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A Point Mutation in the Amino Terminus of TLR7 Abolishes Signaling without Affecting Ligand Binding

Carlo Iavarone,* Katrin Ramsauer,* Andriy V. Kubarenko,† Jason C. Debasitis,* Igor Leykin,* Alexander N. R. Weber,‡ Owen M. Siggs,‡ Bruce Beutler,‡ Pu Zhang,* Gillis Otten,* Ugo D’Oro,§ Nicholas M. Valiante,* M. Lamine Mbow,* and Alberto Visintin*

TLR7 is the mammalian receptor for ssRNA and some nucleotide-like small molecules. We have generated a mouse by N-nitrose-N’-ethyl urea mutagenesis in which threonine 68 of TLR7 was substituted with isoleucine. Cells bearing this mutant TLR7 lost the sensitivity to the small-molecule TLR7 agonist resiquimod, hence the name TLR7\textsuperscript{68I}. In this work, we report the characterization of this mutant protein. Similar to the wild-type counterpart, TLR7\textsuperscript{68I} localizes to the endoplasmic reticulum and is expressed at normal levels in both primary cells and reconstituted 293T cells. In addition to small-molecule TLR7 agonists, TLR7\textsuperscript{68I} fails to be activated by ssRNA. Whole-transcriptome analysis demonstrates that TLR7 is the exclusive and indispensable receptor for both classes of ligands, consistent with the fact that both ligands induce highly similar transcriptional signatures in TLR7\textsuperscript{wt/wt} splenocytes. Thus, TLR7\textsuperscript{68I} is a bona fide phenocopy of the TLR7 null mouse. Because TLR7\textsuperscript{68I} binds to ssRNA, our studies imply that the N-terminal portion of TLR7 triggers a yet to be identified event on TLR7. TLR7\textsuperscript{68I} mice might represent a valuable tool to help elucidate novel aspects of TLR7 biology. The Journal of Immunology, 2011, 186: 4213–4222.

Recognition of invading microorganisms at the cellular level relies on a number of germ line-encoded receptors that initiate the immune response, trigger the clearance of the invading noxious agent, and participate in tissue repair processes. In mammals, TLRs form one of the most prominent families of such receptors and drive early innate and later Ag-specific responses to pathogens (see Ref. 1 for an extensive review of the field). TLRs are type I receptors that evolved to recognize conserved features mainly found in the microbial biosphere. A subset of TLRs (TLR3, 7, 8, and 9) recognize nucleic acids and are endoplasmic reticulum (ER)-resident (2–4). Subcellular compartmentalization and cellular distribution of TLRs seem to be the main determinants for specificity of ligand recognition (exogenous versus endogenous) and quality of the cellular response (proinflammatory, phagocytic, or antiviral) (1, 5). The activity of TLRs is regulated by a variety of proteinaceous cofactors involved in improving ligand recognition [CD36 (6, 7), CD14 (8), MD-2 (9, 10)] or maintaining their correct subcellular localization [gp96 (11), PRATA/B (12, 13), UNC93B (14, 15)].

It is generally accepted that dimerization of the extracellular domain of most TLRs is the triggering mechanism. For example, TLR3 forms stable dimers upon interaction with dsRNA of ∼40 bp (16–18), thus bringing the two intracellular signaling domains in close proximity. In sharp contrast, TLR9 and TLR8 seem to exist as preformed signaling-incompetent dimers in ectopically reconstituted systems (19–21). The current model for TLR9 activation is that nucleic acids bind to two distinct binding sites (21, 22) and induce a conformational change in the ectodomains. In immune cells, this results in complex proteolytic and trafficking events that eventually propagate the triggering signal to its intracellular domain (15, 19, 23). The precise mechanism of action of TLR9 is unknown.

Unique among all TLRs, TLR7 and TLR8 can also be activated by synthetic low m.w. agonists alien to the microbial world. A prime example is imiquimod, an imidazoquinoline marketed as Aldara that is used in the treatment of certain skin diseases, including actinic keratosis, superficial basal cell carcinoma, and external genital warts caused by human papilloma virus (24). Imiquimod is a “pure” TLR7 agonist in humans, that is, it only activates TLR7, however it triggers both TLR7 and TLR8 in some ungulates (20, 25). Resiquimod (R848), a compound closely related to imiquimod, is a dual (TLR7/8) agonist in humans, but it is a pure TLR7 agonist in mice. When R848 is used in combination with poly dT17 (a short DNA homopolymer), it can activate mouse TLR8 (26). There have been reports that TLR8 can sense dsDNA in this species, but this is still controversial (4, 27). Taken together, these results suggest that low m.w. TLR agonists interact with different TLRs in a species-specific manner, and, under certain conditions, they might require nucleic acids to form functional mimics of the natural ligands. However, there is a major gap in our understanding of the exact mechanism of action of TLR7 ligands. This is mainly due to the scarcity of investigative...
tools and the difficult biochemistry around the study of endosomal TLRs.

