Two Tyrosine Residues of Toll-like Receptor 3 Trigger Different Steps of NF-κB Activation*

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Saumendra N. Sarkar, Christopher P. Elco, Kristi L. Peters, Saurabh Chattopadhyay, and Ganes C. Sen

From the Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195

Innate immune response to viral infection is often triggered by Toll-like receptor 3 (TLR3)-mediated signaling by double-stranded (ds) RNA, which culminates in the activation of the transcription factor NF-κB and induction of NF-κB-driven genes. We demonstrated that dsRNA-induced phosphorylation of two specific tyrosine residues, 759 and 858, of TLR3 was necessary and sufficient for complete activation of the NF-κB pathway. When Tyr-759 of TLR3 was mutated, gene induction was inhibited, although NF-κB was partially activated. It was released from 1κB and translocated to the nucleus but failed to bind to the κB site of the target A20 gene promoter. This defect could be attributed to incomplete phosphorylation of the RelA (p65) subunit of NF-κB, as revealed by two-dimensional gel analyses of p65, isolated from dsRNA-treated cells expressing either wild type TLR3 or the Tyr-759 → Phe mutant TLR3. Thus, two phosphotyrosine residues of TLR3 activate two distinct pathways, one leading to NF-κB release and the other leading to its phosphorylation.

Innate immune response to viral infection of mammalian cells is mediated by the products of viral stress inducible genes (1). Many of these genes are transcriptionally induced when cells are treated with double-stranded RNA (dsRNA), a common by-product of virus infection. Several cellular proteins, that can recognize dsRNA and mediate gene induction, have been identified recently. They include the cytoplasmic RNA-helicases, RIG-I and Mda-5, and the membrane-bound protein Toll-like receptor 3 (TLR3) (2–4). The signaling pathways activated by dsRNA receptors, converge to activate the same major transcription factor, NF-κB. Two major transcription factors, namely, NF-κB, IRF-3/IRF-7, and members of the AP-1 family. Consequently, genes induced by the TLR3 and the RIG-I signaling pathways are largely overlapping.

We have been analyzing the TLR3-mediated signaling pathways activated by dsRNA in a HEK293 cell based experimental system. TRIF is the unique adaptor protein that connects TLR3 to the IKK complex which activates NF-κB. It also connects TLR3 to TBK-1, the enzyme that activates IRF-3 (5). We have studied, in detail, the characteristics of signaling to IRF-3 by TLR3 and demonstrated that phosphorylation of two separate tyrosine residues, located in the cytoplasmic domain of TLR3, is essential for complete activation of IRF-3 (6, 7). One phosphotyrosine, 858, triggers TBK-1 activation, leading to partial phosphorylation of IRF-3 causing its dimerization and nuclear translocation. However, this partially phosphorylated IRF-3 cannot bind efficiently to the cognate sites in the promoters of target genes to drive their transcription. For full activation of IRF-3, its further phosphorylation is needed. The second step of phosphorylation of IRF-3 requires the action of PI 3-kinase, although the proximal kinase that directly phosphorylates IRF-3 remains to be identified. Engagement of PI 3-kinase by TLR3 requires ligand-induced phosphorylation of another residue, Tyr-759. Thus, ligand-induced phosphorylation of Tyr-759 and Tyr-858 of TLR3 initiates two signaling pathways, both of which act together upon IRF-3 to completely phosphorylate and activate it.

In addition to IRF-3, TLR3 triggers the activation of another major transcription factor, NF-κB. The activation of NF-κB involves its release from the inhibitory protein 1κB. This occurs when 1κB is phosphorylated by the IKK enzyme complex and eventually degraded (8). Many cytokines and extracellular stresses cause activation of IKKα and/or β. dsRNA-mediated TLR3 activation can lead to the activation of IKKβ in the IKK complex. Either a TRAF complex or RIP1 linked to TLR3 via TRIF can mediate IKK activation. Although released NF-κB can translocate to the nucleus, its full transcriptional potential is only achieved upon phosphorylation of specific Ser residues of its p65 subunit. Two major target residues of p65 phosphorylation are Ser-276 and Ser-536. Phosphorylation of these residues is known to affect the DNA-binding and oligomerization properties (9).

Among the hundreds of genes induced by dsRNA engagement of TLR3, many require the combined actions of several transcriptional factors. However, there are dsRNA inducible genes that are driven by IRF-3 alone, such as ISG56, ISG54, or ISG15. In contrast, NF-κB is exclusively needed for dsRNA-induced genes such as A20 or IL-8. The promoter of A20 does not contain any IRF-3 binding site, and the gene is induced normally in the cells lacking IRF-3. On the other hand, in cells expressing super-repressor 1κB, which blocks NF-κB activation, A20 is not induced, although IRF-3 driven genes normally induced (10). Thus the A20 gene is an excellent marker for analyzing activation of the NF-κB pathway.

