Structural and Functional Analyses of the Human Toll-like Receptor 3

ROLE OF GLYCOSYLATION

Received for publication, September 22, 2005, and in revised form, February 16, 2006. Published, JBC Papers in Press, February 16, 2006, DOI 10.1074/jbc.M510442200

Jingchuan Sun, Karen E. Duffy, C. T. Ranjit-Kumar, Jin Xiong, Roberta J. Lamb, Jon Santos, Hema Masarapu, Mark Cunningham, Andreas Holzenburg, Robert T. Sarisky, M. Lamine Mbowa, and Cheng Kao

From the Departments of Biochemistry and Biophysics and Biology and the Microscopy and Imaging Center, Texas A&M University, College Station, Texas 77843 and Discovery Research, Centocor Research and Development, Inc., Radnor, Pennsylvania 19087

Toll-like receptors (TLRs) play critical roles in bridging the innate and adaptive immune responses. The human TLR3 recognizes foreign-derived double-stranded RNA and endogenous necrotic cell RNA as ligands. Herein we characterized the contribution of glycosylation to TLR3 structure and function. Exogenous addition of purified extracellular domain of TLR3 (hTLR3 ECD) expressed in human embryonic kidney cells was found to inhibit TLR3-dependent signaling, thus providing a reagent for structural and functional characterization. Approximately 35% of the mass of the hTLR3 ECD was due to posttranslational modification, with N-linked glycosyl groups contributing substantially to the additional mass. Cells treated with tunicamycin, an inhibitor of glycosylation, prevented TLR3-induced NF-κB activation, confirming that N-linked glycosylation is required for bioactivity of this receptor. Further, mutations in two of these predicted glycosylation sites impaired TLR3 signaling without obviously affecting the expression of the protein. Single-particle structures reconstructed from electron microscopy images and two-dimensional crystallization revealed that hTLR3 ECD forms a horseshoe structure similar to the recently elucidated x-ray structure of the protein expressed in insect cells using baculovirus vectors (Choe, J., Kelker, M. S., and Wilson, I. A. (2005) Science 309, 581–585 and Bell, J. K., Botos, I., Hall, P. R., Askins, J., Shiloach, J., Segal, D. M., and Davies, D. R. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10976–10980). There are, however, notable differences between the human cell-derived and insect cell-derived structures, including features attributable to glycosylation.

Proteins that recognize pathogen-associated molecular patterns are key factors in the cascade of events from the detection to the elimination of an invading organism. This form of innate immunity is conserved in eukaryotes. For example, the Drosophila melanogaster Toll protein is responsible for resistance to fungal and bacterial infections (3, 4), and plants can encode disease-resistance proteins that are important in determining the outcome of infection (5). The vertebrate pathogen-detecting proteins called Toll-like receptors (TLRs) are key players in the activation of both the innate and adaptive arms of the immune system (6–9).

The TLRs and related pathogen sensors contain leucine-rich repeat motifs that form docking sites for pathogen ligands or adaptors that bind pathogen ligands, the binding of which will activate signal transduction pathway(s) (10–12). TLR3 recognizes double-stranded RNA and may be a part of a redundant sensor system to detect viral infections (13–15). Although specific features in the ligands required to interact with TLR3 remain to be identified, TLR3 is activated by polyinosine-polycytidylic acid (poly(I:C)) and has been reported to be activated by RNAs extracted from necrotic cells (16).

A number of issues concerning TLR3 structure and function remain to be elucidated. For example, TLR3 can apparently act both on the surface of the plasma membrane, as it does in fibroblasts, and by attaching to the membranes of intracellular vacuoles, where it is proposed to act in immature dendritic cells (17, 18). The trafficking of TLRs should be influenced by glycosylation in general, and N-linked glycosylation of TLR2 and TLR4 has been shown to play essential roles in its localization (19, 20). A significant recent advance in TLR3 was the elucidation of a 2.1 Å structure of the soluble ectodomain by Choe et al. (1). Bell et al. (2) independently elucidated a highly similar structure. In both studies, the crystalized ectodomains were produced in a baculovirus expression system and formed a horseshoe-shaped solenoid structure that was extensively decorated with glycosyl modifications, some of which were partially resolved in the structure. Whether the glycosylations are important in TLR3 localization and/or function were not directly addressed in these works (1, 2). However, de Bouteiller et al. (21) showed that a change of Asn-247 to an arginine in TLR3 negatively affected TLR3 activity.