By screening N-nitroso-N'‐ethyl urea (ENU)-induced mutations in mice, we identified a variety of genetic defects in the mammalian innate immune response, including genes involved in TLR function (28). One such gene is Unc93b1, which encodes the 12-spanning ER-resident protein UNC93B. Peritoneal macrophages from Unc93b1 mutant mice were unable to respond to synthetic DNA oligonucleotides, pC, and R848 ex vivo, that is, they had a triple defect in TLR signaling (14). The molecular mechanism for the loss of function was ascribed to a defect in the trafficking of these endosomal TLRs, which is governed by UNC93B (15). Because the analysis of ENU mutagenized mice had proved very successful to elucidate many other aspects of TLR signaling (28), we asked whether the ENU screen produced mice with impaired responses to TLR7 agonists. When the current project started in 2008, we found one mutant strain of C57BL/6 mice that lost the ability of responding to R848 (http://mutagenetics.scripps.edu/home.cfm). We called this mutant strain rsq1 and mapped the mutation to a C-to-T transition at position 391 of the TLR7 transcript, resulting in the conversion of threonine 68 to isoleucine (T68I) in the TLR7 protein. The TLR7<sup>rsq1</sup> mutation resides in the N-terminal portion of TLR7, in close proximity to the first two insertions thought to be relevant for ligand binding (29). Since then, we identified a second inactivating mutation that also affects the proximal ectodomain, rsq2. Rsq2 is an X-linked recessive inactivating mutation that produces the conversion of alanine 128 to a tyrosine in TLR7 protein. In this study, we used the TLR7<sup>rsq2</sup> mutant as a tool to gain insight into the molecular mechanism of TLR7 triggering. We first performed an extensive biochemical characterization of the TLR7<sup>rsq1</sup> and TLR7<sup>rsq2</sup> mutant proteins in transfected 293T cells and identified a crucial role for N66, a putative glycan acceptor site. Abrogation of the N-glycosylation consensus impaired the ability of TLR7 to signal in response to ssRNA and low m.w. TLR7 agonists but not its ability to bind to ssRNA. Whole-genome mRNA expression profiling of 293TTLR7<sup>rsq1</sup> and 293TTLR7<sup>rsq2</sup> cells and splenocytes from the TLR7<sup>rsq1</sup> mice revealed no off-target effects of the two benchmark TLR7 agonists R848 and ssRNA. Taken together, our results suggest that TLR7 is an exclusive receptor for both R848 and ssRNA in mice and that the N-terminal portion of TLR7 is necessary for function but not ligand binding.

**Materials and Methods**

**Reagents and Abs**

All common reagents were from Sigma, unless stated differently. The single-stranded oligoribonucleotide (ssRNA) R0006 (UUGUUUGGUUGU- GUUGGUUGU (30)) and its complementary strand 6000R (ACACAA- CAACACAACAACACA) were purchased from IDT (Coralville, IA) and were fully phosphorothioated. In some experiments, a 3'-biotinylated version of 293TTLR7<sup>rsq1</sup> cells and splenocytes from the TLR7<sup>rsq1</sup> mice retained their ssRNA and low m.w. TLR7 agonists but not its ability to bind to ssRNA. Whole-genome mRNA expression profiling of 293TTLR7<sup>rsq1</sup> cells and splenocytes from the TLR7<sup>rsq1</sup> mice revealed no off-target effects of the two benchmark TLR7 agonists R848 and ssRNA. Taken together, our results suggest that TLR7 is an exclusive receptor for both R848 and ssRNA in mice and that the N-terminal portion of TLR7 is necessary for function but not ligand binding.

**Animals, tissue extraction procedures, and cell differentiation**

Splenocytes were isolated from C57BL/6 mice or TLR7<sup>rsq1</sup> mutant mice (same genetic background) by mechanical dissociation (Miltienyi Biotech, Bergisch Gladbach, Germany). RBCs were lysed in 3 ml ACK lysis buffer (Sigma) for 3 min at room temperature. Cells were cultured in RPMI 1640, 2.5% FCS, and penicillin/streptomycin mix. Bone marrow-derived macrophages (BMDMs) were derived as previously described (31). Treatments were performed overnight in 96-well flat-bottom tissue culture plates at a cell density of 5 × 10<sup>3</sup> cells/well in 200 μl.

**Plasmids and cell lines**

The cDNA for RFP-tagged calreticulin was from Origene. The expression vectors pUNOTLR7, pUNOTLR7HA, and IRF5HA were purchased from Invivogen. IRF5HA was subcloned into the pCDNAs.1 hygro vector backbone (Invitrogen) and used to produce the stable cell line 293TLR7/IRF5HA. The TLR7<sup>rsq1</sup> (N66I) mutant was generated by site-directed mutagenesis using the QuickChange kit according to the manufacturer’s recommended primers and procedures (http://www.stratagene.com/sdmdesigner/default.aspx). pUNOTLR7<sup>rsq2</sup> was cloned by inserting the YFP expression cassette after a 9-aa spacer (AAAGAAAGG). The resulting protein was functional in 293T cells, although it was not as potent as the untagged version. All the constructs have been sequence verified, and additional information on the different plasmids will be provided upon request. Stable 293T cell lines were generated by transient transfection with the required cDNA and selection in blasticidin (5 μg/ml) for pUNO-TLR–based plasmids, hygromycin (250 μg/ml) for IRF5HA, and puromycin (5 μg/ml) for NF-κB–luciferase. All the cells were maintained in DMEM supplemented with 5% FBS (1 Scientific), 2 mM l-glutamine, and 100 μM penicillin-streptomycin (Sigma). Human PBMCs were purified by Ficol-Paque Plus separation (Amersham) from 50 ml of heparinized blood from healthy donors.

