activation of PI3K. First, we determined whether or not MUC1 activates PI3K/Akt signaling in response to flagellin. Because HEK293 cells express endogenous TLR5 (23, 34), the activation of which also induces PI3K/Akt activation (39), we first suppressed TLR5 signaling with a dominant negative mutant of TLR5 (9) before treatment with flagellin. As shown in Fig. 4, treatment of HEK293-MUC1 cells with flagellin resulted in Akt phosphorylation, which was not observed in the HEK293-control cells (which do not express MUC1). These results indicate that the expression of MUC1 was attributable to the flagellin-induced Akt phosphorylation. Next, we wanted to confirm the published results that both MUC1 and PI3K are anti-inflammatory. HEK293-MUC1 or HEK293-control cells were transiently transfected with a NF-κB luciferase reporter plasmid followed by incubation for 24 h before treating with flagellin for 6 h, and activation of NF-κB was measured by luciferase assay as previously described (17). Figure 5 shows that flagellin increased NF-κB activity by 4.5-fold in HEK293-control cells, whereas there was no significant effect of flagellin in HEK293-MUC1 cells, indicating that the proinflammatory effect of flagellin can be completely inhibited by overexpression of MUC1. On the other hand, treatment of cells with
to determine whether Y20 of MUC1 CT is required for the anti-inflammatory activity of MUC1. Cells expressing either CD8/MUC1 or its Y20F mutant were treated with flagellin, and NF-κB activities were monitored by measuring luciferase activity. As predicted, flagellin increased NF-κB activity dramatically in cells transfected with empty vector alone whereas overexpression of CD8/MUC1 almost completely abrogated the flagellin effect (Fig. 6). In addition, there was no significant difference between the wild-type CD8/MUC1 and its Y20F mutant, suggesting that Y20 was not required for the anti-inflammatory effect of MUC1. We then examined whether the anti-inflammatory activity of MUC1 could be increased by stimulation of MUC1 CT with anti-CD8 Ab. As can be seen in Fig. 7, treatment with anti-CD8 Ab further suppressed NF-κB activity compared with control IgG, suggesting a small but significant contribution of MUC1 CT phosphorylation to its anti-inflammatory activity. However, there was no significant difference in the responsiveness to anti-CD8 Ab between cells transfected with pCD8/MUC1 and pCD8/MUC1(Y20F), suggesting that Y20 also was not responsible for the additional suppressive effect of activated MUC1. Taken together, these results indicated that the presence of Y20 is not responsible for the anti-inflammatory activity of either “unstimulated” or “stimulated” MUC1.

**DISCUSSION**

MUC1/Muc1 has been extensively studied as a tumor antigen (7) and has also been shown to play a negative role in TLR5 signaling following flagellin treatment (23). Overexpression of MUC1 in HEK293 cells resulted in complete suppression of flagellin-induced IL-8 release, and NHBE cells grown at an air-liquid interface expressing TLR5 released much greater amounts of IL-8 in response to flagellin when MUC1 had been knocked down compared with MUC1/TLR5-coexpressing cells. The anti-inflammatory role of Muc1 was also demonstrated in vivo. Muc1 null mice showed greater inflammatory responses compared with their wild-type littermates.
following either intranasal instillation of *P. aeruginosa* or its flagellin. In this study, we sought to understand the mechanism of cross talk between MUC1 and TLR5 focusing on PI3K as a potential link between the two flagellin receptors. Our results showed that whereas stimulation of MUC1 by an exogenous ligand clearly resulted in activation of the PI3K-Akt signaling pathway through interaction between the MUC1 CT and PI3K, either pharmacological inhibition of PI3K or disrupting the CT PI3K binding motif failed to abolish the suppressive effect of MUC1 on flagellin-induced proinflammatory responses. Therefore, we concluded that the negative regulation of TLR5 signaling by MUC1 does not involve activation of PI3K.

The role of PI3K in TLR signaling has been extensively studied in dendritic cells in the context of T helper type 1 (Th1) and Th2 responses. The amount of IL-12 produced by stimulation through TLRs is crucial in determining the balance between Th1 and Th2 responses (28, 36). PI3K has been shown to have a negative regulatory role during induction of the Th1 immune response by suppressing the production of IL-12 by dendritic cells (6). In dendritic cells, PI3Ks are activated by many distinct stimuli that can activate various TLRs (6). Although the signal transduction pathways that activate PI3K downstream of TLRs are not completely characterized, the fact that PI3K is activated after triggering of multiple different TLR members suggests the presence of “shared” signaling pathway(s) for TLR-mediated activation of PI3K (6). Recently, the negative regulation by PI3K has also been demonstrated in intestinal epithelial cells as well as PI3K-null mice in which either blockade or absence of PI3K enhanced flagellin-induced proinflammatory responses (39). On the contrary, Rhee et al. (31) demonstrated that blocking PI3K activation reduced IL-8 production induced by flagellin in human colonic epithelial cells, suggesting the positive regulation of TLR5 by PI3K. Our results with HEK293 cells, however, support the negative regulatory role of PI3K because inhibition of PI3K by wortmannin resulted in an enhancement of flagellin-induced NF-κB activation (Fig. 5) as well as stimulation of IL-8 promoter (data not shown). The fact that the inhibition of PI3K in MUC1 expressing cells could not abrogate the suppressive effect of MUC1 on flagellin-induced NF-κB activation (Fig. 5) strongly suggested that PI3K is not responsible for the anti-inflammatory effect of MUC1. This notion was confirmed by an additional experiment in which mutation of the PI3K binding site in the MUC1 CT (Y20F) did not prevent the suppressive effect of MUC1 (Fig. 6).

