Biochemical and Functional Analyses of the Human Toll-like Receptor 3 Ectodomain

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The structure of the human Toll-like receptor 3 (TLR3) ectodomain (ECD) was recently solved by x-ray crystallography, leading to a number of models concerning TLR3 function. The structure revealed four pairs of cysteines that are putatively involved in disulfide bond formation, several residues that are predicted to be involved in dimerization between ECD subunits, and surfaces that could bind to poly(I:C). In addition, there are two loops that protrude from the central solenoid structure of the protein. We examined the recombinant TLR3 ECD for disulfide bond formation, poly(I:C) binding, and protein-protein interaction. We also made over 80 mutations in the residues that could affect these features in the full-length TLR3 and examined their effects in TLR3-mediated NF-κB activation. A number of mutations that affected TLR3 activity also affected the ability to act as dominant negative inhibitors of wild type TLR3. Loss of putative RNA binding did not necessarily affect dominant negative activity. All of the results support a model where a dimer of TLR3 is the form that binds RNA and activates signal transduction.

The recognition of foreign molecules by the innate immune receptors can lead to the activation of a signaling cascade, changes in gene expression, and production of cytokines by effector cells. The consequence of this pathway could dictate the outcome of an immune response through modulation of T- and B-lymphocyte activation in the adaptive immune pathways. At least 11 TLRs have been identified in the human genome. TLR3 recognizes poly(I:C), a synthetic double-stranded RNA (dsRNA) analog, as well as viral dsRNA, presumably formed during viral infection. TLR3 knock-out mice are unable to mount a full response to cytomegalovirus infection and decreased the cytotoxic T cell response after the initial infection in mice. These results support a role for TLR3 in modulating the host immune response against microbial challenge and provide a rationale to better understand the mechanism of action of TLR3.

Upon ligand binding, TLR3 can, through adaptor proteins, activate the transcription factor NF-κB, which translocates to the nucleus to modulate gene expression. The site of action for TLR3 activation is probably in or near intracellular vesicles, although some cell surface expression is observed in human embryonic kidney cells.

The structures of the TLR3 ectodomain have been elucidated by x-ray crystallography by two groups, leading to several predictions about how the structure affects function. The TLR3 ECD is in the shape of a solenoid horseshoe, characteristic of proteins with multiple leucine-rich repeats (LRRs). A number of features in the protein structure could impact TLR3 function. Based on surface charge properties and the location of glycosylations, the region proposed to bind dsRNA was proposed to be free of glycosylation. The structures from the crystal packing suggest that the C-terminal portions of two subunits interact through ionic interactions. TLR3 ECD is also predicted to contain four disulfide bonds near the N and C termini of the solenoid that may stabilize the ECD structure. Last, there are two protruding loops in the TLR3 ECD solenoid that may contribute to TLR3 function.

Analysis of how features of the TLR3 ECD structure impact function is an active area of research. All of the predicted N-linked glycosylation sites in TLR3 ECD have been mutated, and two contribute to TLR3 activity in transfected human embryonic kidney (HEK) 293T cells. De Bouteiller et al. have mutated several of the cysteines putatively involved in disulfide bond formation. However, not all of the cysteines have been examined, and there is no biochemical evidence for these disulfide bonds being present. Bell et al. examined the effects of many mutations throughout the ECD of TLR3 and demonstrated that two residues, H539E and N541A, wild type; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; FACS, fluorescence-activated cell sorting; HEK, human embryonic kidney; FITC, fluorescein isothiocyanate.
are affected for activity in 293T cells and prevented recombinant TLR3 ECD from binding to dsRNA in a gel filtration based assay. A thorough mutational analysis of residues neighboring H539E and N541A had more modest effects on TLR3 activity (13). This work also led to the proposal that RNA binding by the TLR3 ECD leads to dimerization.

In this study, we have made over 80 mutants in TLR3 that are predicted to affect disulfide bond formation, dimerization, and RNA binding and examined their effects in a cell-based assay for TLR3 activation of downstream reporter expression. Biochemical assays for the properties of the TLR3 ECD produced in human cells are also examined. The effects of select mutants that decreased TLR3 activity were examined for effects on protein expression and cellular localization and for the ability to act as dominant negatives of co-transfected wild type (WT) copy of TLR3.

MATERIALS AND METHODS

Reagents—Antisera to TLR3 and poly(I:C) were purchased from Imgenex Inc. (San Diego, CA). Dithiothreitol, iodoacetamide, and trypsin were purchased from Sigma. The water used in all procedures was purified using a Millipore Milli-Q UV Plus purification system. Organic solvents used for mass spectrometry were high pressure liquid chromatography grade, and all of the other chemicals were reagent grade. The fluorescent dye that stains acidic membrane, Lysotracker, was purchased from Molecular Probes, Inc. (Eugene, OR). A 40-bp poly(I:C) was chemically synthesized (gеноMechanix, Gainesville, FL). A 20-bp poly(I:C) was made by treating polyinosinic acid and polycytidylic acid with 1 M NaOH for 5 min, followed by separating the bands on a denaturing gel and cutting out the 20-nucleotide bands with a razor and annealing them.