In this study we have analyzed the characteristics of the signaling pathways leading to A20 gene induction by dsRNA-mediated TLR3 activation. Our results indicate that it is a two-step

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

1 To whom correspondence should be addressed: Dept. of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-0636; Fax: 216-444-0513; E-mail: seng@ccf.org.

2 The abbreviations used are: dsRNA, double-stranded RNA; TLR3, Toll-like receptor 3; IRF, interferon regulatory factor; PI, phosphatidylinositol; wt, wild type; IL, interleukin.
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process, mediated by two signaling pathways initiated by phosphorylation of two specific tyrosine residues of TLR3. One pathway leads to the release of NF-κB from IκB and the other leads to hyperphosphorylation of p65. Both steps are necessary for stable binding of NF-κB to the promoter of the A20 gene resulting gene induction.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Plasmids—HEK293 and its derivative cell lines were maintained as described previously (6). Preparation and treatment of cells with dsRNA (100 μg/ml poly(I):poly(C), unless specified otherwise), and co-immunoprecipitation assay have been described before (7). Human anti-TRIF polyclonal antibody was a kind gift from Dr. Kui Li (11). Human RIP1 (BD Transduction Laboratories), p65 (Upstate Biotechnology), phosphoserines 276 and 536 (Cell Signaling Technology) were purchased and used as directed by the manufacturer.

Assays for Gene Induction—A20 mRNA induction was measured by RNase protection assays using the RPA III kit (Ambion, Austin, TX). Actin and A20 RPA probes have been described previously (10). For reverse transcription-PCR, total cellular RNA was isolated using the RNA-Bee kit (Tel-Test, Friendswood, TX). cDNA was made using total RNA by the SuperScript III First Strand Synthesis System (Invitrogen). Primers were used to amplify a region of IL-8 mRNA corresponding to base pairs 107–293. 5X NF-κB reporter assay has been described before (12).

NF-κB Activation Assays—NF-κB release was visualized by electrophoretic mobility shift assay using whole cell lysates prepared 1.5 h after treatment with dsRNA and analyzed according to the method described previously (13). NF-κB nuclear translocation was measured by detecting p65 in nuclear fraction before and after dsRNA treatment (6). Phosphorylation status of p65 was monitored by analyzing whole cell extracts on two-dimensional gel electrophoresis followed by detection of p65 by Western blotting (6).

Chromatin Immunoprecipitation—In vivo binding of NF-κB to A20 promoter was assayed by chromatin immunoprecipitation. 4×10⁶ cells were serum-starved overnight, before treating with poly(I):poly(C) for 2 h followed by cross-linking with 10% formaldehyde. Chromatin was prepared from cross-linked cells according to (14). 100 μg of total chromatin DNA were immunoprecipitated with anti-p65 antibody followed by de-cross-linking and PCR in presence of [α-³²P]dCTP with A20 promoter primers (15). 10 μg of total chromatin DNA was used for each sample without immunoprecipitation to de-cross-link and PCR for input controls.

RESULTS

Tyr-759 of TLR3 Is Required for NF-κB-dependent Gene Induction by dsRNA—To investigate the role of dsRNA-mediated TLR3 tyrosine phosphorylation on NF-κB-dependent gene induction, we used the tyrosine kinase inhibitor, genistein; in the presence of 20 μM genistein, induction of the NF-κB-dependent A20 and IL-8 genes was inhibited (supplemental Fig. 1, A and B). Similar results were obtained in cells expressing a mutant TLR3, in which the five tyrosine residues in its cytoplasmic domain had been replaced by phenylalanine (supplemental Fig. 1C). Only two of those residues, 759 and 858, were together necessary and sufficient for mediating gene induction (6). A20 mRNA was induced in cells expressing wt TLR3 or a TLR3 mutant (YW), in which three Tyr residues, other than those at 759 and 858, had been mutated. In contrast, another mutant (YM), which also contained only two out of five Tyr residues, 733 and 858, failed to support gene induction (Fig. 1A). These results suggested that Tyr-759 is essential for gene induction. Indeed, mutation of Tyr-759 alone abrogated gene induction (Y759F mutant, Fig. 1A). These conclusions were true for another NF-κB-driven gene, IL-8, as well (Fig. 1B and data not shown). To confirm that the observed difference in gene induction was due to a difference in signaling to the κB site, we tested the induction of a reporter gene driven solely by it, in cells expressing either the wt TLR3 or the Y759F mutant TLR3. As shown in Fig. 1C, dsRNA failed to induce expression of the reporter gene in cells expressing the mutant reporter. These results demonstrated that Tyr-759 of TLR3 was required to activate the NF-κB pathway. In the following experiments, we focused on analyzing the basis for this need.

