Human TLR10 Is a Functional Receptor, Expressed by B Cells and Plasmacytoid Dendritic Cells, Which Activates Gene Transcription through MyD88[1]

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Human TLR10 is an orphan member of the TLR family. Genomic studies indicate that *TLR10* is in a locus that also contains *TLR1* and *TLR6*, two receptors known to function as coreceptors for TLR2. We have shown that TLR10 was not only able to homodimerize but also heterodimerized with TLRs 1 and 2. In addition, unlike TLR1 and TLR6, TLR10 was expressed in a highly restricted fashion as a highly *N*-glycosylated protein, which we detected in B cell lines, B cells from peripheral blood, and plasmacytoid dendritic cells from tonsil. We were also able to detect TLR10 in a CD1a$^+$ DC subset derived from CD34$^+$ progenitor cells which resemble Langerhans cells in the epidermis. Although we were unable to identify a specific ligand for TLR10, by using a recombinant CD4TLR10 molecule we also demonstrated that TLR10 directly associates with MyD88, the common Toll IL-1 receptor domain adapter. Additionally, we have characterized regions in the Toll IL-1 receptor domain of TLR10 that are essential in the activation of promoters from certain inflammatory cytokines. Even though TLR10 expression has not been detected in mice, we have identified a partial genomic sequence of the *TLR10* gene that was present but nonfunctional and disrupted by a retroviral insertion in all mouse strains tested. However, a complete *TLR10* sequence could be detected in the rat genome, indicating that a functional copy may be preserved in this species. ***The Journal of Immunology,*** **2005, 174: 2942–2950.**

The Toll-like receptors are germline-encoded receptors that play an essential role in initiating the immune response against pathogens. The 13 mammalian TLR paralogues, which have now been identified (10 in human and 12 in mice), recognize a wide variety of pathogen-associated molecular patterns (PAMPs)[4] from bacteria, viruses, and fungi, as well as certain host-derived molecules (1). TLRs are type I transmembrane glycoproteins with an extracellular domain composed of numerous leucine-rich repeats and an intracellular region containing a Toll IL-1 receptor (TIR) homology domain (2). The extracellular domain features a remarkable plasticity in terms of ligand recognition, since TLRs can recognize structurally diverse ligands from unrelated sources (3). Ligand specificity has been elucidated for most TLRs (4), thus TLR2 and TLR4 recognize Gram-positive and Gram-negative bacterial cell wall products, respectively; TLR5 recognizes a structural epitope of bacterial flagellin; TLR3, TLR7, TLR8, and TLR9 have been demonstrated to recognize different forms of microbial-derived nucleic acid (1). The TIR domains interact with several TIR domain-containing adaptor molecules (MyD88, TIR domain-containg adaptor protein (TIRAP), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) which activate a cascade of events resulting in transcription factor induction (5). TLR10 remains the only orphan member among the human TLRs. One major hindrance to TLR10 studies is that it lacks a rodent homologue. This has hampered the assignation of a natural or synthetic ligand to TLR10 because the majority of TLR ligands have been defined using mutant or genetically deficient mouse models. As in the case of *TLR7* and *TLR8*, which have a common locus on chromosome X (6), *TLR1*, *TLR6*, and *TLR10* also share a common locus on chromosome 4p14 and are structurally similar to one another (human genome resources; National Center for Biotechnology Information, ncbi.nlm.nih.gov). The ectodomains of TLR1/2 and TLR2/6 form functional pairs to recognize a variety of microbial residues (7) and it has been speculated that TLR10 could potentially act as a TLR2 coreceptor. Similarly to TLR7 and TLR9, the expression profile of TLR10 has been reported to be restricted to B cells and plasmacytoid dendritic cells (PDCs) (4). In addition, Bourke et al. (8) showed that resting B cells stimulated with anti-μ and anti-CD40 Abs or with *Staphylococcus aureus* Cowan I bacteria had increased mRNA expression of TLR9 and TLR10. In this study we further characterized the TLR10 expression pattern on immune cells as well as identifying its ability to form homo- or heterodimers with other TLRs. In addition, we have attempted to question the functionality and signaling ability of TLR10 using a constitutively active version of the molecule. Furthermore, we describe the identification of a rat *TLR10* sequence. The functional

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[4] Abbreviations used in this paper: PAMP, pathogen-associated molecule pattern; TIR, Toll IL-1 receptor; TIRAP, TIR domain-containing adaptor protein; TRIF, TIR domain-containing adaptor-inducing IFN-β; TRAM, TRIF-related adaptor molecule; DC, dendritic cell; PDC, plasmacytoid DC; HA, hemagglutinin; h, human.

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significance of this is unknown, but may open the possibility to study this gene in an immunological model.

Materials and Methods

Cell culture

HEK293, 293T and T98G cell lines (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen Life Technologies). B cell lines were obtained from American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% FBS and 0.5% 2-ME (Sigma-Aldrich), except for B lineage acute lymphoblastic leukemia cell lines pre-ALP and Dunatis which were produced and cultured in our laboratory as described previously (9–11). All cells were cultured at 37°C with 5% CO_2.