Mass Spectrometry—To obtain biochemical evidence for disulfide bonds in TLR3, we used mass spectrometry to examine the recombinant TLR3 ECD purified from human cells, named hTLR3 ECD. An aliquot of hTLR3 ECD was reduced and alkylated, similar to published protocols (14). The other aliquot was diluted with 10 μl of 50 mM ammonium bicarbonate, pH 8. Each aliquot was then thermally denatured at 90 °C for 15 min and digested with sequencing grade modified trypsin at 37 °C overnight (15). The molar ratio of trypsin to protein used was 1:40. Each sample was desalted using a C18 Zip Tip (Millipore) before analysis by mass spectrometry utilizing an ABI 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). The matrix was 4-hydroxy-α-cyanocinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) and mixed 1:1 with the desalted sample. All spectra were taken manually. For the tandem mass spectrometry experiments, the acceleration was set at 1 kV, and the collision gas was atmosphere.

Mutagenesis—The WT TLR3 plasmid was previously described by Sun et al. (10). Site-directed mutations were made using oligonucleotides annealed to the target sequence and the QuikChange kit (Stratagene). Sequences of the oligonucleotides will be made available upon request. Several clones that resulted from the mutational analysis were sequenced to confirm the mutation. Mutant clones with affected activity were sequenced to confirm the presence of the mutation and the absence of unintended changes in the protein.

Molecular Modeling—The crystal structure determined by Bell et al. (2) was used for molecular modeling. Two TLR3 ECD molecules were docked into a dimer based on Bell et al. (13). Its complex with a dsRNA was modeled also based on Bell et al. (13) as well as data obtained from this study. The manual docking was performed in the Quanta molecular modeling environment (version 2000; Accelrys). The result was rendered using Pymol (version 0.99; DeLano Scientific LLC).

Analytical Ultracentrifugation—Sedimentation velocity experiments were performed at 4 °C with a Beckman XL-A analytical ultracentrifuge equipped with an AnTi60 rotor. Data were obtained at 30,000 rpm using a two-channel centerpiece containing 1 mm protein or a buffer for reference. Absorbance was measured at 220 nm. The data were analyzed using the computer program SEDFIT and fitted with a Lamm equation to derive the molar mass and the sedimentation coefficient.

Microscopy—Cells were plated on LabTek II CC2-treated chamber slides (Nunc International, Naperville, IL) and transfected with plasmids in Lipofectamine 2000 (Invitrogen). Each TLR3 mutant was visualized 24 h post-transfection with a Zeiss Axioplan fluorescent microscope via immunofluorescence. Briefly, the cells were removed from the incubator and rinsed with phosphate-buffered saline before fixation with 4% formaldehyde in phosphate-buffered saline and permeabilization with 0.1% Triton X-100. The cells were then incubated at room temperature in the dark for at least 1 h in anti-TLR3 FITC-conjugated monoclonal antibody (Imgenex315A; San Diego, CA). The cells were washed and counterstained with Hoechst 33342 dye (Molecular Probes, Inc., Eugene, OR) before being mounted in a buffered glycerol aqueous mounting medium.

Western Blots—293T cells were transiently transfected with WT TLR3, mutant TLR3, or control pcDNA as described above. Thirty-six hours post-transfection, the cells were lysed using passive lysis buffer (Promega) and sonicated to degrade chromosomal DNA. Equal amounts of proteins from each sample were separated on NuPAGE 4–12% bis-Tris gel (Invitrogen), blotted onto polyvinylidene difluoride membrane, and probed with anti-TLR3 monoclonal antibody IMG315A (Imgenex Inc.). The blots were developed with peroxidase-conjugated secondary antibodies and the ECL-plus Western blotting detection system (Amersham Biosciences).

FACS Analysis—FACS analyses were performed with 293T cells grown in 6-well collagen-coated plates (BD Biosciences) at a concentration of 2 × 10⁶ cells/well. The cells were transfected with 1 μg of the appropriate plasmids using Lipofectamine 2000 (Invitrogen). Eighteen to twenty-four hours after transfection, the cells were harvested and washed twice with ice-cold FACS buffer (1× phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4, 3% fetal bovine serum, and 0.04% sodium azide) before suspension at ~2 × 10⁷ cells/ml in FACS buffer. The cells were stained for 30 min at 4 °C with 1 μg of phycoerythrin-labeled anti-human TLR3 monoclonal antibody (TLR3.7; purchased from eBioscience, San Diego, CA) or a negative control mouse IgG1 control antibody. The antibodies were added to cells grown in 96-well plates and incubated for 30 min on ice in the dark. The cells were washed twice with FACS buffer to remove unbound antibody and then suspended in FACS buffer. Viaprobe (BD Biosciences) was used to exclude
dead cells. The cells were transferred to the appropriate tubes and analyzed using a FACSCalibur machine (BD Biosciences).

RESULTS AND DISCUSSION

NF-κB Activation Assay for TLR3 Function—HEK 293T cells were used to analyze how mutations in TLR3 will affect TLR3 function and localization as described in Sun et al. (10). 293T cells are useful for this assay, since they do not express detectable levels of endogenous TLR3 (Fig. 1A, box). Briefly, cells cultured in 96-well plates to ∼80% confluence were transfected with a mixture of three plasmids: one to express either WT or mutant TLR3, a second to express the firefly luciferase driven from promoter-containing NF-κB binding sites, and a third to express the transfection control, Renilla luciferase, from the herpesvirus thymidine kinase promoter. Poly(I:C) was used as the ligand to induce TLR3-mediated NF-κB activation. Every TLR3 activity assay result in this work was from a minimum of six independently transfected cell cultures.