We expressed the extracellular domain (ECD) of the human TLR3 in human embryonic kidney cells (HEK 293T) and demonstrated that it was modified with N-linked glycosylations. Using the GlcNAc-transferase inhibitor tunicamycin, a concentration-dependent inhibition of TLR3 activity was observed. Systematic mutational analysis of the predicted N-linked glycosylation sites identified two asparagine residues in leucine-rich repeats 8 and 15 that are important for TLR3 activity. The mutant proteins remain expressed at levels similar to wild type. In addition, because our ectodomain was produced in human cells as opposed to insect-derived protein, which
TLR3 Structure and Function

differs in the presence of glycosylations, we independently established the structural framework for the hTLR3 ECD using electron microscopy of single particles and two-dimensional crystals in conjunction with image reconstruction.

MATERIALS AND METHODS

Expression and Purification of hTLR3 ECD—The full-length human TLR3 cDNA, identical to accession number U88879, was amplified from human dendritic cells and cloned into pcDNA3.1. The extracellular domain of TLR3, consisting of amino acids 1–703, was also cloned into pcDNA3.1 to generate the hTLR3-ECD antigen with a C-terminal hexahistidine tag. The protein was expressed and secreted by transiently transfected HEK 293E cells. During the secretion process, residues 1–26 were cleaved, generating a protein containing residues 27–703. The secreted protein was purified from the supernatant of 293E cells using a BD-TALON™ metal affinity resin (BD Biosciences) and further purified by gel filtration chromatography.

Cell-based Assays—HEK 293T cells were harvested from an actively growing culture and plated in CoStar White 96-well plates at 4.4 × 10^4/ml for transfection. When the cells were ~65–90% confluent, they were transfected with a mixture of the Lipofectamine 2000 reagent (Invitrogen) and plasmids pNF-κB-Luc (Stratagene Inc., La Jolla, CA), pFL-TLR3, and phRL-TK (Promega Corp., Madison, WI) that, respectively, code for the firefly luciferase reporter, full-length wild-type pFL-TLR3, and phRL-TK (Promega Corp., Madison, WI) that, respectively, code for the firefly luciferase reporter, full-length wild-type and/or TLR3s with mutations in the ectodomain, and the Renilla luciferase transfection control. The cells were allowed to incubate for 24 h to allow expression from the plasmids. Poly(I:C) (1 μg/ml, unless stated otherwise) was then added to appropriate sets of transfected cells to induce TLR3-dependent NF-κB activity. After another 6 h of incubation, the cells were harvested using the Dual Glo Luciferase Assay System reagents (Promega). Luminescence was quantified using the FLU-Ostar OPTIMA Plate Reader (BMG Labtech, Inc.).

Electron Microscopy and Image Processing—For single particle imaging, 2.5 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22). The two-dimensional crystals were grown using the lipid monolayer approach (23). Briefly, 0.2 μl of a 50-μg/ml solution of TLR3 ECD in Tris-buffered saline (pH 7.4) was either applied to freshly glow-discharged carbon-coated copper grids (G400) or to holey carbon films. The samples were washed with distilled water and stained for 1 min with an aqueous solution of uranyl acetate (1% w/v, pH 4.25) for the G400 grids or stained with 5% (w/v) ammonium molybdate (pH 6.0) containing 0.1% (w/v) trehalose for the holey carbon film according to Harris and Scheffler (22).
TLR3 Structure and Function