Cell preparations

Umbilical cord blood samples, peripheral blood samples, and whole tonsils were obtained according to institutional guidelines. PMBCs were purified from human peripheral blood by Ficoll-Hypaque centrifugation. Monocytes were purified from PBMC by centrifugation over a 50% Percoll gradient followed by immunomagnetic depletion of T, B, and NK cells (12). $CD34^+$ hemopoietic cells and monocytes were isolated from umbilical cord; both populations of cells were used to generate dendritic cells (DCs) and activated as previously described (12–14). Activation of DCs was performed by culturing overirradiated CD40L-expressing L cells in the presence or absence of IL-3 (15). For Western blot analysis, peripheral blood B cells were enriched from whole blood using CD19-coupled magnetic beads (Miltenyi Biotec). PDCs were purified from tonsils as previously described (16) and activated overnight in the presence of IL-3 with or without CD40L-expressing L cells (15).

RT-PCR of human (h) TLR10

Total cellular RNA was isolated using the GdSCN/CsCl gradient procedure (12). Cells were lysed in 4 M guanidine thiocyanate solution and the total RNA was isolated by centrifugation through a 5.7 M CsCl gradient. RNA was treated with DNase I before mRNA purification using the Oligotex-dT kit (Qiagen). Poly(A)$^+$ RNA (2 μg) was used to make first-strand cDNA (Superscript kit; Invitrogen Life Technologies). First-strand cDNAs were prepared after DNase I treatment of 5 μg of total RNA using oligo(dT) primers (Pharmacia) using the Superscript kit. Synthesis of cDNAs was controlled by performing RT-PCR using β-actin primers. RT-PCR was performed using the following primers specific for a 670-bp fragment of the TLR10 cDNA: 5′-GATGGTTGGATGGTCAGATTC (forward primer) and 5′-AAGCCCA CATTTACGCCTATC (reverse primer).

Template DNA was added at 1 ng/μl and amplification was performed using the AmpliTaq enzyme and buffer (PerkinElmer), dNTPs at 0.8 mM, and DMSO at a 5% final concentration. Cycle conditions were 94°C for 1 min 60°C for 2 min, and 72°C for 3 min for 35 cycles.

Northern blot analyses

Human adult and fetal commercial tissue blots were used (MTN blots I–IV; Clontech Laboratories). Hybridization of Northern blots was performed with the 670-bp DNA fragment generated by RT-PCR labeled with [^{32}P]dCTP (Amersham) using the High Prime kit (Boehringer Mannheim). Hybridization was performed overnight in rapid hybridization buffer at 60°C. High stringency washes were at 0.2× SSC and 0.2% SDS for two times for 30 min. Revelation of bands was performed after 15 days exposition on Biomax MR film (Kodak).

Identification of TLR10 rodent homologues

Potential rodent homologues of *TLR10* were identified by the bioinformatics search of the partial rat and mouse genomes using tBLASTn (17). Sequences corresponding to rat and mouse TLR genes that had close homology to human *TLR10*, including areas specific to *TLR10* and not seen in *TLR1* or *TLR6*, were observed. These sequences were completed by amplification from the corresponding genomic DNA, cloning into pCRII-TOPO (Invitrogen Life Technologies), and sequencing using RP and −21 primers. When sequences differed from the public databases, several individual clones were sequenced and the difference was considered a polymorphism if present in at least two separate PCR or in all of the clones