Our assay can respond to up to 75 ng of the TLR3 plasmid in the transfection (Fig. 1A), but our standard assay uses 15 ng of plasmid per transfection to ensure that the signal will not be saturated. The ratio of firefly luciferase to Renilla luciferase activity was calculated for all of the samples induced with poly(I:C) as well as with the buffer alone control. The fold of poly(I:C) activation over the uninduced control was normalized for each transfection and normalized to the WT control assayed in the same experiment. A minimum of 4-fold induction of TLR3 activity by the addition of poly(I:C) was detected in all of our assays (Fig. 1B). As an example, a mutant TLR3 can reduce activity to background (Fig. 1B).

Cysteines Involved in Disulfide Bond Formation—Cysteines that putatively form disulfide bonds to cap the ends of the ECD are as follows: Cys28 and Cys37, Cys95 and Cys122, Cys649 and Cys677, and Cys651 and Cys696 (Fig. 2A) (1, 2). The cysteines are conserved in all species in which the TLR3 orthologs have been

FIGURE 1. A cell-based assay to detect TLR3 activity. A, effects of increasing concentration of plasmid expressing WT TLR3 on the activation of luciferase reporter activity in HEK 293T cells. The luciferase activity is expressed as the ratio of the firefly luciferase driven from promoter containing NF-κB elements over the activity of the Renilla luciferase driven from the herpesvirus thymidine kinase promoter. Activation of the firefly luciferase activity requires poly(I:C), added at 2.5 μg/ml. Inset, a demonstration that HEK 293T cells do not express endogenous TLR3. B, the TLR3 cell-based assay can detect a range of luciferase activity; a 6-fold increase in NF-κB activation is seen with WT TLR3, and a mutant that has a deletion of an internal loop in TLR3 shows a loss of poly(I:C)-induced NF-κB activation.

FIGURE 2. Detection of disulfide bonds in the hTLR3 ECD. A, a hTLR3 ECD structure (Protein Data Bank code 2A0Z) showing the residues near the N- and C-terminal portions of the TLR3 ECD, which participate in disulfide bond formation. B, sequence analysis of the cysteines in TLR3 ECDs from species across a wide phylogenetic range. The cysteine pairs involved in disulfide bond formation are identified by the brackets. C, mass spectra of a tryptic digest of TLR3 protein focusing on the m/z region containing the CTVSHEVACSHLK peptide. The top spectrum is of the peptide containing the disulfide bond. The bottom spectrum is the reduced and alkylated form of the peptide. D, tandem mass spectrometry analysis of the above peptide to confirm the assigned peptide sequence.
identified, suggesting that the disulfide bonds they form are important for TLR3 function (Fig. 2B).

We first attempted to determine whether the formation of the disulfides could be detected in tryptic fragments of TLR3 ECD using mass spectrometry (Fig. 2C). Reduced cysteines are acetylated by iodoacetamide, and the ones involved in disulfide bond formation are not. The mass spectrometry spectra obtained in the nonreduced tryptic digest resulted in a signal at 1526.81 m/z, which corresponds to the modified peptide CTVSHEVADCSHLK if a disulfide was originally present (Fig. 2C, top). To confirm the sequence and structural assignment of this peptide, tandem mass spectrometry was performed (Fig. 2D). Nearly the entire C-terminal y ion series (y2–y9) was observed, confirming the peptide sequence assignment. More significant is the observation that the b series ions b10–b13 were all 2 Da lower than what would be expected for the reduced peptide (16). These results confirm the presence of a disulfide bond between Cys28 and Cys37. The other three pairs of disulfides were not observed despite repeated attempts, suggesting that they may either be present at lower abundances or that the peptides containing these disulfides could not be ionized under the conditions used.

To assess the functional relevance of the disulfide bonds, we mutated each participating cysteine to alanine. Mutants C28A, C37A, C95A, C122A, C649A, C651A, and C696A all resulted in TLR3 activities near background (Fig. 3A). We also examined whether replacements of some of the cysteines with serine or methionines would affect activity. All of the changes made in Cys37, Cys95, Cys122, Cys649, Cys651, and Cys696 resulted in activities at or near the background level. Therefore, the cysteines involved in disulfide bond formation are required for TLR3 function. In contrast, mutations of cysteines that are not predicted to participate in disulfide bond formation (Cys242, Cys356, or both) had only minimal effects on TLR3 activity (Fig. 3A).