Absence of TLR3 Tyr-759 Does Not Affect Adaptor Binding, NF-κB Release, and Nuclear Translocation—TRIF is the critical adaptor molecule for TLR3 signaling (5, 16). Its ligand-induced recruitment to TLR3 was not impaired in cells expressing the

FIGURE 1. TLR3 tyrosine phosphorylation is essential for NF-κB mediated gene induction. A, impairment of A20 mRNA induction by dsRNA in HEK293 cells stably expressing wt and mutant TLR3. Total RNA from dsRNA treated (+) and untreated (−) cells were used to quantitate A20 mRNA and actin mRNA by RNase protection assay. B, absence of IL-8 mRNA induction by dsRNA in 759F cells. Total RNA from dsRNA treated and untreated cells were used to quantitate IL-8 mRNA and HRPSL (human ribosomal protein S12) mRNA by reverse transcription-PCR. C, luciferase reporter assay of NF-κB promoter activity. A luciferase reporter gene under transcriptional control of 5X NF-κB promoter was transiently transfected to either wt or 759F mutant TLR3-expressing cells. Luciferase activity was measured after 6 h of dsRNA treatment.
Y759F mutant (Fig. 2A). The same was true for another adaptor protein, RIP-1 (Fig. 2B). Moreover, in mutant TLR3-expressing cells, dsRNA treatment caused the release of NF-κB from IκB, as evidenced by its ability to bind to the cognate DNA element in a gel-shift assay (Fig. 2C). The released NF-κB was translocated efficiently to the nucleus, as revealed by the presence of the p65 subunit of NF-κB in the nuclear fractions of dsRNA-treated cells expressing either the wt or the mutant TLR3 (Fig. 2D). The nuclear fractions were virtually free of cytoplasmic contaminations (Fig. 2D, bottom panel). These results indicated that Tyr-759 of TLR3 is not needed for the steps of TLR3 activation, which lead to the recruitment of TRIF and RIP-1 and the resultant activation of the IKK complex, causing phosphorylation of IκB and the release and nuclear translocation of NF-κB.

In the absence of TLR3 Tyr-759, p65 Is Not Completely Phosphorylated, Resulting in Its Defective Binding to the κB Site in the Gene Promoter—To investigate why nuclear NF-κB could not drive gene induction in cells expressing the mutant TLR3, we measured its ability to bind to the κB site in the promoter of the A20 gene, using chromatin immunoprecipitation assays. As shown in Fig. 3A (left panel), p65 was bound tightly to the promoter in dsRNA-treated cells expressing wt TLR3, but not in the corresponding cells expressing the mutant TLR3. Same amounts of A20 promoter were present in the preparations from all samples, before immunoprecipitation (Fig. 3A, right panel).

To seek a biochemical basis for this observation, we examined the phosphorylation status of p65, which is known to affect its transcriptional activity. Like many NF-κB-activating agents, dsRNA caused phosphorylation of residues Ser-276 and Ser-536 of p65 in cells expressing wt TLR3. More importantly, these phosphorylations also occurred in cells expressing the mutant receptor (Fig. 3, B and C), indicating that Tyr-759 was not needed for activating the corresponding kinases. However, a more global analysis of the phosphorylation status of p65 revealed remarkable differences. Two-dimensional gel analysis can identify differentially phosphorylated species of the same protein by virtue of the differences in their isoelectric points. In untreated cells, expressing either wt TLR3 (first panel) or Y759F mutant (third panel), p65 was phosphorylated on multiple residues (Fig. 3D). Upon dsRNA treatment of cells expressing the wt receptor, it was strongly phosphorylated on additional residues, as revealed by the shift to the acidic side of the gel (second panel, Fig. 3D). As expected, phosphatase treatment of the proteins, before gel analysis, caused shifting of the p65 species to the basic side of the gel, because of the removal of acidic phosphate residues from them (data not shown). In cells expressing the Tyr-759 mutant, dsRNA treatment caused only an intermediate level of p65 phosphorylation (fourth panel, Fig. 3D). These results indicate that incomplete phosphorylation of p65 may be the cause of its failure to bind to the A20 gene.
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promoter in dsRNA-treated cells that express the mutant TLR3.