This study also demonstrates that the anti-inflammatory effect of MUC1 is mainly attributable to its CT domain and not its EC domain because CD8/MUC1, which is devoid of the MUC1 EC domain, exhibited almost a complete inhibition (84%) of the stimulatory effect of flagellin (Fig. 6). Activation of the MUC1 CT with anti-CD8 Ab showed a small (~20%) but significant suppression of flagellin-induced NF-κB activation compared with the IgG control, suggesting that tyrosine phosphorylation may further increase the suppressive effect of MUC1 (Fig. 7). However, given the presence of continuous phosphorylation and dephosphorylation of the MUC1 CT domain even in unstimulated cells (40), it is possible that phosphorylation on MUC1 CT could be extremely important for its anti-inflammatory function.

What are the possible mechanisms for the cross talk between MUC1 and TLR5? It has been shown that transcription of IL-8 is positively regulated by NF-κB and AP-1 through their specific binding to the IL-8 promoter (32). AP-1 activation is mostly mediated by MAPKs such as JNK, p38, and ERK1/2 in TLR signaling (14). Based on a report showing that PI3K plays a major role in MEK-independent, prolonged MAPK activation by PDGF signaling in Swiss 3T3 fibroblasts (10), a PI3K-MAPK-AP-1 signaling pathway might exist in inflammatory cells. However, this signaling pathway does not seem to work in TLR signaling based on the recent report by Yu et al. (39) showing that flagellin-induced PI3K activation suppresses the activity of MAPKs (p38 and ERK1/2) resulting in reduced IL-8 production. Furthermore, the ability of MUC1 to activate ERK1/2 (10, 22) yet dramatically suppress IL-8 production seems to support neither the PI3K-MAPK-AP-1 pathway for inflammation nor its interaction with this signaling pathway. On the other hand, the ability of MUC1 to suppress a TLR5-induced increase in NF-κB activity independent of PI3K activity (Fig. 5) seems to point to the NF-κB signaling pathway as a possible site of interaction. In addition to PI3K, other negative regulatory mechanisms for TLR signaling have been described. Kobayashi et al. (16) showed that IL-1 receptor-associated kinase-M (IRAK-M) is induced upon TLR stimulation and negatively regulates TLR signaling by preventing dissociation of IRAK and IRAK-4 from MyD88 and formation of IRAK-TNF receptor-associated factor 6 (TRAF6) complexes. Blockade of IRAK release by a mechanism that did not require protein synthesis has also been reported by Mizel and Snipes (26). More recently, Sun et al. (35) demonstrated that inhibition of TLR5-associated IRAK-4 activity is likely to be the cause of flagellin-induced tolerance in polarized intestinal cells. As an another negative regulatory mechanism, Kinjo et al. (15) and Nakagawa et al. (29) independently demonstrated that the suppressor of cytokine-signaling-1 (SOCS1/JAB) is rapidly induced by lipopolysaccharide (LPS) and negatively regulates LPS signaling using SOCS1-null mice and the cells derived from these mice. Thus, whereas PI3K functions at the early phase of TLR signaling and modulates the magnitude of the primary activation, inhibition of IRAK activity and expression of SOCS-1 are induced by TLR signaling.

**Fig. 7.** Activation of MUC1 induces further suppression of TLR5 signaling regardless of the presence of Y20 in the MUC1 CT. The experimental procedures were identical to those in Fig. 6 except that cells were treated with anti-CD8 Ab or control IgG in addition to flagellin. Relative luciferase activity is expressed as the percent of control values with the mean luciferase activity of flagellin treatment group being set at 100%. Each data point represents the mean ± SE (n = 3). *P < 0.05.
and function during the second or continuous exposure to stimulation. Because the anti-inflammatory effect of MUC1 was present in response to both LPS and flagellin (23), which activate TLR4 and TLR5, respectively, the site of action of MUC1 is likely downstream of these TLRs where a common signaling pathway is shared between these two TLRs.

In summary, this study demonstrated that expression/activation of MUC1 resulted in recruitment and activation of PI3K followed by phosphorylation of Akt. Although PI3K was a negative regulator of TLR5 signaling, it was not responsible for MUC1-induced negative regulation of TLR5. Possible interactions with the shared TLR signaling pathway are currently being investigated in our laboratory.

ACKNOWLEDGMENTS

We thank Dr. Erik Lillehoj (University of Maryland Baltimore) for critical review and excellent editing of this manuscript.

Grants

This work was supported by National Heart, Lung, and Blood Institute Grants R01 HL-47125 and HL-81825.

references