Mutations in the disulfide-forming cysteines could affect several properties of TLR3, including its expression, stability, and/or intracellular localization. To examine whether TLR3 expression was affected, lysates from transfected cells were subjected to Western blots with a TLR3-specific monoclonal antibody. All cysteine mutants were expressed at levels comparable with WT (Fig. 3B). To examine whether the mutant proteins are affected in their intracellular localization, we immunostained transfected HEK 293T cells and observed that TLR3 was primarily localized to intracellular acidic organelles in a punctate distribution, consistent with the results of Funami et al. (17). The spots co-localized with acidic vesicles that can be stained with LysoTracker (data not shown). Mutant C242A, which is not implicated in disulfide bond formation and suffered no significant loss of activity in the cell-based assay, has an

| Plasmid | PIC | % |
|---------|-----|---|
| WT TLR3 | -   | 21 |
| WT TLR3 | +   | 100 | 2 |
| C242A   | +   | 89 | 9 |
| C365A   | -   | 104 | 4 |
| C242A/C365A | + | 104 | 4 |
| C28A    | +   | 39 | 13 |
| C37A    | +   | 30 | 9 |
| C37S    | +   | 28 | 11 |
| C37M    | +   | 30 | 10 |
| C95A    | +   | 33 | 11 |
| C122A   | +   | 20 | 9 |
| C122S   | +   | 18 | 4 |
| C122M   | +   | 18 | 4 |
| C649A   | +   | 20 | 1 |
| C649S   | +   | 26 | 2 |
| C649M   | +   | 24 | 2 |
| C651A   | +   | 20 | 2 |
| C651S   | +   | 21 | 2 |
| C696A   | +   | 23 | 1 |
| C28A/C122A | + | 24 | 2 |
| C37A/C651A | + | 23 | 1 |

**FIGURE 3. Effects of the mutations in the cysteines involved in the disulfide bond formation.** A, a summary of the effects of mutations on TLR3 activity. All mutant names contain the amino acid, its position in TLR3, and the residue to which it is mutated. All activities are normalized to the WT TLR3 assayed in the same experiment. B, Western blot analysis of select mutants to analyze whether the mutation affects the expression of the protein. pcDNA is the plasmid vector used to express TLR3 or mutant TLR3s. C, in situ localization of TLR3 stained with FITC-labeled monoclonal antibody specific for TLR3. The names of the samples are shown on the left, and the types of image taken are shown above the micrographs. D, results of FACS analysis of several cysteine mutants looking at the cell surface fluorescence. The monoclonal antibody recognizing TLR3, TLR3.7, was purchased from eBioSciences Inc. (San Diego, CA). The distribution of the cells is graphed, with the fluorescence intensity denoted on the horizontal axis. The shaded regions are signals from an immunoglobulin isotype of the monoclonal antibody recognizing TLR3. pc, pcDNA; WT, wild type TLR3.
TLR3 Structure-Function

We also examined the cell surface distribution for mutants C37A and C122A by fluorescence-activated cell sorting. C37A and C122A were reduced for cell surface expression (Fig. 3D). We concluded that all cysteines involved in disulfide bond formation in TLR3 are important for activity, and the mutants are expressed at levels comparable with WT, but some are affected in their intracellular localization. This change in localization could contribute to the loss of TLR3 activity.

Loop1—Structures that project out from the central solenoid structure of TLR3 ECD could provide features important for TLR3 function. A loop within the LRRs of TLR9 has been hypothesized to interact with the ligand, CpG DNA (18). TLR3 has two loops in the ECD solenoid. The first, called Loop1, resides in LRR12 (residues 335–343) and is rich in serine residues (Fig. 4, A and B). An examination of the sequence of Loop1 revealed that it is variable in its sequence and length. For example, whereas the mammalian Loop1 is composed of eight residues, the equivalents from fish have only six residues (Fig. 4B).

We replaced the central six residues of Loop1 (SISLAS) with the six-residue sequence, CCPGCC, that could bind the FlAsH dye (19). Our intention was to fluorescently label TLR3 by its binding to the FlAsH dye. However, this construct, L1–TCM, did not bind the FlAsH dye, perhaps due to steric constraints. Nonetheless, the construct was as active as the WT for NF-κB reporter activity (Fig. 4C). Next, we changed four of the residues in Loop1 from QSISLASL to QSTALTSH in a construct named L1–4M. Again, more than 85% of the TLR3 activity was retained. Last, we deleted Loop1 altogether (ΔL1) and found the resultant construct to retain greater than 80% of the WT TLR3 activity (Fig. 4C). Western analysis showed that the proteins were made similar to WT (Fig. 4D). In localization experiments, ΔL1 formed intracellular specks in a manner indistinguishable from WT (Fig. 4E). These results demonstrate that Loop1 is not essential for TLR3 function.

Loop2—The second loop in TLR3 ECD resides within LRR20 (residues 547–554) (Fig. 5). These results demonstrate that Loop1 is not essential for TLR3 function.

FIGURE 4. Examination of the role of Loop1 in TLR3 function. A, a partial model of TLR3 showing the relative location of Loop1. B, a comparison of the sequences in Loop1 from different species. The residues that are different from the human Loop1 sequence are shown in boldface type. The residues that are apparently deleted are shown as dashes. C, activity assay of WT TLR3 and mutations in Loop1 of TLR3. D, Western blot analysis examining whether the mutations in Loop1 affects protein expression. E, in situ localization of the mutant deleted for Loop1 in comparison with WT. The presence of TLR3 in punctate spots, the nuclei, and merging of the two results are shown as identified above the micrographs. Bar in the lower micrograph, 20 μm.

