Double-stranded RNA Signaling by Toll-like Receptor 3 Requires Specific Tyrosine Residues in Its Cytoplasmic Domain

Double-stranded RNA (dsRNA) is a potent regulator of gene expression in mammalian cells. It is thought to be a critical viral gene product that modulates host response in virally infected cells (1). In cell culture, the addition of exogenous dsRNA causes rapid induction of more than a hundred genes, many of which are also induced by type I interferons (IFN) and virus infection (2). Among the most well studied dsRNA-induced genes is the human IFN-β gene. Its promoter is complex, and its induction requires the coordinate activation of members of the NFκB, AP-1, and IRF families of transcription factors (3, 4). In contrast, the human 561 gene has a simple promoter containing only IFN-stimulated response elements and no κB sites (5). This gene can be induced strongly by IFN or dsRNA. Moreover, microarray analyses have shown this gene to be one of the most highly induced genes in virus-infected cells (2, 6, 7). The encoded protein, P56, binds to the translation initiation factor eIF3 and inhibits protein synthesis (8). We have extensively used the 561 gene and its promoter to analyze the relevant dsRNA-signaling pathway (5, 9). Our studies demonstrate that neither IFN nor the Jak/STAT pathway of IFN signaling is required for 561 mRNA induction by dsRNA (5). In contrast, the transcription factor IRF-3 is critical for this induction process. Using mutant cells partially responsive to dsRNA, we established conclusively that activation of NFκB, AP-1, p38, or JNK is not required for IRF-3 activation and consequent 561 mRNA induction. Thus, dsRNA uses a distinct IRF-3 pathway for activation of genes such as 561 (9).

Recently, the Toll-like receptor 3 (TLR3) has been shown to mediate dsRNA response. Using TLR3-deficient cells and mice, Alexopoulos et al. (10) have demonstrated that TLR3 is required for NFκB activation and mitogen-activated protein kinase activation by dsRNA. The TLR family of transmembrane receptors is an integral part of our innate immune system. They recognize, with high specificity, diverse pathogen-associated molecular patterns and elicit intracellular signals to cause cellular inflammatory responses (11–13). Although there are partial overlaps among the genes induced by different TLRs, their signaling pathways are thought to be distinct. However, the complete signaling pathway has not been elucidated for any TLR. In this study, we have investigated the role of TLR3 in the specific dsRNA-elicited signaling pathway that causes induction of the 561 gene. In the course of this investigation we have made the unexpected observation that specific tyrosine residues of the cytoplasmic domain of TLR3 are essential for this signaling pathway.

MATERIALS AND METHODS

Cells, Reagents, and Plasmids—HER293 cell line was maintained in high glucose DMEM supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml gentamicin, and 50 μg/ml streptomycin. Anti-human TLR3 monoclonal antibody was purchased from Biocarta (San Diego, CA). Cycloheximide and genistein were purchased from Calbiochem. The FLAG-TLR3 cDNA construct was obtained from Dr. Xiaoxia Li (Cleveland Clinic Foundation). The TLR3 expression construct (FLAG-TLR3) we used in this study was constructed by removing the original TLR3 signal peptide up to Phe 17 and replaced with the signal peptide from pre-protrypsin, followed by a FLAG epitope tag (10) and was cloned in pcDNA3 (Invitrogen). The ΔTIR-TLR3 construct (ΔYYYY) was made by PCR amplification of FLAG-TLR3 containing up to amino acid 750 of TLR3. All the Tyr mutants of TLR3 were generated by the mega-primer PCR method using appropriate primers and FLAG-TLR3 as the template. Mutations were confirmed by sequencing.