Production and characterization of the human TLR3 ECD. A, purified hTLR3 ECD (residues 27–704) electrophoresed on a NuPAGE 4–12% bis-tris gel (Invitrogen). The molecular mass of the hTLR3 ECD (0.05 and 0.25 μg) is calibrated against the Benchmark protein ladder (designated M) (Invitrogen). B, expression of the TLR3 ectodomain (residues 1–704) in a rabbit reticulocyte lysate (Promega). This protein, designated RRL ECD, lacks glycosylation. C, a Western blot demonstrating that 293T cells do not contain detectable full-length TLR3. TLR3 is identified by the right of the gel image, and a protein nonspecifically recognized by mAb MG315A (Imgenex Inc.) is identified by N.S. D, the recombinant hTLR3 ECD could inhibit TLR3-dependent signaling in 293T cells. Results of the cell-based assay were determined 20 h after the addition of poly(I:C) to 1 μg/ml of final concentration. Each symbol represents the firefly/Renilla luciferase ratio for one culture of cells tested, with the horizontal line representing the average of all the individual wells. Poly(I:C) was preincubated in the presence and absence of TLR3 ECD in medium for 30 min at 37 °C before this mixture was added to the cells.

Eight classes were selected to generate a first three-dimensional reconstruction with C1 symmetry using STARTANY. This first three-dimensional reconstruction served as a starting point for iterative refinement until the reconstruction was stable as judged by Fourier shell correlation. The three-dimensional volume of the final reconstruction was displayed with the program Chimera (26).

RESULTS

Purified Recombinant hTLR3 ECD Can Affect Signaling by Full-length TLR3—We expressed and purified amino acids 27–704 of the extracellular domain of human TLR3 in 293T cells to initiate characterization of its structure and function. The recombinant protein contained six additional histidine residues at the C terminus to facilitate purification and is henceforth named hTLR3 ECD. In SDS-PAGE, purified hTLR3 ECD migrated as a mass of ~110 kDa, much larger than the predicted mass of ~75 kDa (Fig. 1A). The difference is likely to be because of posttranslational modifications. To confirm that this is the case, we translated residues 1–704 of TLR3 in rabbit reticulocyte lysates and found that the resultant protein migrated at the expected mass (Fig. 1B). These results indicate that hTLR3 ECD is heavily modified, likely by glycosylation, a feature that we will examine below.

To examine the function of the hTLR3 ECD and how mutations affected TLR3 activity, we used a cell-based assay that measures TLR3-dependent activation of gene expression. Briefly, the assay was performed by transiently transfecting 293T cells with a mixture of three plasmids: one expressing wild-type TLR3, a second reporter plasmid in which firefly luciferase sequence was expressed from a promoter containing two NF-κB binding sites, and a third control plasmid that constitutively expresses the Renilla luciferase under the control of the Herpes Simplex Virus thymidine kinase promoter. The assay thus detects TLR3 function by the amount of reporter firefly luciferase activity produced through activation of the NF-κB transcription factor. The Renilla luciferase activity was used to detect any potentially toxic effects of our manipulations. HEK 293T cells are suitable for these assays because they do not produce detectable TLR3 activity (21) and do not contain detectable TLR3 in Western blots (Fig. 1C). Furthermore, this reporter assay is strictly dependent on the transient expression of TLR3 (data not shown) and specifically dependent on the addition of poly(I:C) (Fig. 1D). A typical assay will increase the ratio of firefly luciferase to Renilla luciferase by 4- to 7-fold (Fig. 1D).

When hTLR3 ECD was added to the reporter assay to a final concentration of 12.5–100 μg/ml, we observed a concentration-dependent inhibition of wild-type TLR3-induced NF-κB activity (Fig. 1D), demonstrating that hTLR3 ECD can effectively compete with wild-type human TLR3 for poly(I:C)-induced NF-κB activation. These results indicate that hTLR3 ECD is suitable for additional biochemical analyses.

hTLR3 ECD Is Glycosylated—Mass spectrometry was used to examine the mass of hTLR3 ECD with greater precision. The purified hTLR3 ECD ionized as a large number of peaks that ranged from 105 to 115 kDa, indicating that it exists in a highly heterogeneous state (data not shown). A similar proportion of the extra mass in the ectodomain of TLR2 and TLR4 is due to glycosylation (19).