FIGURE 5. Examination of the role of Loop2 on TLR3 function. A, a model of a section of TLR3 showing the location of Loop2. B, a comparison of the sequences in Loop2 from different species. The residues that are different from the human Loop2 sequence are shown in black. C, activity assay of WT TLR3 and mutations in Loop2 of TLR3. D, Western blot analysis examining whether the mutations in Loop2 affect protein expression. E, in situ localization of the mutant deleted for Loop2 in comparison with WT TLR3. The presence of TLR3 in punctate spots, the nuclei, and merging of the two results are shown as identified above the micrographs. Bar in the lower micrograph, 20 μm.
not. We note that the amount of poly(I:C) used in these assays cross-linked to poly(I:C), whereas bovine serum albumin was added to hTLR3 ECD at an equal molar ratio to provide an internal control. The hTLR3 ECD was cross-linked with an equal mixture of hTLR3 ECD and bovine serum albumin (BSA). The phosphor image of the cross-linked products is shown in the left image. A, cross-linking between TLR3 ECD and poly(I:C) as a function of pH. Poly(I:C) was radiolabeled by kinasing with \(^{\gamma}-\text{P}\)ATP and T4 polynucleotide kinase. The cross-linking was performed with an equal mixture of hTLR3 ECD and bovine serum albumin (BSA). The phosphor image and the Coomassie Blue-stained gel images are on the left and right, respectively. B, effect of poly(I:C) length on RNA cross-linking as a function of pH. Poly(I:C) of 40 and 20 bp were radiolabeled separately and used as probes. The phosphor image and the Coomassie Blue-stained gel images are on the left and right, respectively. The RNAs used are shown above the lanes in which the RNA was added. The effect on cross-linking to the 20-bp poly(I:C) was quantified above the gel image. C, competition for hTLR3 ECD binding to poly(I:C) by competitor RNAs added to the reactions at 2–4-fold the level of 20-bp poly(I:C). The RNAs used are shown above the lanes in which the RNA was added. The effect on cross-linking to the 20-bp poly(I:C) was quantified above the gel image.}

**FIGURE 6.** Poly(I:C) binding by TLR3 ECD in vitro. A, a complex between the TLR3 ECD and poly(I:C) as a function of pH. Poly(I:C) was radiolabeled by kinasing with \(^{\gamma}-\text{P}\)ATP and T4 polynucleotide kinase. The cross-linking was performed with an equal mixture of hTLR3 ECD and bovine serum albumin (BSA). The phosphor image and the Coomassie Blue-stained gel images are on the left and right, respectively. B, effect of poly(I:C) length on RNA cross-linking as a function of pH. Poly(I:C) of 40 and 20 bp were radiolabeled separately and used as probes. The phosphor image and the Coomassie Blue-stained gel images are on the left and right, respectively. C, competition for hTLR3 ECD binding to poly(I:C) by competitor RNAs added to the reactions at 2–4-fold the level of 20-bp poly(I:C). The RNAs used are shown above the lanes in which the RNA was added. The effect on cross-linking to the 20-bp poly(I:C) was quantified above the gel image.

highly conserved (Fig. 5B). When the tetracysteine motif was inserted into the apex of Loop2, construct L2-TCM resulted in a protein that retained 82% of the activity of the WT (Fig. 5C). Replacement of the Loop2 sequence in TLR3 with the comparable sequence from Takifugu in construct L2-Fugu retained 75% of TLR3 function, confirming that there is some flexibility in the Loop2 sequence. However, a deletion of Loop2 in construct ΔL2 resulted in activity near background (Fig. 5C). Both the level of ΔL2 protein and its intracellular distribution were similar to WT (Fig. 5E). Additional examination of Loop2 along with other defective mutants will be presented below.

**Poly(I:C) Binding by TLR3 ECD**—TLR3 responds to dsRNA that could be generated during viral infection. dsRNA binding could occur either directly or through an accessory protein, such as CD14 (12). Assays for dsRNA binding by the TLR3 ECD are limited. Choe et al. (1) demonstrated an electrophoretic mobility shift of TLR3 ECD upon poly(I:C) binding, whereas Bell et al. (2) observed a complex between the TLR3 ECD and RNA in a gel filtration assay. We used a UV cross-linking assay to examine hTLR3 ECD interaction with a 40-bp poly(I:C) fluorescently labeled at the 5’ terminus with Texas Red. Since TLR3 is localized to acidic vesicles, we also assessed whether the pH of the reaction would affect TLR3 interaction with poly(I:C). Bovine serum albumin was added to hTLR3 ECD at an equal molar ratio to provide an internal control. The hTLR3 ECD was cross-linked to poly(I:C), whereas bovine serum albumin was not. We note that the amount of poly(I:C) used in these assays could induce TLR3-dependent activation of NF-κB activity. Furthermore, cross-linking to poly(I:C) was most effective at acidic pH (Fig. 6A).

It has been shown that TLR9 interacts with its ligand, non-methylated CpG DNA, in a pH-dependent manner (23). TLR9 interacts weakly with CpG DNA at physiological pH. However, at acidic pH similar to the one found in endosomal/lysosomal vesicles, the interaction was stronger and specific to nonmethylated CpG DNA. Taken together with our observation that TLR3 ECD interaction with poly(I:C) is stronger at acidic pH, it suggests that TLR3 and TLR9 signaling is initiated from acidic vesicles.