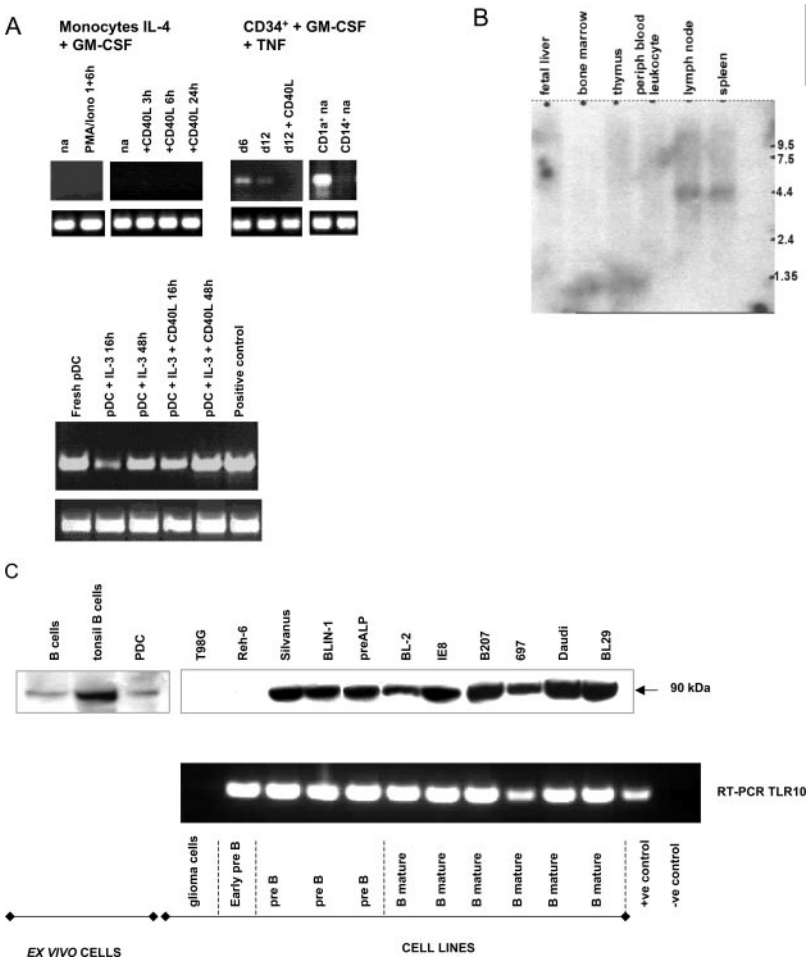

FIGURE 1. *A, upper panel,* Mono-derived DCs in the presence of IL-4 and GM-CSF activated with PMA and ionomycin or CD40L at different time points, $CD34^+$-derived DCs in the presence of TNF-α and GM-CSF activated with CD40L, and analysis of $CD14^+$ and CD1a subsets. *A, lower panel,* Ex vivo tonsil-derived PDCs incubated in the presence of IL-3 or with CD40L-expressing L cells. *B,* Northern blot analysis of TLR10 from human tissues using primers specific for the 670-bp fragment of TLR10 that was also used for RT-PCR analysis. Western blots of TLR10 on ex vivo cells and cell lines. *C,* Analysis of human ex vivo B cells and B cell lines using a monoclonal hTLR10 Ab. RT-PCR was performed using the same B cell lines analyzed for protein expression of hTLR10. The positive control used was pDISPLAY-TLR10 and a negative control consisted of a PCR with no added cDNA.

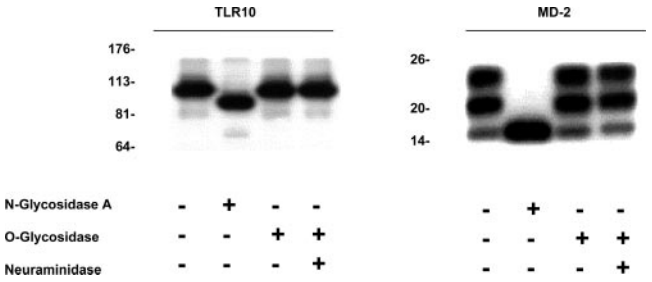

FIGURE 2. TLR10 is an *N*-glycosylated protein. HA-tagged TLR10 or MD-2 was transfected into HEK293T cells and 48 h after transfection cells were harvested, washed, and lysed. Lysates were treated with *N*-glycosidase F, *O*-glycosidase, and *O*-glycosidase plus neuraminidase for 16 h at 37°C.

from a given reaction. Genomic DNA from two rat strains, Sprague Dawley and Lewis, was used as was DNA from nine unrelated mouse strains. Tissue samples (tail biopsies) of C57/BL/6 and 129sv mice were obtained from Charles River Breeding Laboratories and DNA was extracted using the Qiagen DNeasy genomic DNA extraction kit (Qiagen). Genomic DNA from inbred mouse strains FVB/NJ, NZB, PL/J, NOD/Lt (*Mus musculus*), wild inbred strains Cast/Ei (*Mus musculus castaneus*), and SPRET/Ei (*Mus spretus*) was procured from The Jackson Laboratory. Genomic DNA from Swiss mice was obtained from Promega. Amplification from the 5′ end of the mouse *TLR10* coding sequence using the primers 5′-GATGTCAACA GAAGCCTGGGC (forward primer) and 5′-GACCATCAGCCAAGTT GTGTTG (reverse primer) gave bands of slightly different sizes from each strain of mouse. PCR amplification was performed for 35 cycles at 92°C for 1 min, 55 or 60°C for 1 min, and 72°C for 2 min. Identical results were achieved at amplification temperatures of 55 and 60°C. Sequences were cloned in pCRII-TOPO (Invitrogen Life Technologies) and sequenced.

Plasmid constructions

Flag-tagged hTLR2, hTLR4, and hemagglutinin (HA)-tagged hTLR3 were kind gifts from Prof. Alberto Mantovani (Universita degli Studi di Milano, Milan, Italy). Other hTLRs and hMD-2 were amplified either from human genomic DNA or cDNA from PBMC, produced as above, and cloned into the pDISPLAY (Invitrogen Life Technologies) eukaryote expression vector which contains the Ig κ signal peptide and the NT-HA tag. We made use of a *Not*I site downstream of the multiple cloning site to eliminate the c-*myc* epitope and the transmembrane domain. Alternative pDISPLAY vectors which encode c-*myc* or flag epitopes were produced in our laboratory by substitution of double-stranded oligonucleotides at the HA site of the vector. These were used to produce Flag-TLR10 and c-*myc*-MD-2. Constitutively active CD4TLR3, 4, and 10 were constructed by fusing cDNA encoding the extracellular domain of murine CD4 to the transmembrane and cytoplasmic domain of human TLR10, TLR4, and TLR3 (18). Mutants were generated in the TIR domain of CD4TLR10 construct using the Gene Editor mutagenesis kit (Promega). Primers used to introduce mutations or deletions are as follows: 5′-CTACTTTGACCATGGCAAAAGC (P674H); 5′-CTTCATTGAGAATCGATATAAGTCC (KS688–689NR); AAAAAGCATAGATCTAATGGCCCAAG (Y754stop); and 5′-TGTTA ATGTATGAGCTCCCAGAGAAATG (L780stop).

MyD88 and DNMyD88NT Flag (deletion of the death domain) were amplified from human cDNA and cloned into the pCMVNT-Flag vector. NF-κB, ENA-78, and IL-4 luciferase constructs were cloned as previously described (18).

Western blotting and immunoprecipitations of HEK293T cells

HEK293T cells were seeded into six-well plates and the following day 500 ng of the respective construct was transfected using FuGENE (Roche). Twenty-four hours after transfection for CD4TLRs and 48 h for full-length TLRs, cells were lysed in mild lysis buffer (MLB) containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 20 mM NaF, and 0.3 mM Sodium Orthovanadate.