**Western blotting, RNA–TLR7 binding assays, coimmunoprecipitations, and deglycosylation assay**

Cells were lysed in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM PMSF, and freshly added protease inhibitors) for 10 min on ice. Lysates were cleared by centrifugation at 13,000 rpm for 5 min and postnuclear whole-cell lysates (WCLs) were used immediately or stored frozen. For the RNA capture experiments, biotinylated ssRNA (R0006; 10 μg/6 × 10<sup>6</sup> cells) was mixed with WCLs containing TLR7HA or TLR7<sup>rsq1</sup> HA and 20 μl of packed avidin–agarose beads/sample (Pierce). Reactions were incubated in rotation at 4˚C for 1 h, and RNA–protein complexes were collected by centrifugation, washed three times in lysis buffer, resuspended in 30 μl SDS-sample buffer, boiled, and subjected to SDS-PAGE. Presence of TLR7 in the precipitates was assessed by anti-HA Western blotting. Binding to TLR7HA and TLR7<sup>rsq1</sup> HA was also determined using an ELISA-like assay in which plastic-adsorbed RNA was used to capture TLR7 from WCLs containing the HA-tagged protein and 1 μg/ml of an HRP-conjugated rat mAb anti-HA Ab (Roche). Biotinylated RNA was plated in titrated amounts on avidin-coated and preblocked white plates for 10 min (Thermo Scientific). After washing of the excess unbound oligos in PBS-0.05% Tween 20, WCLs from TLR7<sup>rsq1</sup> cells (diluted 1:3 in citrate buffer pH 7; Sigma) were applied to the streptavidin-captured oligos for 60 min. After three additional washes, a luminogenic HRP substrate (Pierce) was added to the wells and the light emitted measured using standard luminometry. Then, 100 μl of RNase I (NEB) was added to the lysates to eliminate endogenous cellular RNA, which can bind to TLR7, confounding lysis. Endo glycosidase H (Endo-H) resistance/sensitivity was used to determine whether proteins underwent trans-Golgi maturation as described in Ref. 32. Briefly, 20 μl WCLs from the indicated cell lines were boiled in 5 mM DTT and treated with 1 μl of either Endo-H or Endo-F for 1 h at 37˚C (NEB). Reactions were stopped by adding SDS-sample buffer, and the shift in mobility was assessed by Western blotting as described above.

**NF-κB–luciferase reporter gene assay**

Adherent 293T cells stably expressing the indicated TLR and the NF-κB–luciferase reporter cassette were induced in 96-well format (50,000 cells/well) with the indicated amount of stimulus. After 16 h, spent medium was removed by aspiration, and the cells were lysed in 50 μl of a 1:1 dilution of Cell-Glo (Promega) in water. Emitted light was quantitated using a luminometer and the values plotted as the average of duplicates ± SD (luciferase units). In some experiments, the readings were divided by prestimulated control and plotted as fold induction (relative luciferase units).

**RNA isolation, labeling, and microarray analysis**

Total RNA was extracted using QIAShredder/ RNeasy columns according to the manufacturer's instructions (Qiagen). RNA labeling, hybridization, and scanning were performed using methods, reagents, software, and hardware
purchased from Agilent Technologies. Briefly, 400 ng RNA was retrotranscribed and labeled using Cy3 (for the samples treated with DOTAP, DMSO, PBS, or left untreated) or Cy5 (LPS, R848, R0006) and column purified (Qiagen). The efficiency of Cy5 and Cy3 dyes incorporation was assessed by flurometry. Reactions with yields >0.835 μg RNA and yields >8 pmol dye/μg cRNA were used for hybridization. Equal amounts of labeled Cy5 and Cy3 cRNAs were hybridized onto 4 × 44 Whole Human or Whole Mouse Genome Microarray. After scanning, images were analyzed using the Feature Extraction 10.5.1.1 software. Data were transferred to the Base 2.1.4.1 software for microarray data management and analysis (33). For each spot, local background was subtracted, and spot intensities were normalized by the mean fluorescence intensity for each channel. The average intensity ratio of each spot from experimental replicates was estimated by geometric mean, and the accuracy and statistical significance of the observed ratios were determined by using Student’s t test. Genes with a p value <0.05 and average intensity ratios ≥2 (log, ratio ≥1) were considered differentially expressed. Hierarchical clustering was performed with the Tigr MultiExperiment Viewer (TMEV) 4.4.1 software (34) on the transformed log2 ratio. The complete set of microarray data was submitted to the Array Express database EMBL-EBI (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-3051.