Since commercial preparations of poly(I:C) are heterogeneous in mass, we prepared poly(I:C) of 40 and 20 bp for the cross-linking assay. Both were cross-linked to hTLR3 ECD (Fig. 6B). Last, to determine whether hTLR3 ECD specifically recognized poly(I:C), we examined whether cross-linking to the radiolabeled 20-bp poly(I:C) could be competed with other potential ligands. The competitors used were unlabeled poly(I:C) of 20 or 40 bp, two small interfering RNAs of 21 bp, a highly structured RNA of 13 nucleotides (20), and a 33-nucleotide single-stranded unmodified RNA named −21/13 (21). The two preparations of poly(I:C) were effective competitors when present at 2–4-fold above the labeled ligand, reducing the radiolabeled complex to less than 40% of the reaction lacking a competitor (Fig. 6C). The two small interfering RNAs were weaker competitors, reducing poly(I:C) cross-linking to approximately one-third. The structured and single-stranded RNA were the worst competitors. These results provide biochemical evidence that hTLR3 ECD could specifically recognize poly(I:C) in the absence of accessory proteins.

Several residues in the TLR3 ECD were proposed to contact dsDNA (1). We made alanine mutants of most of the predicted residues as well as the basic residues near the Loop2 of TLR3 ECD. All of the single amino acid changes were largely unaffected in TLR3 activity (Fig. 7A). Combinations of two and three mutations in TLR3 did have some effect, but activity remained at more than half of the level for WT; therefore, none of the individual residues appear to be critical for TLR3 activity. Western blots showed that several of the single and multiple mutants had expression levels and in situ localization similar to that of WT (Fig. 7, B and C).

Bell et al. (13) proposed an asparagine-rich surface on the side and C-terminal third of the TLR3 ECD solenoid as the RNA-binding surface. Mutations of two residues, His539 and Asn541, had severe effects on TLR3 activity (Fig. 7B). Additional examination of Loop2 along with other defective mutants will be presented below.
helix, residues Glu\textsuperscript{442} and Lys\textsuperscript{467}, which are predicted to be involved in TLR3 oligomerization (1), are at the other side of the RNA molecule and could contact poly(I:C) and/or be affected by poly(I:C) binding.

We made mutants H539E and N541A and other mutations in the putative RNA-binding surface in an effort to confirm and extend the results of Bell \textit{et al.} (13). In agreement with their results, mutants H539E and N541A had TLR3 activity near background (Fig. 8B). Furthermore, adjacent mutations not previously tested by Bell \textit{et al.} (2), N466A and N540A, also reduced TLR3 activity to background level.

Some changes at the same positions in TLR3 that we tested had different effects from those reported by Bell \textit{et al.} (13), possibly due to the identity of the altered residue. It was reported that N515D and N516L did not affect TLR3 activity (13). We found that N515A and N516A reduced TLR3 activity to 47% and to 36% of WT. Also, mutant N572A had 55% activity of the WT. Last, R489A and N517A reduced TLR3 activity to nearly background, whereas Bell \textit{et al.} (13) reported that R489A and N517A had more than 50% activity. Western blots of these mutant proteins showed that they are produced in 293T cells, although some, such N515A, were present in slightly lower amounts compared with WT and could have contributed to decreased TLR3 activity (Fig. 8C).

Although some effects of our mutant analysis differed from the quantifications reported by Bell \textit{et al.} (13), both sets of results demonstrate that charged surface characterized by enriched asparagines in LRR17 to -20 on the side of the solenoid is critical for TLR3 function.

**TLR3 Oligomerization**—As noted above, the putative RNA-binding patch is spatially close to the putative dimerization domain in TLR3 ECD, suggesting a relationship between these
two activities. The TLR ECD could exist as a dimer in both three- and two-dimensional crystal lattices (1, 2, 10). However, that observation could be due to the high protein concentrations needed for cryo-crystal formation. Therefore, we examined whether hTLR3 ECD could exist in an oligomeric state at lower protein concentrations using dynamic light scattering analysis. The mass of hTLR3 ECD in solution was between 172 and 230 kDa, demonstrating that hTLR3 ECD can exist as a dimer in the absence of the ligand. As a third method to confirm the molecular mass of the native ECD, we subjected it to sedimentation analytic centrifugation. In comparison with the reference, the mass of hTLR3 ECD was determined to be 160 kDa (Fig. 9A). When tested in sodium acetate buffer from pH 6.0 to 4.8, the mass of hTLR3 ECD in solution was between 172 and 230 kDa, demonstrating that hTLR3 ECD can exist as a dimer in solution in the absence of ligand and at a pH typically found in an acidic vesicle. We also subjected hTLR3 ECD to gel filtration chromatography in comparison with molecular mass markers, and it eluted with a peak at 196 kDa, confirming that hTLR3 ECD exists predominantly as a dimer in vitro (data not shown). As a third method to confirm the molecular mass of the native ECD, we subjected it to sedimentation analytic centrifugation.

In comparison with the reference, the mass of hTLR3 ECD was determined to be 160 kDa (Fig. 9B), consistent with the mass being a dimer in the absence of the ligand.