Development of 293/TLR3 and 293/TLR3-mutant Cell Lines—HER293 cells were seeded into 100-mm dishes and transfected using Lipofectamine 2000 (Invitrogen) with 6 μg of the appropriate plasmid DNA. Cells were selected in DMEM containing 530 μg/ml G418 (Research Products International, Mt. Prospect, IL). Individual colonies were picked, expanded, and confirmed for TLR3 expression by Western blotting with anti-TLR3 monoclonal antibody (final concentration: 2 μg/ml). For Western blotting, 2.5 × 10^6 cells were lysed in lysis buffer containing 300 mM NaCl, 20 mM Tris-Cl (pH 7.5), 5 mM β-mercaptoethanol, 0.2% Triton X-100, 10% glycerol, and protease inhibitors (complete EDTA-free protease inhibitor tablets, Roche Molecular Biochemicals). 150 μg of total protein lysates were used for Western analysis. Stable cell lines were propagated and maintained in complete DMEM supplemented with 530 μg/ml G418.

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dsRNA Signaling by TLR3

RESULTS

TLR3-dependent Induction of 561 mRNA—The human kidney cell line 293 does not express TLR3, and addition of dsRNA to its culture medium did not cause induction of the 561 mRNA, but the same mRNA was induced strongly in the 293 cell line expressing TLR3 (Fig. 1A). 561 mRNA was not detectable in untreated 293/TLR3 cells, but it was rapidly induced after dsRNA treatment. This induction was direct and did not require the synthesis of any new protein. 561 mRNA was induced by dsRNA equally well in the presence of cycloheximide, a potent inhibitor of protein synthesis (Fig. 1B).

Role of Specific Tyrosine Residues of TLR3 in dsRNA Signaling—There is information in the literature demonstrating the dependence of dsRNA signaling on protein-tyrosine kinase activity (15). We observed that the induction of 561 mRNA by dsRNA was blocked by genistein, an inhibitor of tyrosine kinases (data not shown). This observation suggested to us that tyrosine residues present in the cytoplasmic domain of TLR3 might be required for its ability to transmit dsRNA-elicited signals. The putative cytoplasmic domain of TLR3 has 130 residues, out of which five are tyrosines (Fig. 2A). One tyrosine, tyrosine 733, is situated very close to the transmembrane do-

Fig. 1. TLR3-mediated 561 mRNA induction by dsRNA. The levels of 561 mRNA (filled arrow) and actin mRNA (open arrow) were measured by RPA. A, 293 and 293/TLR3 cells were left untreated (−) or treated with poly(I)poly(C) for 6 h (+). RPA was performed to measure 561 mRNA and actin mRNA levels. B, 293/TLR3 cells were pretreated with 50 μg/ml cycloheximide for 30 min, where indicated, before treating with poly(I)poly(C) for 6 h, in the presence of cycloheximide. RPA was done to measure 561 mRNA induction.

Ribonuclease Protection Assay (RPA)—RNA was isolated with RNA Bee (Tel-Test, Friendswood, TX). RPs were performed with the RPA III kit (Ambion, Austin, TX) following the manufacturer’s protocol. The 561 and actin RPA probes had been described previously (9). For each sample 20 μg of total RNA was used for RPA. Protected mRNA levels were visualized by autoradiography as well as quantified using PhosphoImager (Amer sham Biosciences).

561-Luciferase Transient Transfection Assay—The 561-luciferase reporter construct contains the −3 to −654 nucleotides of the P56 promoter (14) cloned into the Sac/l/HindIII restriction sites of the pGL3B vector (Promega, Madison, WI). The pRL-SV40 vector coding for Renilla luciferase (Promega) was used as the internal control for normalization of transfection efficiency. HER293 cells were transfected in six-well dishes using 5 μl of Lipofectamine 2000 (Invitrogen) with 50 ng of total RNA. Protected mRNA levels were confirmed by Western blotting of the extracts.

Detection of Phosphotyrosines in TLR3—Four × 10⁵ cells were treated with poly(I)poly(C) for 1 h or left untreated for control. Lysates were made by incubating cell suspensions on ice for 30 min in lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100, 10 mM NaF, 2 mM dithiothreitol, 1 mM Na₃VO₄, 12.5 mM β-glycerophosphate, and protease inhibitors. Equal amounts of proteins were immunoprecipitated with 20 μl of anti-FLAG M2 antibody agarose (Sigma) for 4 h. Following four washes with the lysis buffer, beads were boiled in 1× SDS-PAGE loading buffer and analyzed on SDS-PAGE. Phosphotyrosines were detected by Western blotting with PY20 antibody (1:2000, Phar mingen). Blots were stripped and reprobed with anti-FLAG M2 antibody for detecting TLR3.