Subcellular fractionation

Subcellular fractionation was carried out using reagents and protocols provided in an ER isolation kit (Sigma). Briefly, 293T cells stably expressing TLR7 or TLR7\(^{-1}\) were plated on three 150-mm tissue culture dishes at a density of 9 × 10\(^6\) cells/dish. After 16 h, the cells were harvested, resuspended in hypotonic buffer at a volume three times that of the packed pellet, and mechanically disrupted using a Wheaton Dounce tissue grinder with a type A pestle. Post-nuclear supernatants were diluted in an equal volume of isotonic isolation buffer. Cell extracts were then fractionated on a discontinuous iodixanol gradient by centrifugation at 100,000 × g for 16 h. Fractions were collected, solubilized in Triton X-100, and subcellular fractionation was not due to the lack of TLR7 expression, and both pattern and level of expression of TLR7\(^{-1}\) and TLR7 were comparable by confocal analysis (Fig. 1C). As a consequence of the lack of intracellular signaling, TLR7\(^{-1}\) triggering by both R848 and ssRNA (another TLR7-dependent stimulus)

Results

TLR7\(^{-1}\) is not functional

TLR7\(^{-1}\) mice were originally identified because of the failure of their peritoneal macrophages to respond to R848. Analogously, BMDMs from TLR7\(^{-1}\) mice failed to trigger IκB, p38, and JNK in response to R848, confirming that signaling downstream of TLR7 is impaired (Fig. 1A). The signaling defect of TLR7\(^{-1}\) appeared to precede IRAK1 activation, as R848 treatment of BMDMs derived from the TLR7\(^{-1}\) mouse failed to induce the degradative pathway that is triggered by IRAK1 auto/antiphosphorylative events (Fig. 1B) (44). The defect in intracellular TLR7 signaling was not due to the lack of TLR7 expression.
did not induce cytokines in BMDMs (Fig. 2A), splenocytes, and DCs (data not shown). Moreover, splenic B cells from the TLR7 rsq1/animals failed to upregulate costimulatory and activation markers (CD86 and CD69) in response to TLR7 stimuli (Fig. 2B). Conversely, TLR7 rsq1/ cells always responded normally when treated with other TLR ligands, such as LPS or ssDNA. TLR7 wild-type (solid bars) or TLR7 rsq1/ mice are born in a normal Mendelian ratio and appear normal and healthy. Immunologically, they do not show differences in the frequencies of T cells (CD3+ and either CD4+ or CD8+), B cells (CD122+), monocytes (CD14+), and DCs (CD11c+) in either the bone marrow or the spleen (Table I).

To elucidate the molecular defect of the TLR7(rsq1) mutation and to gain insight into the mechanism of action of TLR7, we constructed the human version of mTLR7 rsq1/ to gain insight into the mechanism of action of TLR7, we constructed the human version of mTLR7 rsq1/ and asked whether the human ortholog of TLR7 rsq1/ is functional in 293T cells. Human 293TLR7(rsq1) cells did not induce the NF-κB–luciferase reporter gene in response to both R848 (45) and ssRNA (3, 30) (Fig. 3A).

Next, we tested whether other signaling pathways were impaired. As shown in Fig. 3B, 293TLR7 cells induced p38 and JNK in response to R848, whereas 293TLR7(rsq1) cells did not, which cor-

![FIGURE 2. Lack of cytokine secretion and cellular activation in BMDMs and splenic B cells of TLR7 rsq1/ mice. A, BMDMs from TLR7 rsq1/ (solid line) and TLR7 wt (dashed lines) were stimulated with the indicated concentrations of LPS, ssRNA, or R848 for 16 h. Supernatants were harvested and analyzed by supernatant cytokine ELISA. B, Splenocytes from TLR7 wild-type (solid bars) or TLR7 rsq1/ (open bars) were stimulated with R848 (10 μM), R0006 (5 μg/ml), the immunostimulatory CpG oligonucleotide ODN1826 (1 μM), and carriers alone (DMSO and DOTAP) for 22 h. Cells were then harvested and labeled with lineage-specific markers and analyzed by flow cytometry. Plotted are the mean CD86 values (left panel) and percentage CD69 positivity (right panel) of the CD3+, CD4+, CD8+, CD11c+, CD19+, MHC+ population. Results represent the average of two mice plus range.