Residues Glu442 and Lys467 and also Lys547 and Asp575 were predicted to form salt bridges between TLR3 subunits as a part of the interaction between TLR3 subunits (1). A number of amino acid substitutions were made to test this prediction. Mutants K467A and K467E only reduced TLR3 activity to 76 and 60% of WT, respectively (Fig. 9C). Mutants E442A and E442D retained more than 62% of WT TLR3 activity, but changing Glu442 to a lysine reduced activity to 25%. Localization of E442K in 293T cells showed that it is expressed similar to WT (Fig. 9D). Also, K547A and D575A mutants had negligible effect on activity of the protein as measured by NF-κB activation (Fig. 9C). However, we note that a double mutant E442K/K467E had 66% of the WT TLR3 activity. This suggests that the reduction of activity seen with E442K can be partially compensated with the K467E mutation. These results identify that a negatively charged residue at position 442 is important for TLR3 function, but our results do not support the idea that these residues form simple salt bridges, since neither E442A nor K467A reduced TLR3 activity significantly. It is possible that some changes at this position could be better compensated by a network of interactions involving two ECD molecules, as proposed by Choe et al. (1).

Given the difficulty in assessing the oligomerization state of TLR3 in cells, we used a genetic assay to assess whether mutant versions of TLR3 could suppress the activity of WT TLR3 (i.e. to act as a dominant negative). Although the mechanistic basis for the dominant negative activity of a mutant TLR3 is not understood, two likely possibilities exist. 1) A mutant TLR3 is unaffected for binding to the WT TLR3 but cannot carry out other activities needed to activate gene expression. Therefore, the mutant protein traps the WT in an inactive state. 2) The mutant protein exists as monomer and retains the ability to bind and titrate ligands and/or accessory factors away from the WT. Given that the TLR3 ECD could form a dimer (Fig. 9A and B), we believe that the first model is more likely to apply.

To examine the basis for dominant negativity/dimer formation, we used ΔTIR, a dominant negative version of TLR3 that lacks the TIR domain (17). Since ΔTIR is inactive for TLR3 activity, all of the output of the assay is from the co-transfected WT TLR3. At a 2- or 6-fold molar excess of the WT TLR3, ΔTIR suppressed TLR3 activity to 26 and 12%, compared with an assay containing WT TLR3 challenged with comparable amounts of the empty vector (Fig. 10A). If the dominant negative effect of ΔTIR occurs by titrating away the ligand poly(I:C), then increasing poly(I:C) concentration should at least partially reverse the dominant negative effect. To test this, a 4- or 8-fold higher concentration of poly(I:C) was added to the cells, and no significant change in the dominant negative effect of ΔTIR was observed (Fig. 10B), suggesting that the effect is not due to ΔTIR titrating away the ligand. Similar results were obtained even when ΔTIR was present at a 1:1 ratio to that of WT (data not shown).

If the dominant negative activity were due to protein-protein interaction, then mutations that affect RNA binding without affecting protein-protein interaction would be dominant nega-
TLR3 activity, we tested it for dominant negativity only when it lacked the ΔTIR domain. K467EΔTIR inhibited TLR3 activity to 27% at a 6-fold molar excess, suggesting that mutation K457E did not affect dominant negativity (Fig. 10D).

Another mutant that affected TLR3 activity dramatically was ΔL2. Both ΔL2 and ΔL2ΔTIR were poor dominant negatives (Fig. 10, C and D). Based on these results, we propose that Glu442 and Loop2, both of which are near the poly(I:C) binding surface, are required for interactions between TLR3 subunits.

The above result suggests that there may be a functional link between RNA binding and TLR3 dimerization. Therefore, we tested whether changes of the residues in/near the charged surface that contacts the dsRNA will affect TLR3-TLR3 interaction and thereby act in a dominant negative manner. Mutants R489A, N517A, N540A, and N541A had different abilities to inhibit the activity of WT TLR3, ranging from R489A, N517A, and N541A, which were able to partially retain dominantly negative activity, to N540A, which is a poor dominant negative. These results suggest that some of the residues in the asparagine-rich surface of the TLR3 ECD that putatively contacts poly(I:C) can participate in protein-protein interaction to result in a dominant negative phenotype. There appears to be an intimate relationship between RNA binding by TLR3 and dimerization.

CONCLUSIONS

In this work, we analyzed several features of the TLR3 ECD that were predicted by structural analysis. These characterizations build on those from de Bouteiller et al. (11) and Bell et al. (13) and examined the models for TLR3 ECD function proposed by Choe et al. (1) and Bell et al. (3). We find that the recombinant TLR3 ECD protein can be demonstrated to contain at least one disulfide bond that involves Cys28 and Cys37, as determined by mass spectrometry analysis. Furthermore, all of the cysteine mutants involved in disulfide bond formation are essential for TLR3 activity. We have also demonstrated that Loop1 within LRR12 of the TLR3 ECD is dispensable for TLR3 activity. In fact, Loop1 may be useful as a place to insert a specific tag to follow TLR3 localization. We also demonstrated that hTLR3 ECD can be cross-linked to poly(I:C) in pH conditions similar to that of acidic vesicles and that non-dsRNAs are poor competitors for this cross-linking between TLR3 and poly(I:C). Also, TLR3 ECD appears to exist as a dimer in solution in the absence of ligand.

Ruiz et al. (23) have shown that TLR9 interacts with its ligand, nonmethylated CpG DNA, in a pH-dependent manner.