Western blot analysis of purified B cells from blood, tonsil, and B cell lines

Cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1% Nonidet, 0.5% sodium deoxycholate, and 0.1% SDS. Protease inhibitors aprotinin, leupeptin, and PMSF (Roche) were added. For immunoprecipitations, cells were lysed in MLB

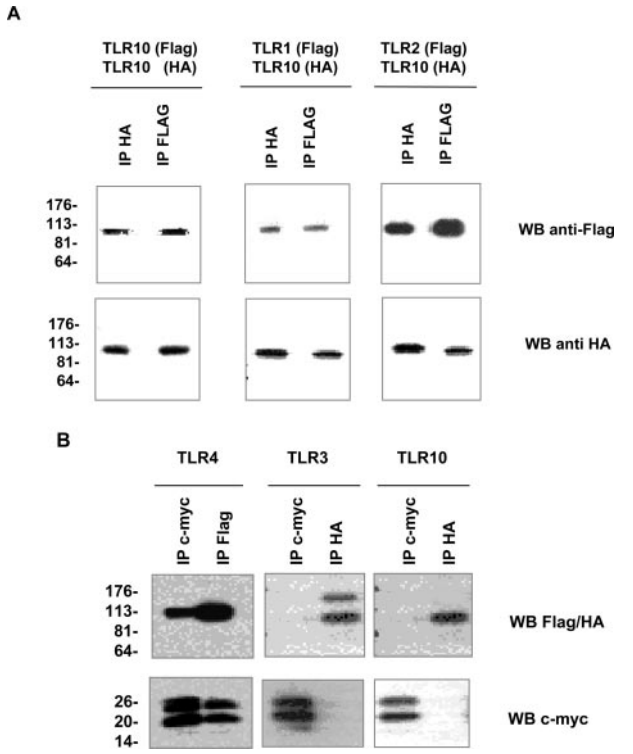

FIGURE 3. *A,* TLR10 homodimerizes and heterodimerizes with TLR2 and TLR1. TLR10 tagged with Flag or HA was cotransfected with the conversely tagged TLRs in HEK293T cells and harvested 48 h after transfection. Immunoprecipitations and Western blots were performed with Flag and HA Abs to examine and confirm dimerization in both senses. *B,* Coimmunoprecipitation of MD-2 with TLRs. MD-2-c-*myc* was cotransfected in HEK293T cells with Flag-tagged TLR4, HA-tagged TLR3, or HA-tagged TLR10. Immunoprecipitations and Western blots were performed with Flag, HA, and c-*myc* Abs to examine and confirm dimerization in both senses.

and 40 μg of total protein was immunoprecipitated with 1.5 μg of the respective Ab for 2 h or overnight at 4°C in the presence of protein G-Sepharose. Beads were washed four times in MLB and 4× lithium dodecyl sulfate loading buffer was added. In general between 20 and 40 μg of total cellular protein (determined by Bradford assay; Bio-Rad) were used for SDS-NuPAGE and immunoblotting (Invitrogen Life Technologies). After incubation with primary Abs, reactive proteins were detected with peroxidase-conjugated anti-mouse secondary Abs (Jackson ImmunoResearch Laboratories) and ECL (Amersham).

Analysis of glycosylation of transiently transfected TLR10 and MD-2

For *N*-glycosylation analysis, 48 h after transfection the cells were lysed in 0.1 M phosphate (pH 7), 0.05% (w/v) SDS, 1% β-ME, 1% Igepal, and 50 mM EDTA (Nonidet P-40 buffer) and the lysates were treated with *N*-glycosidase F at 40 U/ml, *O*-glycosidase at 40 mU/ml, and *O*-glycosidase plus neuraminidase at 40 mU/ml for 16 h at 37°C.

Reporter assays

HEK293 cells were transiently transfected using FuGENE (Roche) with 100 ng of reporter construct along with 25 ng of CD4TLR expression vectors and, in certain experiments, in the presence or absence of 50 ng of MyD88DN into 24-well tissue culture plates with cells at 50% confluency. In addition, 1 ng of a construct directing expression of Renilla luciferase (under the control of the constitutively active CMV promoter) was used to normalize the transfection efficiency. Cells were harvested and analyzed for luciferase activity 24 h after transfection.

Luciferase assay

Cells were harvested and analyzed simultaneously for firefly and Renilla luciferase activity (to normalize) using the PerkinElmer Firelite reporter assay reagents.