To visualize the glycosyl groups on hTLR3 ECD directly, we separated hTLR3 ECD by SDS-PAGE and stained the gel with a glycosylation-specific fluorescent dye, SYPRO Ruby (Fig. 2A). Treatment with NANAse or O-glycosidase, which releases α (2, 3)-linked sialic acids and unsubstituted Ser- or Thr-linked GalGalNac, respectively, did not alter the electrophoretic mobility of hTLR3 ECD or abolish binding by SYPRO Ruby dye. PNGlycosidase F, which releases the N-linked glycans, not only decreased the molecular mass of hTLR3 ECD from ~115 to ~90 kDa (Fig. 2A) but also reduced the amount of bound dye. An ~90-kDa band was detected in amounts similar to the input when stained with Coomassie Blue (data not shown). These results confirm that hTLR3 ECD contains a significant amount of glycosylation and further demonstrate that the majority of the removable glycosyl groups are N-linked. The fact that the mass of the protein was not reduced to the unmodified 75 kDa, the residual staining with Ruby Red, and the heterogeneous peaks in mass spectrometry suggest that additional modifications other than N-linked glycosylations are present.

Glycosylation of TLR3 Is Required for TLR3 Function—Inhibitors of protein glycosylation were used to examine whether glycosylation is required for TLR3 function in 293T cells (Fig. 2, B–D). Tunicamycin inhibits the addition of N-acetylglucosamine, the first sugar of N-linked glycosylation, whereas swainsonine affects the processing of the terminal mannoses by lysosomal mannosidase II that could lead to more complex glycosylation structures (27, 28). Both compounds were added between 0.2 and 5 μg/ml. These concentrations do not exhibit toxicity as determined by the normal expression of Renilla luciferase expressed from the constitutive thymidine kinase promoter (data not shown). Tunicamycin inhibited TLR3-dependent induction of the NF-κB reporter in a concentration-dependent manner (Fig. 2B). However, swainsonine did not apparently affect TLR3 signaling even at 5 μg/ml, a concentration that should be sufficient to inhibit glycosylation (Fig. 2C) (29). Another mannosidase II inhibitor, deoxymannojirimycin, also did not affect TLR3 activity in our assay, suggesting that TLR3 activity is

6 C. Kao, data not shown.
not affected by modifications that require mannosidase II. The differential effects of tunicamycin and swainsonine have been reported for a number of glycoproteins (30, 31).

To determine whether treatment with tunicamycin or swainsonine affected the level of TLR3 expression, transfected HEK297 cells were analyzed by Western blot. Tunicamycin addition to the cultures significantly decreased the amount of full-length TLR3, whereas the addition of swainsonine to 5 μg/ml did not have an obvious effect on TLR3 levels (Fig. 2D). The decrease in TLR3 levels was consistently observed in at least six cultures in this experiment. The assay result is represented as the ratio of firefly luciferase (indicating TLR3 signaling) to Renilla luciferase (transfection control). The presence or absence of poly(I:C) (PIC) at 10 μg/ml is denoted by + or −, respectively. C, effects of swainsonine on TLR3 activity in the cell-based assay. D, Western blot and FACS analysis of TLR3 expression in transiently transfected 293T cells, as affected by tunicamycin or swainsonine. TLR3.7 was used for the FACS analysis but not the Western blots because it does not recognize denatured TLR3 (17). Western blots were treated with the monoclonal antibody IMG315A from Imgenix Inc. Tunicamycin or swainsonine was added to a final concentration of 5 μg/ml.
were also mutated. For these four residues, Choe et al. (1) observed electron densities associated with N-glycosamini for residues Asn-124 and Asn-252 and Bell et al. (2) observed glycosylations associated with Asn-70 and Asn-252, but not Asn-124. We do not have evidence that Asn-388 is associated with glycosylations. Plasmids containing the mutant constructs were individually transfected into HEK 293T cells, and the effects on the NF-κB reporter activity were assessed. Changes in the four asparagines that do not match the consensus N-linked glyco- glycosylation sites did not significantly affect TLR3-dependent activation of the reporter assay, although the mutation at Asn-252 decreased NF-κB activity slightly in this assay and had modest effects in other assays (Fig. 3A and data not shown). However, changes in two of the five conserved potential N-linked glycosylation sites, N247A and N413A, consistently reduced TLR3-mediated NF-κB activation to half or less of the wild-type level (Fig. 3C). We also tested both the N413A and N265A mutations at a range of plasmid concentrations from 5 to 20 ng/transfection and found that the defect relative to wild type was observed at all concentrations tested (data not shown). The change at Asn-247 is in agree- ment with the observations of de Bouteiller et al. (21), who characterized a change of this residue to an arginine and found the resultant protein to be nonfunctional. A version of TLR3 containing both the N247A and the N413A mutations reduced TLR3 activity to background (Fig. 3C).