FIGURE 10. Assessing the ability of TLR3 variants to act as dominant negatives. A, analysis of mutant ΔTIR and Y759F to activate TLR3 activity and to act as dominant negatives. In all of these assays, "1×" denotes that the plasmid is present at 15 ng/transfection. The dominant negative assays were performed with 2 and 6 times this concentration. B, a demonstration that increasing poly(I:C) induction could not reverse the dominant negative effect of ΔTIR. C, a summary of the assays for dominant negativity by several mutations that are defective for TLR3 activity. The mutants selected for analysis are also ones that are expressed well, as determined by Western blots. D, a summary of the result from selected mutants built into the context where the TIR domain was deleted (ΔTIR). This construction is useful to confirm the dominant negative result, since the mutants cannot activate NF-κB in the absence of the TIR domain, thus reducing the background for the assay.

TIR3 Structure-Function

A

```
| Compet. | Fold | %   |
|---------|------|-----|
| None    | 1.0  | 100 |
| WT      | 1.0  | 100 |
| ΔTIR    | 1.0  | 100 |
| Y759F   | 1.0  | 100 |
```

B

```
| Compet. | Fold | %   |
|---------|------|-----|
| None    | 1.0  | 100 |
| WT      | 1.0  | 100 |
| ΔTIR    | 1.0  | 100 |
| Y759F   | 1.0  | 100 |
```

C

```
| Compet. | Fold | %   |
|---------|------|-----|
| None    | 1.0  | 100 |
| WT      | 1.0  | 100 |
| ΔTIR    | 1.0  | 100 |
| Y759F   | 1.0  | 100 |
```

D

```
| Compet. | Fold | %   |
|---------|------|-----|
| None    | 1.0  | 100 |
| WT      | 1.0  | 100 |
| ΔTIR    | 1.0  | 100 |
| Y759F   | 1.0  | 100 |
```
TLR9 interacts weakly with CpG DNA at physiological pH. However, at acidic pH similar to that expected in endosomal/lysosomal vesicles, the interaction was stronger and specific to nonmethylated CpG DNA. Taken together with our observation that TLR3 ECD interaction with poly(I:C) is stronger at acidic pH, it suggests that TLR3 and TLR9 signaling is initiated from acidic vesicles.

Perhaps the most interesting aspect of this work is that there is an overlap in the TLR3 ECD residues that are required for poly(I:C) binding with those required for dominant negativity, the mechanistic basis of which is probably due to the binding between a nonfunctional protein WT TLR3 through their respective ECD domains. Using the dominant negative assay, mutations that severely affected TLR3 activation of downstream reporter activity can be separated into those that retain the ability to be dominant negatives and those that do not. Interestingly, some, but not all, of the putative RNA-binding surfaces in TLR3 are required for dominant negative effect of TLR3. Also, Loop2 in LRR20 is required for dominant negativity, suggesting a role in protein-protein interaction. Choe et al. (1) predicted a number of residues that are important for protein-protein interactions between two ECDs. Our mutational analysis supports their claims for the interactions between TLR3 ECD subunits. Most of the residues, including His539 and Asn541, that are suggested to bind RNA by Bell et al. (13) have a considerable overlap between the activities of RNA binding and dimerization.

Two models have been proposed for how ligand binding is related to dimerization. Bell et al. (13) proposed that ligand binding induces dimerization. Choe et al. (1) proposes that two subunits of TLR3 ECD could interact without the ligand but upon ligand binding would change conformation and lead to activation of the TIR domains. Although our results largely support the identification the RNA-binding residues shown by Bell et al. (13), we also observed that the TLR3 ECD could form dimers in the absence of ligand and that putative RNA-binding mutants could act as dominant negatives, which we interpret to mean that it is capable of protein-protein interaction in cells before induction with ligand. Therefore, our model concerning the relationship of the two activities is more akin to the model of Choe et al. (1).
In summary, our data suggest that TLR3 can exist in an oligomerized state in the absence of ligand mostly through Loop2 interactions. However, the ligand binding will cause rearrangement in the dimer, leading to lateral sliding of the two molecules toward each other, whereas the two molecules adjust to accommodate the dsRNA (Fig. 11A). In this ligand-bound form, residues Glu442 and Asn517 (and probably others) will interact primarily with the dsRNA to stabilize the complex. The resultant conformational change due to the sliding of the protein subunits may stimulate the interaction of the TIR domains, the resultant conformational change due to the sliding of the protein subunits may stimulate the interaction of the TIR domains, the subsequent dimerization of which will lead to the activation of the signal transduction pathway (Fig. 11B).

Recently, Gay et al. (24) proposed that a series of conformational changes is required for signaling by the Drosophila Toll protein and the Toll-like receptors. The conformational changes could be initiated by the ligand binding to a preexisting nonfunctional dimer. In the case of ligand-induced Toll, dimerization has been observed both in vitro and in vivo (25, 26). Our model with TLR3 suggests that Toll and the TLRs share a common mechanism for signaling through conformational changes. However, it is important to note that this signaling mechanism does not rule out a role for RNA chaperones to help transfer dsRNA to TLR3. CD14, NFAT proteins, or RNA helicase A, which have all been reported to bind poly(I:C) (12, 27), could chaperone dsRNA to TLR3 or modulate TLR3 interactions with subunits and/or with RNA.

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