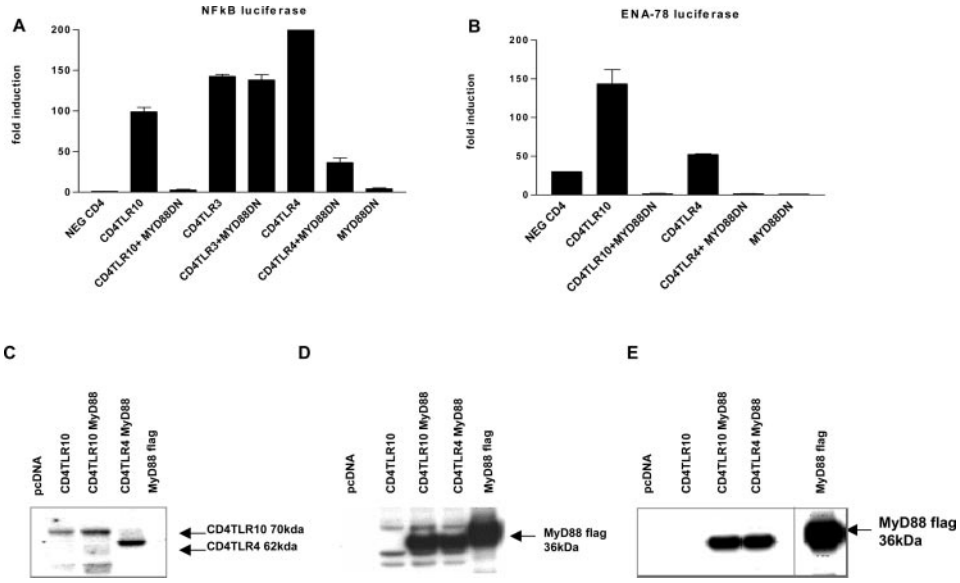

FIGURE 4. TLR10 interacts directly with MyD88: cotransfection of CD4TLR constructions with DNMyD88 and luciferase reporters NF-κB (*A*) or ENA-78 (*B*). HEK293 cells were harvested 24 h after transfection. Three independent experiments were performed, results generally deviated by <10% of the mean value. HEK293T cells were transfected with control and CD4TLR constructs in the presence or absence of MYD88NTFlag and harvested 24 h after transfection. *C,* Western blot control of MyD88NTFlag expression. *D,* Immunoprecipitation control of CD4TLR using the CD4 Ab. *E,* Immunoprecipitation using CD4 Ab, immunoprecipitated complexes were revealed with the Flag tag of MyD88NTFlag. 293T cells transfected only with MyD88NTFlag was used as a control for the Flag Ab.

Abs used

Human TLR10 clone 158C1114 (Imgenex), mCD4 (BD Pharmingen, France), M2 mouse anti-Flag (Sigma-Aldrich), mouse anti-HA (Roche), mouse anti-*myc* (clone 9E10; Roche), anti-CD3 (UCHT1; BD Pharmingen, France), and anti-CD28 (CLB-CD28/1; Sanquin).

Results

TLR10 is expressed by B cells and PDCs

Fig. 1*A* showed that DCs derived from monocytes in the presence of IL-4 and GM-CSF did not express TLR10. $CD34^+$-derived DCs expressed TLR10 during the early stages of differentiation but this disappeared upon culture with CD40L-expressing L cells. Culture of $CD34^+$ progenitor cells in GM-CSF and TNF gives rise to two populations of cells. The $CD14^+$ subset has been described as being similar to dermal DCs or circulating DCs, whereas the $CD1a^+$ subset resemble Langerhans cells in the epidermis (14). Interestingly, TLR10 expression was almost exclusively restricted to the $CD1a^+$ subset. As described by Kadowaki et al. (19), we also confirmed expression of TLR10 on PDCs (Fig. 1*A, lower panel*). We observed that the expression of TLR10 on PDCs isolated from tonsil remained relatively constant after maturation with IL-3 and CD40L. We were able to detect TLR10 expression in immunological tissue. An mRNA of 4.4 kb was detected by Northern blot analysis (Fig. 1*B*), uniquely in lymph node and spleen,