FIGURE 3. Lack of signaling in 293T cells reconstituted with human TLR7 rsq1/ A, 293T stably expressing wild-type human TLR7 (diamonds) or human TLR7(rsq1) (squares) were stimulated overnight with R848 at the indicated concentrations (left panel) or ssRNA (right panel) mixed 1:1 (w/w) in DOTAP. The activity of the NF-κB–luciferase reporter gene was recorded by luminometry. Results are representative of three independent experiments, and readings are the average ± SD of triplicate readings. B, 293T cells stably expressing TLR7(rsq1) or TLR7 were left untreated or were treated with R848 (10 μM) or TNF-α (10 ng/ml). After 30 or 60 min, cells were lysed, and phospho-IκB, IkB, phospho-p38, phospho-JNK, and tubulin were determined by Western blot on whole-cell extracts. Results are representative of two similar gels, and all the panels derive from the same input sample, run on different gels or day.

TABLE I. Comparison of cell populations in the spleens and bone marrow of TLR7 wt and TLR7 rsq1/ mice

<table>
<thead>
<tr>
<th>Cell Population and Location</th>
<th>% TLR7 wt (SD)</th>
<th>% TLR7 rsq1/ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>54.5 (0.70)</td>
<td>53.03 (1.22)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>40.03 (0.71)</td>
<td>41.83 (1.62)</td>
</tr>
<tr>
<td>B cell</td>
<td>50.43 (3.79)</td>
<td>46.46 (4.88)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>2.23 (0.31)</td>
<td>2.55 (0.06)</td>
</tr>
<tr>
<td>NK cells</td>
<td>8.26 (1.84)</td>
<td>6.39 (1.67)</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>32 (3.62)</td>
<td>33.4 (5.44)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>49.5 (4.16)</td>
<td>51.23 (6.30)</td>
</tr>
<tr>
<td>B cell</td>
<td>52.27 (1.86)</td>
<td>43.5 (3.68)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>3.96 (0.16)</td>
<td>3.62 (0.73)</td>
</tr>
<tr>
<td>NK cells</td>
<td>3.43 (0.10)</td>
<td>3.49 (1.05)</td>
</tr>
</tbody>
</table>

T cells were gated based on their scatter characteristics and CD3+ expression. Shown is the percentage of CD4+ and CD8+ cells in the total CD3+ cells. B cells were gated by CD19 and B220 expression. Monocytes were gated by CD14 expression, and NK cells were gated by CD49b expression. Data in the table represent the average of percentage of cells harvested from spleens and bone marrow for three individual mice.
roborated the observations from primary cells. A general signaling defect in 293TLR7<sup>rsq1</sup> cells could be excluded because they responded to TNF-α.

An intact N-glycosylation target sequence in position 66–68 is required for proper TLR7 function

We noted that T68 belongs to an N-glycosylation consensus site [NXT/S (46, 47)], which is predicted to be disrupted in TLR7<sup>rsq1</sup>. A second N-glycosylation is predicted to involve N69. To gauge the potential importance of glycosylation at N66, we analyzed the sequence alignments of all currently available TLR7 sequences. As shown in Fig. 4A, the region harboring the two predicted glycosylation sites (N66 and N69) in LRR1 is extremely well conserved from mammals to fish. Only <i>Takifugu rubripes</i> (puffer fish) is missing the asparagines at position 66. Based on the three-dimensional model of TLR7 (21), T68 is found at the end of the TLR7 ectodomain in the first LRR β-strand, whereas N66 is at the end of the turn leading into it (48). We sought to assess if glycosylation at N66 or N69 was possible in the stereochemical context of the asparagines’ side-chain conformations found in the three-dimensional model by using GlyProt (see Materials and Methods for details). The modeling reveals that glycans could be added to both N66 and N69 in human and murine TLR7, respectively (Fig. 4). We then asked whether mutating the acceptor asparagine 66 would lead to the same effect as T68I. The resulting mutant cannot be glycosylated at position 66. As shown in Fig. 5A, TLR7<sup>N66A</sup>, like TLR7<sup>rsq1</sup>, was incapable of activating NF-κB in 293T transfectants, supporting our model that an N66-linked glycan could be the reason for the loss of function phenotype observed in these mutants. The inability of TLR7<sup>rsq1</sup> to signal was not specific to R848 or ssRNA, and other adenine-based low m.w. TLR7 agonists failed to activate TLR7 as well (data not shown). Taken together, the in silico analysis and the mutagenesis experiments suggest that the defect in TLR7<sup>rsq1</sup> might be due to the suppression of the carbohydrate-substituted N-glycosylation site in position 66.

**TLR7<sup>rsq1</sup> localizes to the ER like its wild-type counterpart**

Next we asked whether the wild-type or the mutated TLR7 differed in their subcellular localization in 293T cells. To visualize the subcellular localization of TLR7 and TLR7<sup>rsq1</sup> in live cells, we generated 293T cell lines stably expressing YFP-tagged versions of TLR7 and TLR7<sup>rsq1</sup> (see Materials and Methods for details). The modeling reveals that glycans could be added to both N66 and N69 in human and murine TLR7, respectively (Fig. 4). We then asked whether mutating the acceptor asparagine 66 would lead to the same effect as T68I. The resulting mutant cannot be glycosylated at position 66. As shown in Fig. 5A, TLR7<sup>N66A</sup>, like TLR7<sup>rsq1</sup>, was incapable of activating NF-κB in 293T transfectants, supporting our model that an N66-linked glycan could be the reason for the loss of function phenotype observed in these mutants. The inability of TLR7<sup>rsq1</sup> to signal was not specific to R848 or ssRNA, and other adenine-based low m.w. TLR7 agonists failed to activate TLR7 as well (data not shown). Taken together, the in silico analysis and the mutagenesis experiments suggest that the defect in TLR7<sup>rsq1</sup> might be due to the suppression of the carbohydrate-substituted N-glycosylation site in position 66.