Expression and Localization of Mutant TLR3 Proteins—The lack of TLR3 activity with mutations N247A and N413A could be either because of an effect on protein expression, protein localization, or on a specific activity such as ligand binding. To examine the expression of the mutant TLR3 proteins, we first performed a Western blot with mAb IMG315A as detailed under “Materials and Methods.” B, TLR3 expression as analyzed by FACS analysis using the anti-human TLR3 monoclonal antibody TLR3.7 or an isotype control antibody. The plasmids used to transfect the 293T cells are shown in bold. The quantifications of the histogram are shown.

FIGURE 4. Expression of wild-type and glycosylation mutants of TLR3. A, a representative Western blot examining the expression of TLR3 in 293T cells. The cells were transfected with the plasmids listed above the gel image. The lysates were probed with mAb IMG315A as detailed under “Materials and Methods.” B, TLR3 expression as analyzed by FACS analysis using the anti-human TLR3 monoclonal antibody TLR3.7 or an isotype control antibody. The plasmids used to transfect the 293T cells are shown in bold. The quantifications of the histogram are shown.

with wild-type TLR3 showed MFI levels over 27. All mutants, includ- ing N247A and N413A, had MFI >18, indicating that all of the proteins were expressed on the cell surface at levels comparable with the WT TLR3 (Fig. 4B). The background level for permeabilized cells was higher, likely because of the monoclonal antibody recognizing other proteins nonspecifically (see Fig. 1C and Ref. 17). However, cells transfected to express the mutant proteins had significantly higher MFI compared with the control (Fig. 4B), confirming the results of the Western blots that these mutant proteins are expressed. The effects of the mutations thus cannot be attributed simply to a defect in the lack of expression.

Electron Microscopy of hTLR3 ECD—Because hTLR3 ECD protein was produced in human cells, it is reasonable to expect that it would be more extensively glycosylated in comparison with the protein crystal- lized by Choe et al. (1) and Bell et al. (2). Furthermore, most of the glycosyl groups were not well resolved in the x-ray structures. We used electron microscopy to investigate possible molecular interactions and the quaternary structure of hTLR3 ECD in solution and on a lipid sur- face. To this end, two-dimensional crystals were grown on lipid mono- layers. Several different types of two-dimensional crystals were observed and imaged. Fourier transformations of selected crystalline areas displayed readily discernible reflections to 3 nm resolution. The major two-dimensional crystal type is shown in Fig. 5, A and B. This type of crystal displayed a p22,2, symmetry (a = 26.5 nm, b = 12.5 nm, γ = 90°) and featured a subunit of hTLR3 ECD interacting through a terminus to the central portion of a second subunit. The other crystal type (Fig. 5, C and D) displayed p2 symmetry (a = 9.7 nm, b = 18.2 nm, γ = 109.9°), with each asymmetric unit having two horseshoe-shaped molecules related by a 2-fold axis. Furthermore, there is a notable deficit in the middle of the horseshoe-shaped molecule that was not observed in the structure of Choe et al. (1). This central deficit could not be seen in the other structure because of the overlap between the two subunits. We noted that the interactions between the two monomers in both crystal forms are different from the dimer proposed by Choe et al. (1) and Bell et al. (2). In addition, the fact that at least two different ways of crystal packing were observed suggests that the interactions between

FIGURE 5. Two-dimensional crystals of the hTLR3 ECD grown on lipid monolayers. A and C, electron micrographs of negatively stained two-dimensional crystals of hTLR3 ECD. The insert shows a representative Fourier transformation. B, projection map of the two-dimensional crystal with p22,2, symmetry. D, projection map of the two-dimen- sional crystal with p2 symmetry.
TLR3 ECD molecules can be variable. In the p22 crystal, two modes of interaction between the molecules exist even within one-unit cells: (i) molecules are packed in parallel "filaments" of alternating polarity consisting of horseshoe-shaped TLR3 molecules in a flip-flop arrangement where one terminal domain of a horseshoe interacts with the central domain of another molecule, and (ii) two molecules interact via their corresponding terminal domains, thereby forming centers of 2-fold symmetry. A more complete understanding of the native quaternary structure of TLR3 must await the analysis of full-length protein in the lipid bilayer.