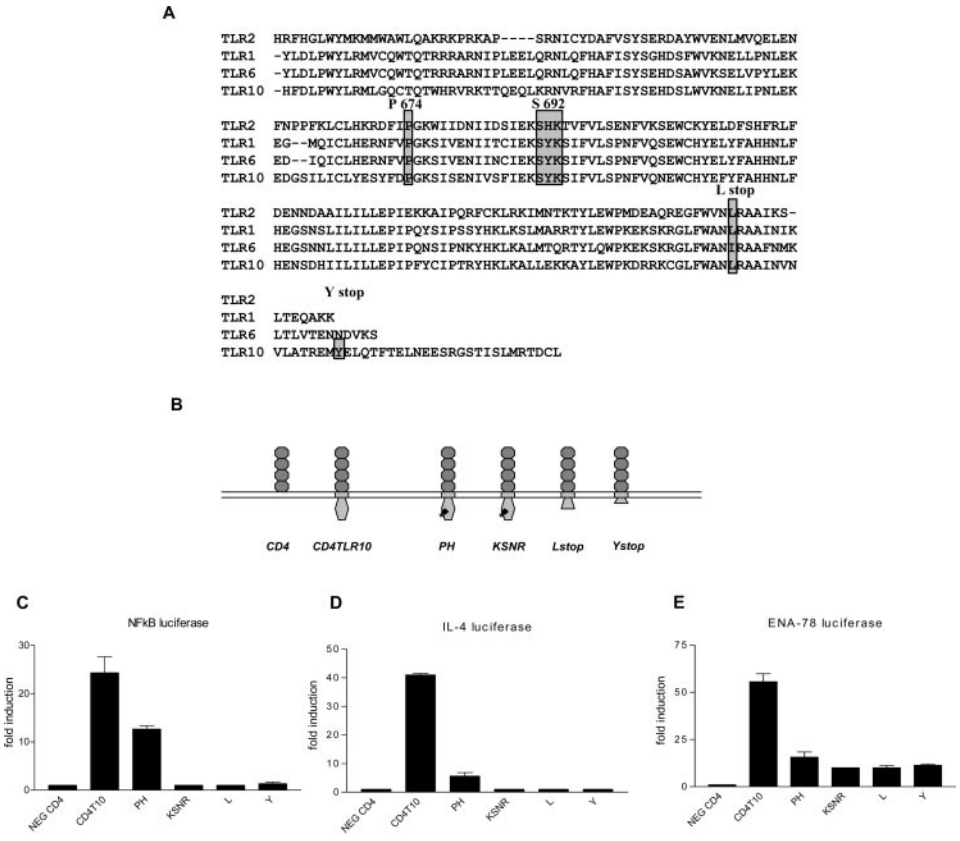

FIGURE 5. *A,* CD4TLR10 mutants vs gene reporter activity: Alignments of the TIR domains of TLR1, TLR2, TLR6, and TLR10 shows the mutations and deletions generated in the TIR domain of TLR10. Sites of mutations are highlighted in gray. *B,* Figure representing the mutations and deletions generated in the TIR domain of TLR10. In dark gray are the areas representing the extracellular domain of murine CD4. Shown in light gray is the transmembrane domain and TIR domain of human TLR10. Sites of mutations are shown in black. The L and Ystop mutants were made in the carboxyl-terminal where stop codons were introduced at amino acid positions 779 and 753, respectively. CD4TLR10 mutants vs luciferase reporters. Cotransfection of CD4TLR10 plasmids with NF-κB (*C*), IL-4 (*D*), and ENA-78 (*E*) reporters into HEK293 cells. Changes in luciferase activity were measured 24 h after transfection. Three independent experiments were performed; results generally deviated by <10% of the mean value.

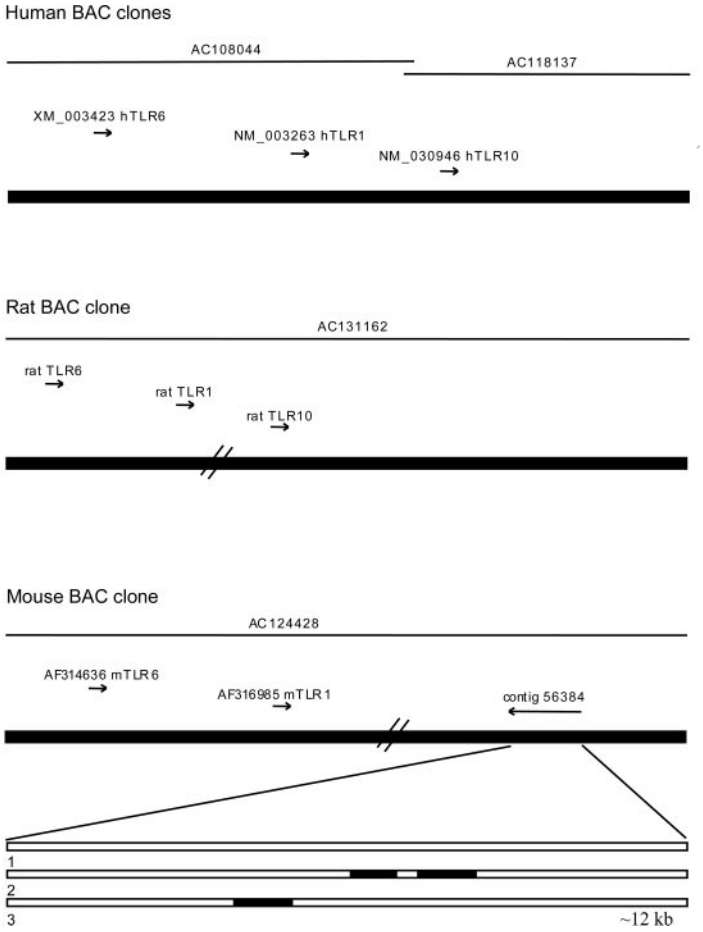

FIGURE 6. (*continues*)

FIGURE 6. *A,* The genomic structure at the *TLR1*, *TLR6*, and *TLR10* locus in mouse, rat, and humans. Since the noncoding exons cannot be predicted for the rodent sequences, genes are shown as a simple bar covering the single coding exon; however, the three exons of the human TLR10 could be predicted correctly from the human genomic sequence. BAC clones AC108044 and AC118137 show a clear overlap and are contiguous. The mouse sequence is shown as the genomic clone containing *TLR10*-like sequences. These have been shown as three open reading frames covering different reading frames due to the insertions of repetitive and retroviral sequences in this genome. For the human and rat sequences, the entire open reading frame is coded on a single exon. Sequence upstream of the start codon indicates that the rat gene, like the human gene, has at least one intron 5′ of the coding sequence. *B,* Alignments of amino acid sequences of the human, mouse, and rat TLR10 proteins. The potential signal peptide and transmembrane domains are boxed. Potential *N*-glycosylation sites are circled. The mouse C57BL/6 sequence is shown; sequencing of all other strains gave essentially the same peptide sequence, but showed alterations in the repetitive and noncoding regions. Missing sequence from the mouse is due to phase changes in the genomic sequence and sequential loss of the nonfunctional gene.

probably reflecting the high expression in B cells. Using a TLR10 Ab, we detected the presence of a 90- to 100-kDa protein in B cell lines (Fig. 1*C*). An early pre-B cell line, Reh-6, was negative for TLR10, whereas all of the pre-B, B, and mature B cell lines tested expressed TLR10 protein. The glioma cell line T98G was used as a negative control. In addition, we also detected expression of TLR10 protein on ex vivo B cells purified from PBMCs and tonsil as well as PDCs from tonsil.

hTLR10 is N*-glycosylated at multiple sites and associates with other receptors in the TLR family*