**FIGURE 4.** T68 of TLR7 in the content of primary and tertiary structure of TLR7. A, Alignment of TLR7 LRRNT and LRR1 region sequences from all available species (list of species is given in Materials and Methods). T68 is 100% conserved. B, Mapping of conservation of surface residues on the model of human TLR7 ectodomain. Green regions correspond with highly conserved residues and red regions with nonconserved. C and D, Models of the extracellular domains of human and mouse TLR7 (in surface representation) with all possible glycans (orange sticks and semitransparent surfaces representation). E and F, Close-ups of the LRR1 of human and mouse TLR7, respectively. T68 is highlighted by red sticks, whereas N66 and N69 with linked glycans are in orange.
FIGURE 5. TLR7 requires an intact N-glycosylation consensus sequence at positions 66–68. A, 293T transiently expressing the indicated TLR7 mutant (absissa) and the NF-κB–luciferase reporter gene were left untreated (nil) or were stimulated with R848 (5 μM). Four hours later, the activity of the NF-κB–luciferase reporter gene was recorded by luminometry. Results are representative of three independent experiments, and readings are the average ± SD of triplicate readings. RLU, relative luciferase units. B, The amounts of the HA-tagged TLR7 mutants used in the experiment in A were assessed by anti-HA Western blotting.

of human TLR7 and TLR7rsq1. Both wild-type and mutant TLR7 colocalized with a transiently transfected ER marker (Fig. 6A, calreticulin–RFP merged panels), showing that both receptors are similarly localized to the ER (Fig. 6A). To characterize further the subcellular localization of the two receptors, we assessed whether a fraction of the receptors underwent Golgi maturation and escaped the typical ER-restricted compartmentalization of TLR7, TLR8, and TLR9. Golgi-matured proteins acquire Endo-H resistance and therefore do not display a mobility shift when treated with Endo-H (32). As shown in Fig. 6B, both TLR7 and TLR7rsq1 were Endo-H sensitive, suggesting they did not go through the secretory pathway. To map in more detail the subcellular localization of TLR7 and TLR7rsq1, we fractionated the cellular lysates in discontinuous iodixanol gradients. Both TLR7 and TLR7rsq1 partitioned with the ER-resident protein calnexin (Fig. 6C). Taken together, these results show that the loss of function of human TLR7rsq1 is not due to its lack of expression or gross changes in its subcellular localization. Moreover, they confirm the concept that despite being type I glycoproteins with a leader signal sequence, both TLR7 and TLR7rsq1 are ER-resident proteins that are not translocated to the cell surface.

**TLR7rsq1 is dominant negative**

Data in the literature support the notion that nucleic acid-recognizing TLRs exist as preformed dimers and that the triggering of the downstream signaling cascade follows the rearrangement of the extracellular domains in the dimer (19–21). We therefore asked whether TLR7rsq1 acts as dominant negative by pairing with the wild-type molecule. 293T cells stably expressing untagged TLR7 and an NF-κB–luciferase reporter gene were transiently transfected with an empty vector or with HA-tagged versions of TLR7 or TLR7rsq1. The ectopic expression of TLR7rsq1 reduced TLR7 response to R848, whereas wild-type TLR7 enhanced it (Fig. 7, left panel). The transiently expressed proteins were expressed at similar levels (data not shown). To determine whether the impairment affected signaling cascades other than NF-κB, we tested whether TLR7rsq1 interfered with type I IFN production. When 293T cells are reconstituted with TLR7 and IRF5, they secrete IFN-β in response to TLR7 agonists (49). Similar to that observed for NF-κB, coexpression of TLR7rsq1 and TLR7 decreased IFN-β secretion (Fig. 7, right panel), whereas coexpression of TLR3 did not interfere with TLR7 signaling (data not shown). Our working model is that the inactive TLR7rsq1 associates with wild-type TLR7 in situ, thereby preventing the formation of signaling-competent dimers.