Role of Specific Tyrosine Residues of TLR3 in dsRNA Signaling—There is information in the literature demonstrating the dependence of dsRNA signaling on protein-tyrosine kinase activity (15). We observed that the induction of 561 mRNA by dsRNA was blocked by genistein, an inhibitor of tyrosine kinases (data not shown). This observation suggested to us that tyrosine residues present in the cytoplasmic domain of TLR3 might be required for its ability to transmit dsRNA-elicited signals. The putative cytoplasmic domain of TLR3 has 130 residues, out of which five are tyrosines (Fig. 2A). One tyrosine, tyrosine 733, is situated very close to the transmembrane do-

Fig. 2. Requirement of specific Tyr residues in the cytoplasmic domain of TLR3 for the induction of 561-luciferase by dsRNA. A, positions of the five tyrosine residues in the cytoplasmic domain of TLR3 are shown. B, these Tyr were mutated individually or in combinations to Phe to study their roles in TLR3-mediated induction of 561-luciferase expression by dsRNA. 293 cells were co-transfected with expression vectors of wt or mutant TLR3, the 561-luciferase reporter gene, and the normalization control pRL-SV40. Cells were treated with dsRNA, where indicated, for 6 h before measuring luciferase levels. On the left, the mutual statues of the five Tyr residues are shown. All samples, except one, were treated with dsRNA. The bars on the right show normalized -fold inductions of luciferase activity. The error bars are from three independent experiments, each done in triplicates. C, the relative importance of Tyr residues in signaling is shown. Tyr⁷³⁹ plays the most crucial role followed by the lesser but equally important roles of Tyr⁷⁵⁵ or Tyr⁷⁵⁶, Tyr⁷⁵⁶ and Tyr⁷⁶⁴ have the least important roles.

dsRNA treatment. This induction was direct and did not require the synthesis of any new protein. 561 mRNA was induced by dsRNA equally well in the presence of cycloheximide, a potent inhibitor of protein synthesis (Fig. 1B).
main and another, tyrosine 858, is located distally. Three other tyrosine residues, tyrosines 756, 759, and 764, are clustered in a region about 30 residues away from the transmembrane domain. We wanted to examine the roles of these five tyrosine residues in TLR3 signaling by mutating them conservatively to phenylalanine residues individually or in combination.

A reporter assay was developed to test the function of these mutant proteins. For this purpose, a luciferase reporter gene driven by the promoter of the 561 gene was co-transfected with an expression vector of wt or mutant TLR3 to 293 cells, and the level of luciferase expression was measured after dsRNA treatment. That wt and mutant TLR3 proteins were expressed to similar levels was ensured by Western blot analysis of transfected cell extracts (data not shown). When wt TLR3 was used, there was little expression of luciferase in cells not treated with dsRNA (Fig. 2B, line 1). However, the expression level was elevated about 20-fold when cells were treated with dsRNA (Fig. 2B, line 2). A mutant TLR3, from which most of the cytoplasmic domain had been deleted (residues 751–860 deleted), failed to signal even in the presence of dsRNA (Fig. 2B, line 3). The above characteristics of the reporter assay established its validity and the obvious need for the cytoplasmic domain of TLR3 for its ability to signal.