We also performed a three-dimensional reconstruction of hTLR3 ECD using a single-particle analysis. Two different sample preparation methods were used. First, we used glow-discharged continuous carbon films to absorb hTLR3 ECD, followed by staining of the molecules with 1% uranyl acetate (Fig. 6, A and B). The second method suspends the sample over holey carbon film followed by embedding and negative staining with trehalose/ammonium molybdate (Fig. 6B). Although the contrast is lower, the latter method better preserves the proteins in a state closer to their native conformation (22).

Well separated single particles of ~9-nm diameter were readily discernible with both sample preparation methods (Fig. 6, A and B). The observed structures appeared to be monomeric in solution and have a characteristic horseshoe shape. Classification and average of ~2000 particles by multistatic analysis and multireference alignment with IMAGIC-5 software revealed heterogeneity of hTLR3 ECD in terms of the sizes and shapes of hTLR3 ECD (Fig. 6B, inset). For example, some molecules exhibit a more opened horseshoe structure, whereas in others the termini portions appear to be almost in contact with each other (Fig. 6B). Although some of these variations may be because of subtle differences in the orientations of the molecules relative to the electron beam, others may be caused by different conformation and perhaps differences in the patterns of glycosylation between molecules. This degree of conformational flexibility would not be difficult to detect when the protein is in a more rigid crystal lattice. In addition, it should be noted that some peanut-shaped particles with two distinct density maxima were occasionally observed (Fig. 6A, inset). These projections likely represent side-on views.

The three-dimensional reconstruction using the data obtained from single particles negatively stained with uranyl acetate and C1 symmetry resulted in a structure with ~2.5-nm resolution (Fig. 6, C and D). The outer diameter of the final structure is 9–9.5 nm, and the inner boundary of the horseshoe measures ~3.5–4 nm. The extra densities in our hTLR3 ECD are likely contributed from glycosylation. However, higher resolution structures would be needed to accurately locate the sites of glycosylation.

**FIGURE 6. Single-particle analysis of the hTLR3 ECD.** A, electron micrograph of negatively stained hTLR3 ECD on continuous carbon film. The inset contains a gallery of class averages. Box size is 16 × 16 nm. Top row, raw particles; middle row, class averages; bottom row, re-projections from the three-dimensional reconstruction. B, electron micrograph of negatively stained hTLR3 ECD on holey carbon film. The inset contains a gallery of the class averages of the single particles. C, Fourier shell correlation of refinement process and EOTEST. The inset is an asymmetric triangle showing the distribution of the particles’ orientations. D, surface display of the three-dimensional reconstruction of hTLR3 ECD. For surface rendering a threshold corresponding to a molecular mass of 100 KDa was used.
TLR3 Structure and Function

Lectin-hTLR3 ECD Interaction—We used electron microscopy and single-particle analysis to directly examine the extent of glycosylation in hTLR3 ECD. This approach required that we identify a lectin that will bind hTLR3 ECD. Seven commercially available lectins (Vector Laboratories, Burlingame, CA) were tested for the ability to detect decreasing amounts of hTLR3 ECD spotted on membranes. The *Ricinus communis* lectin (120 kDa) recognizes hTLR3 ECD better than others and was selected for this analysis. It was mixed at a 2:1 ratio with the hTLR3 ECD for 1 h and stained with uranyl acetate. Particles that have additional density from the hTLR3 ECD were collected, and class averages were obtained. The results show that the *R. communis* lectin was able to bind at multiple positions on the outer perimeter of the horseshoe structure (Fig. 7A), providing experimental evidence confirming that glycosyl groups exist at multiple sites of the hTLR3 ectodomain.