**TLR7rsq1 and TLR7 bind similarly to ssRNA**

Structural evidence demonstrates that the N-terminal portion of TLR3 interacts with RNA (50), and TLR9 seems to use its N-terminal region to sense DNA (21). Because the rsq1 mutation is located in the N terminus of TLR7, we surmised that the rsq1 defect might be related to impaired ligand binding. However, the results in Fig. 8A show that TLR7 and TLR7rsq1, but not TLR3

FIGURE 6. TLR7 and TLR7rsq1 are localized to the ER. A, 293 cells stably expressing TLR7YFP (upper panels) or TLR7rsq1YFP (bottom panels) were transiently transfected with the RFP-tagged ER marker calreticulin (CALR–RFP). Cells were then imaged by confocal microscopy. Shown are the signals from the green (left panels), red (middle panels), and merged YFP and RFP (right panels) fluorescence (original magnification ×40). B, Whole-cell extracts from 293 cells stably expressing TLR7HA and TLR7rsq1HA were treated with Endo-H or Endo-F and subjected to Western blotting for the HA epitope. Shown is the mobility shift due to the complete removal of N-linked glycans in both proteins. C, 293TLR7 (left panels) or 293TLR7rsq1 (right panels) were lysed and subjected to iodixanol discontinuous gradient fractionation. Fractions were then probed by Western blotting for the indicated subcellular localization markers: tubulin (cytoplasm), LAMP1 (lysosomes), and calnexin (ER).

FIGURE 7. TLR7rsq1 is dominant negative. 293T cells stably expressing the NF-κB–luciferase reporter and IRF5 were transiently transfected with the indicated plasmids: empty vector (dashed line), human TLR7 (squares), and human TLR7rsq1 (triangles). The cells were stimulated with titrated amounts of R848. After an overnight incubation, the NF-κB reporter activation was determined by luminometry (left panel). Results are plotted as in Fig. 3A. IFN-β in the supernatants was determined by MesoScale Discovery analysis (right panel). These results were reproduced in three separate experiments.
nor TLR4 [data not shown], were equally efficient in binding an activating biotinylated ssRNA as revealed by avidin pulldown experiments from lysates containing the HA-tagged receptors (51). Under identical conditions, TLR7 and TLR7rsq1 bound weakly to the nonstimulatory ssRNA sequence 6000R, confirming specificity of the assay (Fig. 8B). To determine potential differences in affinity, we tested the binding of TLR7 to ssRNA over a wide range of concentrations in a quantitative assay. WCLs from the 293T cells stably expressing HA-tagged TLR7 and TLR7rsq1 were used to probe titrated amounts of immobilized biotinylated ssRNA. Wild-type and mutant TLR7 bound similarly to RNA (Fig. 8B) suggesting that the T68I mutation does not involve a ligand binding site. A detailed characterization of the TLR7 binding thermodynamic and specificity will be presented elsewhere. Because TLR7rsq1 colocalized with TLR7, was able to interfere with its signaling, and displayed similar ligand-binding characteristics, we conclude that TLR7rsq1 is missing a feature that is critically linked to signal transduction but not to its ability to bind to itself or its ligand(s).

TLR7rsq1 cells do not modulate gene expression in response to TLR7 ligands

To exclude formally that TLR7rsq1 had any residual activity and that both ssRNA and small molecules have no TLR7-independent effects, we performed whole-genome microarray analysis to compare TLR7rsq1 with TLR7wt induced transcription profiles in

**FIGURE 8.** TLR7rsq1 binds to ssRNA. A, Protein extracts from 293T cells or 293T cells stably expressing human TLR3HA, TLR7rsq1HA, or TLR7HA were incubated with biotinylated ssRNA (bio-R0006), and the complexes were collected using streptavidin–Sepharose beads. The presence of HA-tagged TLRs in the pellets was assessed by Western blotting. The shown experiment is representative of four similar independent experiments. B, WCLs from 293T cells stably expressing human TLR7rsq1 or TLR7HA were left untreated (lane 1) or incubated with bio-R0006 or its complementary (nonstimulatory) bio-6000R. Samples were then processed as in A. C, WCLs from 293T, 293TLR7HA, and 293TLR7rsq1HA cells were used to probe RNA plated on avidin-coated plates in ELISA-like experiments. An HRP-conjugated anti-HA Ab was mixed with the lysate before the incubation and the signal from the captured TLR7HA–anti-HA complex measured by luminometry using luminogenic HRP substrates. Plotted is the average of the light units from duplicate points. Biotinylated ssRNA (R0006) was plated in 2-fold dilution (x-axis, maximum concentration 1.4 μM). This experiment is representative of three experiments.