DISCUSSION

We have demonstrated previously that for initiating dsRNA-mediated signaling, that leads to IRF-3 activation, phosphorylation of two specific Tyr residues located in the cytoplasmic domain of TLR3 is required. Signals generated from phosphotyrosine 858 lead to the activation of TBK1, which phosphorylates IRF-3, causing its dimerization and nuclear translocation. However, without the second signal generated by phosphotyrosine 759 of TLR3, IRF-3 is not fully activated. Although present in the nucleus, the partially activated IRF-3 fails to bind to the interferon stimulated response elements in the promoters of target genes and drive their transcription. The biochemical basis of the second step of IRF-3 activation is its further phosphorylation by a PI3-kinase-mediated pathway (6).

Results presented here demonstrate that a similar two-step activation pathway is also operative for complete activation of NF-κB by TLR3 activation. The same two tyrosines of TLR3, 759 and 858, are necessary and sufficient for fully activating NF-κB. Both tyrosines initiate distinct, but complementary, branches of signaling. When Tyr-759 was mutated, dsRNA-binding to TLR3 elicited only one of these signals leading to the release of NF-κB from IκB and its nuclear translocation. However, NF-κB failed to induce gene transcription because it was not fully activated due to the incomplete phosphorylation of p65. Although activation of both IRF-3 and NF-κB were driven by two signals elicited from Tyr-858 and Tyr-759, there were two major differences. First, both steps of IRF-3 activation were activated by its sequential phosphorylation. In contrast, for NF-κB activation the first step was mediated by phosphorylation of IκB and the resultant release of NF-κB. The second step involved phosphorylation of p65, a component of the NF-κB complex. These observations are consistent with the literature that both release from IκB and its own phosphorylation are required for NF-κB to acquire its full potential as transcription factor (17–22). The second critical difference between the IRF-3 and NF-κB pathways was that while PI 3-kinase activity was essential for mediating the second step of activation of IRF-3, it was dispensable for NF-κB activation. In contrast to the IRF-3-driven ISG56 mRNA transcription, NF-κB-driven A20 mRNA transcription was not inhibited by LY294002, a potent inhibitor of PI 3-kinase (data not shown). It is known that two different kinases, TBK1 and IKKα/β, mediate the first step of activation of IRF-3 and NF-κB, respectively. It appears that the same is true for the second step as well, one requires PI 3-kinase, the other one does not. We conclude that phosphorylated Tyr-759 recruits an as yet unidentified adaptor protein, which in turn recruits PI 3-kinase and a different kinase, to initiate two independent signaling cascades for the second steps of IRF-3 and NF-κB activation, because neither of the pathways have the terminal kinases that phosphorylate IRF-3 and p65 been identified yet. The reason for which nuclear NF-κB fails to induce gene transcription in dsRNA treated cells expressing the Y759F TLR3 mutant protein is unclear. This could be due to the failure of incompletely activated p65 to interact with co-activators or the core complex of the RNA polymerase. This idea is consistent with the observed hypo-phosphorylation of p65 because the phosphorylation status of NF-κB is known to determine its ability to interact with CBP/p300 and HDAC-1 (22).

Many activators of NF-κB, including IL-1, tumor necrosis factor-α, and lipopolysaccharide, are known to trigger phosphorylation of p65. Our results show that the same is true for dsRNA signaling by TLR3. Four Ser residues of p65 at 276, 529, and 536 have been identified as potential targets of phosphorylation (9). Among those, we tested the phosphorylation status of two Ser-276 and Ser-536. Both were phosphorylated in response to dsRNA treatment. These phosphoryations occurred even in cells expressing the 759F mutant of TLR3 indicating that the first step of signaling was sufficient for not only the release of p65 but also its phosphorylation at those sites. Many protein kinases have been implicated in different systems to mediate phosphorylation of p65. Among them are TBK1, IKKe, IKKα/β, and PI 3-kinase, all of which are known to be activated by TLR3 signaling. In fact, blocking PI 3-kinase activity by the inhibitor LY294002 inhibited p65 Ser-536 phosphorylation in response to TLR3 activation (data not shown). However, it did not block A20 gene induction, indicating that Ser-536 phosphorylation was not required for p65 activation by dsRNA. On the other hand, results from the cells expressing the TLR3 759F mutant indicated that Ser-276 and Ser-536 phosphorylation were not sufficient for p65 activation because despite their phosphorylation there was no gene induction. Biochemical analyses demonstrated that p65 was strongly phosphorylated, presumably at multiple sites, in dsRNA-treated cells expressing wt TLR3. This process was severely inhibited in cells expressing the mutant TLR3, although Ser-276 and Ser-536 were still phosphorylated. These data suggest that the second signal generated from Tyr-759 of TLR3 is essential for hyperphosphorylation of p65 and its functional activation. The specific sites that are phosphorylated and the kinases that phosphorylate them remain to be identified.

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