We created a HA-tagged version of hTLR10; this construct was expressed in HEK293T cells which gave reproducible intracellular expression (data not shown). Western blotting of cell lysates showed a single protein of ~91–100 kDa, similar in size to endogenous TLR10 (Fig. 2). We treated cell lysates with *N*-glycosidase A, *O*-glycosidase, and *O*-glycosidase plus neuraminidase. MD-2 was used as a positive control since the glycosylation sites of this protein have been previously described (20). As shown in this experiment, MD-2 displayed two glycosylated forms of 20 and 26 kDa, respectively, which could be completely deglycosylated to reveal a single nonglycosylated form of 14 kDa. *N*-linked glycosylation of MD-2 and TLR4 has been shown to be essential for LPS recognition (21) TLR10 showed *N*-linked, but not *O*-linked glycosylation (Fig. 2). Deglycosylated TLR10 shows a size of ~80 kDa. Coimmunoprecipitation studies (Fig. 3*A*) revealed the association of TLR10 with itself as well as TLR2 and TLR1; we were unable however to observe a consistent association with TLR6 in two of the three experiments performed. TLR2 has already been shown to associate with TLR1 or TLR6 via the extracellular domains of these receptors (23–26). The fact that TLR10 associated with itself revealed a capacity to form homodimers.

MD-2 is an accessory molecule that associates with TLR4 (27). The coexpression and association of TLR4/MD-2 has been shown to be essential for the recognition of LPS by TLR4 (28). Coimmunoprecipitation experiments (Fig. 3*B*) showed that TLR4 associated with MD-2, but that TLR3 and TLR10 do not.

TLR10 activates immune system promoters

To evaluate the basic signaling potential of the TLR10 TIR domain, we generated a constitutively active construct by fusing the mouse CD4 extracellular portion with the TLR10 transmembrane and TIR domain. Previous experiments have shown that CD4TLR10 was able to activate NF-κB, ENA-78, and other gene promoters (18). In this study, we show for the first time that the signaling activity of TLR10 requires the adapter MyD88 (Fig. 4, *A* and *B*) but not TIRAP, TRAM, or TRIF (data not shown, using DN versions of TIRAP and TRAM and small-interfering RNA for TRIF). We observed that DNMyD88 entirely blocked the signaling ability of CD4TLR10, partially CD4TLR4, but not CD4TLR3 to induce NF-κB and ENA-78 promoters. Furthermore, coimmunoprecipitation studies were performed to determine whether the TIR domain of TLR10 interacts directly with MyD88. These experiments show that CD4TLR10 interacted with MyD88 and with the control CD4TLR4 (Fig. 4, *C–E*), indicating for the first time MyD88 as the adapter molecule involved in the signalization of TLR10.

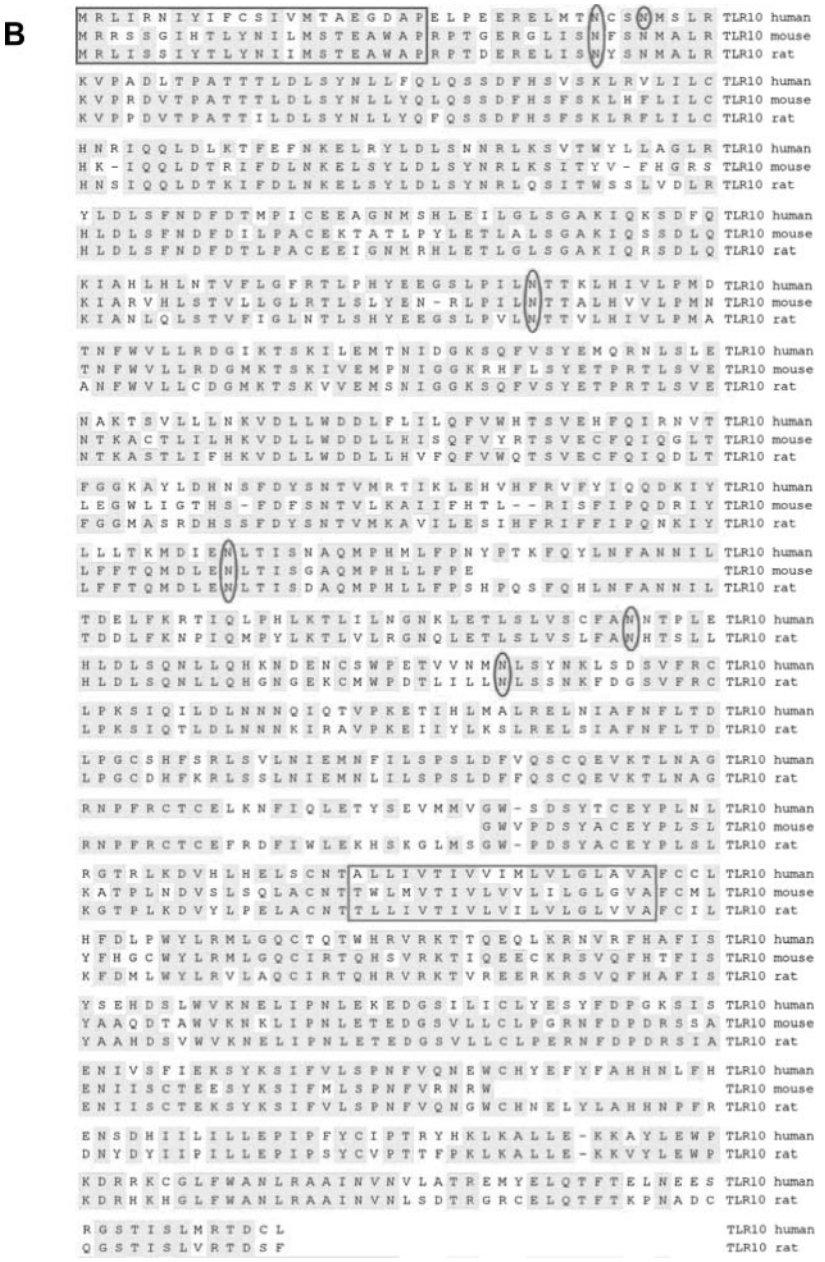

FIGURE 6. (*continued*)