A critical experiment of this series is shown in line 4 of Fig. 2B. When all five Tyr residues were mutated to Phe, the receptor failed to signal even in the presence of dsRNA. This result demonstrated an absolute need for the presence of the Tyr residues in the cytoplasmic domain of TLR3 for its ability to signal. The 5F mutant, with all five residues mutated, was subsequently used as the starting material for restoring one or more Tyr residues at a time. Restoration of only Tyr731 had no effect (line 5, Fig. 2B). On the other hand, restoration of Tyr759 by itself elicited a small, but significant, level of signal (line 6, Fig. 2B). The 561-luciferase activity was restored fully when amino acid 858 was restored back to Tyr as well (line 7, Fig. 2B), although the other three residues (733, 756, 764) remained mutated. Other combinations containing 759 and another Tyr residue and three Phe residues had intermediate activities (lines 8–10, Fig. 2B). In contrast, if the two Tyr residues did not include the 759 moiety, the mutant proteins were completely inactive (line 11, Fig. 2B and data not shown). The same was true for proteins containing three out of five Tyr residues restored. The proteins were active only if the 759 residue was a Tyr (lines 12–14, Fig. 2B). Finally, a series of mutants, which contained single Tyr to Phe mutations, was tested. Mutants Y756F (line 16, Fig. 2B) and Y764F (line 18, Fig. 2B) were completely active, whereas Y733F (line 15, Fig. 2B) and Y858F (line 19, Fig. 2B) were slightly less potent than the wt protein. As expected, the Y759F mutant (line 17, Fig. 2B) was the least active, although about 20% activity remained. The above series of reporter assays strongly indicated that the Tyr residues of the TLR3 cytoplasmic domain are essential for dsRNA signaling to the 561 gene. Moreover, there is a distinct hierarchy among the five Tyr residues with respect to their contributions to signaling (Fig. 2C). The Tyr at 759 is the most critical residue, its mutation to Phe strongly diminished the ability of the receptor to signal (line 17, Fig. 2B).

Although the above results were quite convincing, they were generated by transient reporter assays, which sometimes do not reflect completely the properties of the resident gene. For this reason, we established new 293 cell lines expressing either the wt or various mutant TLR3 proteins. Clones expressing comparable levels of the wt and a mutant protein were matched and used for testing induction of the 561 mRNA in response to dsRNA (Fig. 3). The wt-18 clone and the 5F-24 expressed similar levels of the wt and the five Tyr to Phe mutant TLR3 proteins, respectively (Fig. 3A). As expected, 561 mRNA was strongly induced by dsRNA in the wt-18 cells, but no induction could be detected in the 5F-24 cells (Fig. 3B). Another set of cell lines, wt-11, FFYYYF-9, YFFFFY-9 and 759F-30, expressed the wt or the mutant proteins to similar levels (Fig. 3C). Again, the wt-11 clone strongly expressed 561 mRNA in a dsRNA-dependent manner, as did the cells expressing the FFYYYF mutant. In contrast, cells expressing the YFFFFY or the 759F mutants were unresponsive (Fig. 3D). These results provided definitive evidence for the need of the cytoplasmic Tyr residues of TLR3, especially the one at 759, for mediating dsRNA-elicited signaling to cause transcriptional induction of the 561 mRNA.

**Ligand-induced Tyrosine Phosphorylation of TLR3**—Finally, we wanted to examine whether the cytoplasmic Tyr residues of TLR3 were phosphorylated. Results presented in Fig. 4 show that the wt TLR3 gets Tyr-phosphorylated in its cytoplasmic domain. wt-18 and 5F-24 cells stably expressing the wt and the 5F mutant TLR3 proteins, respectively (Fig. 3), were treated with dsRNA for 1 h, and TLR3 proteins were immunoprecipitated and Western blotted with anti-phosphotyrosine antibody. A phosphotyrosine-specific protein band appeared at the TLR3 position, only after dsRNA treatment of wt-18 cells. (Fig. 4A, lane 3), but not in 5F-24 cells (Fig. 4A, lane 4). A slightly faster migrating phosphorylated protein of unknown identity was present in all samples. Comparable amounts of TLR3 proteins were immunoprecipitated from all extracts containing TLR3 (Fig. 4B). These results demonstrated that TLR3 undergoes Tyr phosphorylation in its cytoplasmic domain upon dsRNA stimulation of the cells.