Modeling of the hTLR3 ECD—As shown above, our mutagenesis data have implicated that mutations at Asn-247 and Asn-413 significantly affected TLR3 function and that a third residue, Asn-252, may have a modest effect on TLR3 function. The locations of these residues were modeled based on the structure of the TLR3 ECD solved by Bell et al. (2). Asn-252 is pointed into the concave surface of TLR3 ECD (Fig. 7B). Asn-247 were found to lie in the outer convex surface, and Asn-413 was closer to the inner concave surface of the molecule.

Lastly, given that the structure of the hTLR3 ECD derived from our studies appears to be somewhat larger than the molecules reported by Bell et al. (2) and Choe et al. (1), we superimposed the structures derived from x-ray crystallography with our lower resolution structures (Fig. 7C). We observed that the electron microscopy structure had the largest difference from those from x-ray crystallography in the diameter of the solenoid, especially near the middle of the protein. We speculate that this difference may be because of differences in glycosylation between the protein expressed in insect and human cells.

DISCUSSION

We have analyzed the structure and function of the human TLR3 ectodomain and determined that the protein can be expressed in human cells in a highly glycosylated form, with N-linked glycosylations contributing significantly to additional mass of the hTLR3 ECD. Additionally, treatment with tunicamycin, which inhibits N-linked glycosylation, abolished reporter signaling by a process stimulated by poly(I:C), likely by reducing the amount of TLR3 expressed. Furthermore, mutations in two of the thirteen potential glycosylation sites in hTLR3 ECD prevented signaling but did not apparently affect expression or cell surface localization. Lastly, we used negatively stained electron micrographs and molecular modeling to visualize hTLR3 ECD.

The structure for the hTLR3 ECD we observed is a solenoid-shaped horseshoe, in agreement with the structures of Choe et al. (1) and Bell et al. (2). There are some differences that may be attributable to our preparation of the protein from human cells. One feature is a prominent shallow notch that is approximately at the middle of the structure (Fig. 5C). A second feature is that our structure has more variable conformation (Fig. 6D). This was missed in the structure of Choe et al. (1) because portions of the termini, especially the C termini, were not resolved in their structure. However, Bell et al. (2) also observed a slightly larger C-terminal portion of their structure. A third feature is that our structure appears to be thicker around the solenoid, perhaps because of the shape of the protein in solution or a higher amount of glycosylation. This claim is consistent with our observation that the *R. communis* lectin, which recognizes N-linked glycosylations, associates with a number of sites in the outer perimeter of hTLR3 ECD. Lastly, the most prominent difference is that we observed quite variable packing in two-dimensional crystals and a higher degree of flexibility in the molecule (Fig. 5). It is likely that hTLR3 ECD may be more flexible in solution in lipid surface two-dimensional crystals than in the three-dimensional crystals. The variable packing that we observed and the predominant monomeric structures of the hTLR3 ECD in single-particle analysis...
suggest that it is premature to assign a specific dimeric structure to the ectodomain of TLR3. We also noted that the ectodomains of TLR2 and TLR4 are expressed in monomeric forms in baculovirus vectors in Sf9 insect cells (19, 20). It remains possible that ligand binding or the presence of the intact molecule could impact TLR3 oligomerization.

Of the eleven human TLRs, TLR2 and TLR4 are the best characterized for the effects of glycosylation on cell surface expression. The ectodomains of TLR2 and TLR4 expressed in insect and human cells (19) were found to migrate at the positions of ~110-kDa bands in SDS-PAGE. This is comparable with the situation we observed with the hTLR3 ECD. Furthermore, N-linked glycosylation is the primary form of glycosylation in TLR2, 3, and 4. Also, whereas the ectodomains of TLR2 and TLR4 have four glycosylation sites that are all important for secretion of the proteins in transfected HEK cells, single amino acid changes in all of the potential glycosylation sites we tested in hTLR3 ECD, including the key ones at Asn-247 and Asn-413, retained some binding or the presence of the intact molecule could impact TLR3 vectors in Sf9 insect cells (19, 20). It remains possible that ligand binding or the presence of the intact molecule could impact TLR3 vectors in Sf9 insect cells (19, 20).

The wild-type TLR3, a potential explanation for reduced TLR3 function in vivo. In particular, we thank L. San Mateo and A. Bassiri for help in FACS analysis and V. Sarojini for mass spectroscopy analysis.