**FIGURE 9.** Whole-genome microarray analysis of TLR7rsq1 mutant. 293TLR7 and 293TLR7rsq1 cells were treated with 10 mM R848 or 5 mg/ml R0006 for 4 h. Cy5- and Cy3-labeled cranes were hybridized onto 4 × 44 Whole Human Genome Microarray. The differentially expressed genes (log2 |1|, p value ≤0.05) are shown in the heat map. Hierarchical clustering of the genes was performed using TMEV. For fold inductions, refer to Table I. A, Cluster analysis of genes upregulated by R848 (left panel) and ssRNA (right panel). B, Cluster analysis of genes upregulated in 293TLR7 wild-type cells by R848, ssRNA, or both.
293T transfectants and primary cells from mutant and wild-type mice. In the first set of experiments, 106 differentially regulated genes with a threshold of $\log_2 -1$ and with a $p$ value $\leq 0.05$ in at least one condition were identified. All of the genes modulated by R0006 and R848 treatment were TLR7 dependent (Fig. 9A). No genes were significantly upregulated or downregulated in either 293TLR7<sup>rsq1</sup> (Fig. 9A) or in 293T cells (data not shown). Analysis of the gene clusters modulated in 293TLR7 cells showed that the majority of significant Gene Ontology (GO) terms are related to immune responses (e.g., IL-1β, IL-8, TNF-α). A group of cytokines and chemokines like CXCL members, secreted effector molecules like pentraxin3 (Ptx3), as well as CD83 and CD44 were upregulated in response to both R848 and ssRNA. Fig. 9B reveals that there are genes that seemed to be regulated by only one compound or the other. Given the small number of genes modulated and the different nature of the two stimuli, small drug-like molecule versus immunostimulatory synthetic ssRNA complexed with a cationic lipid, these differences do not seem to be significant and possibly related to different kinetics and potency (see later). Hence, the transcriptome analysis of ectopically reconstituted 293T cells indicates that TLR7<sup>rsq1</sup> does not produce any significant signaling event. In a second set of experiments, a whole-genome transcriptional analysis was performed on treated splenocytes from the TLR7<sup>rsq1</sup> and the C57BL/6 mice: 1562 genes were differentially regulated with a $p$ value $\leq 0.05$ and a fold change $\geq 4$-fold in splenocytes treated with R848, ssRNA, and LPS (as control). No significant difference between TLR7<sup>rsq1</sup> mice and their wild-type counterparts was observed in the genes induced by treatment with the TLR4 agonist LPS. GO analysis showed that the majority of genes regulated by TLR7 ligands were related to immunological functions. As shown in Fig. 10A (immune genes), Fig. 10B (chemokines), and Fig. 10C (cytokines), there was a striking lack of genes modulated by R848 or ssRNA in the TLR7<sup>rsq1</sup> splenocytes, suggesting that both the small molecule and ssRNA are not sensed by mechanisms other than TLR7. Because of the overall similar transcriptional signature, these seemingly completely unrelated classes of ligands seem to share the same mechanism of action.

**Discussion**

The T68I conversion in the first LRR of the extracellular domain of TLR7 has been identified because cells from mice bearing this mutation are unable to respond to R848 (52). In addition to being insensitive to low m.w. TLR7 agonists (three distinct chemotypes, data not shown), we found that TLR7<sup>rsq1</sup>-bearing cells fail to signal upon treatment with short synthetic ssRNA oligonucleotides as well. Despite extensive investigation, we were unable to identify the exact molecular defect caused by the T68I mutation. However, our studies reveal several novel aspects of TLR7 biology. Because the TLR7<sup>rsq1</sup> mutation does not affect binding of TLR7 to RNA (Fig. 8A), it is unlikely that the region encompassing T68 participates directly in the binding to R848 or RNA. Accordingly, the modeling of TLR7 reveals that this mutation resides in the glycan-rich face of TLR7, which, based on available
TLR-liganded structures, should not contain the ligand binding site. It is interesting to note that residues in the N terminus of TLR9 have been identified that impair CpG DNA signaling but not binding (21). Hence, ligand binding and signal transduction seem to be separate events for both TLR9 and TLR7, which is in contrast to other TLRs for which dimerization induced by their respective ligands is the activating event. For example, the propagation of an out-in activating signal by the intracellular domain of TLR4 can be achieved by Ab cross-linking (10, 53), by inducing its aggregation via the extracellular domain from different TLRs (5), or by using extracellular moieties known to produce dimeric structures (54). Given the ability of TLR7\(^{\text{mut}}\) to bind to RNA (Fig. 8), its ability to interfere with TLR7 signaling (Fig. 7), its ability to colocalize (Figs. 2, 6) and bind to wild-type TLR7 data (not shown), we infer that the loss of a putative N-linked polysaccharide does not result in a gross misfolding of the entire receptor. Rather, because glycosylation has been shown to affect the induction of compact \(\beta\)-turns (55) and N66 is directly located in the turn leading up to the LRR \(\beta\)-strand (Fig. 4), the lack of the sugar moiety could result in the structural perturbation of a defined region that is critical to TLR7 function. Mutagenesis studies in murine TLR7 and TLR9 illustrated the sensitivity of these receptors to subtle changes (21). Although not formally demonstrated for TLR7, the structural perturbation induced by the lack of the N-glycosylation in position 66 might affect a conformational change required for triggering the dimeric receptor complex, as reported for TLR9 (19), or impact the ability of TLR7 to pair with a luminal signaling partner, such as a protease (23) or a chaperone protein (56).

Whatever the nature of this event is, TLR7 produces remarkably similar responses to seemingly unrelated classes of TLR7 ligands: purine-like low m.w. TLR7 agonists and ssRNA. The few differences found in the transcriptional signature of ssRNA and R848 in 293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid-293TLR7 cells might be ascribed to the different kinetics of activation of TLR7 by a cell-permeable small molecule versus a lipid.

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References