**DISCUSSION**

Transcriptional induction by dsRNA of the 561 gene and other IFN-stimulated genes is mediated by members of the IRF family, primarily IRF-3 (1, 16). This signaling pathway is independent of dsRNA-mediated activation of IκB kinase, JNK, or p38 kinases, and it does not require the activities of transcription factors such as NFκB or AP-1 (9). Alexopoulou et al. (10) have shown that the 293 cells are devoid of the latter.
responses to dsRNA unless TLR3 is expressed in them by transfection. Here, we have established the same requirements for another dsRNA-elicited signaling pathway, which leads to 561 gene activation. To validate direct induction of the 561 gene by dsRNA in 293 cells, we performed the cycloheximide experiment (Fig. 1B). Since the 561 gene and other genes of this family can also be induced by type I IFN, there was a possibility that the observed induction of 561 mRNA was mediated by intermediate IFN synthesis. However, if that were the case, the induction would have been inhibited by blocking new protein synthesis. In other cell types, such as HT1080 and G4 using genetic and biochemical approaches, we have previously shown that the 561 gene can be induced by dsRNA without any involvement of IFNs or their receptors (5, 9). Here, the same conclusion was formally established for 293/TLR3 cells as well. Thus, the 561 gene can be considered as a primary response gene for dsRNA in these cells. The fact that tyrosine kinase inhibitors could block 561 mRNA induction led us to study the role of Tyr residues of TLR3 in dsRNA signaling. Our study has established a possible connection between that susceptibility and TLR3 tyrosine residues.

The transient reporter assay shown in Fig. 2 has been used by us before in other cells (5, 7). In the 293 cells it was quite robust and reproducible with a low background. More importantly, it was totally dependent on the expression of transfected TLR3, thus offering us a convenient and rapid assay for mutant TLR3 functions. Taking advantage of this assay, we could establish the relative functional importance of the five tyrosine residues of the cytoplasmic domain of TLR3. It was clear that Tyr\textsuperscript{759} was the most critical residue, but for maximal activity of the promoter, the presence of another Tyr was needed. For this accessory function, the most membrane-proximal (733) or the promoter, the presence of another Tyr was needed. For this accessory function, the most membrane-proximal (733) or the most distal (858) Tyr residue was most effective, whereas the two other Tyr residues present near the Tyr\textsuperscript{759} (756 and 764) were less effective. The conclusions drawn from the reporter assays were confirmed by examining induction of the resident 561 gene in cell lines derived from 293 cells that expressed wt or mutant TLR3 proteins. Cells expressing the 5F and the YFFFY mutants were totally unresponsive, whereas those expressing the FFYYF mutant were as responsive as the wt cells (Fig. 3). Cells expressing the Y759F mutant were also unresponsive. The Y759F mutant, however, had some residual activity in the transient reporter assay (Fig. 2B, line 17). This minor difference could be attributed to high overexpression of the transfected proteins in the transient assays. It could also be a genuine difference between the responses of the chromosomal gene and the transfected reporter gene driven by an arbitrarily truncated promoter. Thus, the lack of response of the resident 561 gene in cells expressing the Y759F protein is the more physiologically relevant result.

We observed Tyr phosphorylation of wt-TLR3, but not of 5F-TLR3, in response to dsRNA treatment (Fig. 4). We do not know at this time which specific Tyr residues get phosphorylated nor do we know how rapidly it happens. It is tempting to speculate that the phosphotyrosine moieties serve as the docking sites for protein kinases or adaptor proteins, thereby starting a cascade of signaling events leading to activation of the relevant transcription factors. Drawing upon the knowledge of growth factor receptors, it is also conceivable that different tyrosine residues of TLR3 cytoplasmic domains attract different protein partners and hence initiate different independent signaling pathways (17). Extensive genetic and biochemical investigations will be required in the future to examine these possibilities. Irrespective of the mechanism, it is clear from this study that cytoplasmic tyrosine residues and their phosphorylation play an essential role in TLR3 signaling. This observation is highly significant, because tyrosine phosphorylation was not thought to be connected to the primary signaling pathway of any member of the TLR family. The only exception is TLR2, which has recently been shown to contain phosphotyrosine residues in its cytoplasmic domain (18). In that case, however, the critical tyrosine residues have not been identified, but presumably they are required for the assembly of the signaling complex.

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