Acknowledgments—We thank A. Del Vecchio and members of the Kao and Mbow laboratories for constructive comments and suggestions throughout this work. In particular, we thank L. San Mateo and A. Bassiri for help in FACS analysis and V. Sarojini for mass spectroscopy analysis.

REFERENCES

1. Cho, J., Kelker, M. S., and Wilson, I. A. (2005) Science 309, 581–585
2. Bell, J. K., Botos, I., Hall, P. R., Askins, J., Shioloa J., Segal D. M., and Davies, D. R. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10976–10980
3. Lemaitre, B. (2004) Nat. Rev. Immunol. 4, 521–527
4. Zambon, R. A., Nandakumar, M., Vakharia, V. N., and Wu, L. P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 7257–7262
5. Bélhadjir, Y., Subramaniam, R., and Danl, J. L. (2004) Curr. Opin. Plant Biol. 7, 391–399
6. Rifkin, I. R., Leadbetter, E. A., Busconi, L., Vigiliani, G., and Marshak-Rothstein, A. (2005) ImmunoL. Rev. 204, 27–42
7. Takeda, K., and Akira, S. (2005) Int. Immunol. 17, 1–14
8. Underhill, D. M., and Gantner, B. (2004) Microbes Infect. 6, 1368–1373
9. Mbow, L. M., and Sarisky, R. T. (2005) Drug News Perspect. 18, 179–184
10. McGretrick, A. F., and O’Neill, L. A. (2004) Mol. Immunol. 41, 577–582
11. Schroder, M., and Bowie, A. G. (2005) Trends Immunol. 26, 462–468
12. Moynagh, P. N. (2005) Trends Immunol. 26, 469–476
13. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Nature 413, 752–758
14. Karioko, K., Bhuyan, P., Capodici, J., and Weissman, D. (2004) J. Immunol. 172, 6545–6549
15. Edelmann, K. H., Richardson-Burns, S., Alexopoulou, L., Tyler, K. L., Flavell, R. A., and Oldstone, M. B. (2004) Virology 322, 231–238
16. Karioko, K., Ni, H., Capodici, J., Lamphier, M., and Weissman, D. (2004) J. Biol. Chem. 279, 12542–12550
17. Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., and Seya, T. (2003) J. Immunol. 171, 3151–3162
18. Nishiya, T., and DeFranco, A. L. (2004) J. Biol. Chem. 279, 19008–19017
19. Weber, A. N., Morse, M. A., and Gay, N. J. (2004) J. Biol. Chem. 279, 34589–34594
20. da Silva Correia, J., and Ulevitch, R. J. (2002) J. Biol. Chem. 277, 1845–1854
21. de Bouteiller, O., Merck, E., Hasan, U. A., Hubac, S., Benguiu, B., Trinchieri, G., Bates, E. E., and Caux, C. (2005) J. Biol. Chem. 280, 165–175
22. Harris, J. R., and Scheffler, D. (2002) Micron 33, 461–480
23. Hoppert, M. (2003) in Microscopic Techniques in Plant Biotechnology, Wiley-VCH, Weinheim, Germany
24. Hovmöller, S. (1992) Ultrastructural Ecology 41, 476–501
25. Ludtke, S. J., Baldwin, P. R., and Chiu, W. (1999) J. Struct. Biol. 128, 82–97
26. Petersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) J. Comput. Chem. 25, 1605–1612
27. Elbein, A. D. (1984) CRC Crit. Rev. Biochem. 16, 21–49
28. Moremen, K. W. (2002) Biochim. Biophys. Acta 1573, 225–235
29. Guo, H.-B., Lee, I., Kammar, M., and Pierce, M. (2003) J. Biol. Chem. 278, 52412–52424
30. Patel, R. N., Attur, M. G., Dave, M. N., Patel, I. V., Stuchin, S. A., Abramson, S. B., and Amin, A. R. (1999) J. Immunol. 163, 3459–3467
31. Jans, D. A., Jans, P., Luius, H., and Fahrenholz, F. (1992) Arch. Biochem. Biophys. 294, 64–69
32. Karioko, K., Buckstein, M., Ni, H., and Weissman, D. (2005) Immunity 23, 165–175