Mutations in the TLR10 TIR domain alters its signaling potential

The TIR domain is essential for signaling and TLR10 also contains the classical regions of particular signaling importance. Most TIR sequences have a conserved proline, which when mutated to histidine renders the protein unable to signal, probably due to an inability to associate with MyD88. Interestingly TLR3, the only TLR which does not associate with MyD88 has alanine at this position. Fig. 5*A* shows the positions where the mutants were made. Additionally, we noted a conserved potential serine protein kinase C phosphorylation site at position 692 in the TLR10 sequence. This sequence is conserved in all of the TLR TIR domains. The sequence was mutated from KSYK to NRYK to remove the serine residue. We also made truncation mutations to remove parts of the nonconserved carboxy-terminal of the TLR10 TIR domain (Lstop and Ystop). Figure *5B* schematically represents where deletions and stop codons were made in the carboxyl-terminal to mutate or shorten the open reading frame of the TLR10. We next examined the CD4TLR10 mutants in their ability to activate the NF-κB, ENA-78, and IL-4 promoters. Transfection of HEK293 cells with CD4TLR10 alone induced all three reporters (Fig. 6, *C–E*). Mutation of the conserved proline decreased luciferase activity of all three promoters. Mutation of the putative phosphorylation site at S692 caused a complete drop in reporter activity for IL-4 and NF-κB. Deletions L and Y in the TIR domain reduced NF-κB activity but a greater decline was observed with EN8–78 and IL-4 reporters; however, the Y deletion suppressed reporter activity of all promoters. Comparable levels of CD4TLR10 and mutant expression in HEK293T cells was detected by Western blotting using a mouse anti-CD4 Ab (data not shown).

A homologue of TLR10 is present in rat, but not in mouse

We determined whether other species had homologues of TLR10. Genomic DNA from humans, mouse, and rat were digested with *Eco*RI, *Xba*I, *Bam*HI, and *Not*I. Using the 670-bp h*TLR10* probe, single bands were observed for the human DNA, but not for the

other species. Attempts at cross-species PCR using 20-mer oligonucleotides chosen at overlapping sites in the N-terminal coding region of h*TLR10* with little homology to *TLR1* and *TLR6* did not yield any specific amplification (data not shown). We then used bioinformatics analysis to search for sequences with homology to h*TLR10* in the genomic databases. Coding sequences similar to the h*TLR10* protein were detected in mouse and rat BAC clones. Since the rat genomic clone contained sequences from the 5′ and 3′ coding sequence, PCR amplification from genomic DNA from the rat allowed us to identify the entire coding exon of this gene. However, for the mouse sequence only partial sequences could be found. Comparison of the human, rat, and mouse amino acid sequences allowed us to conclude that these sequences represent the rodent homologues of *TLR10* (Fig. 6*A*). Interestingly, we detected sequences very similar to *TLR1* and *TLR6* on the same genomic clones as *TLR10*, suggesting that in the mouse, rat, and human the *TLR1*, *TLR6*, and *TLR10* genes are clustered. The human and rat TLR10 proteins each showed a putative signal peptide (Fig.6*B*, boxed), a transmembrane region (Fig. 6*B*, boxed), and a conserved cytosolic TIR domain. Human and rat TLR10 show, respectively, seven and six potential sites for *N*-glycosylation (circled), of which six are conserved between the two proteins (Fig. 6*B*). Analysis of the genomic DNA sequences revealed that the mouse *TLR10* gene is a nonfunctional gene, with numerous gaps, insertions, and phase changes, and with the TIR domain replaced by a retrovirus-like sequence. Amplification of mouse genomic DNA from nine unrelated mouse strains using PCR primers designed to amplify sequences around the peptide signal (forward primer) and the putative transmembrane domain (reverse primer) gave single bands (data not shown). Sequencing of these bands revealed sequences very similar to that detected on the BAC clone (C57BL/6 strain), with differences in certain repeated regions. Notably the inbred wild strains, CAST/Ei and SPRET/Ei, showed a shorter sequence and contained fewer repeats with respect to the C57BL/6 mice. This suggests that the mouse *TLR10* gene has been lost by retroviral insertion and amplification of repeat regions after the separation of the mouse and rat lineages.

Discussion

TLR10 is the only remaining orphan member among the human TLRs. Research on this receptor has been hindered by the absence of a rodent homologue, a step that has considerably aided the identification of ligands for most TLRs. In this study we aimed to advance the field by defining an immunological model and assess receptor functionality even in the absence of a ligand. We showed that TLR10 had no mouse homologue due to the interruption of the mouse *tlr10* gene by a retroviral sequence. We hypothesize that this insertion had most likely occurred early in the evolution of the mouse lineage as seen by the fact that inbred wild mouse strains such as *M. spretus* or *M. musculus castaneus* of diverse geographical origins have similar vestigial *tlr10* gene sequences to those of highly inbred laboratory strains of *M. musculus*. These sequences represent the only mouse *tlr10* gene, shown at least for the C57BL/6 genome, in which *tlr10* can be isolated at the same locus as the *tlr1* and *tlr6* genes and in the same arrangement as seen for the human and rat copies of the three genes. Our data imply that although mice do not have a functional TLR10, it is probable that the *tlr10* gene in rat is expressed and functional. Recently, Hubert et al. (29) have isolated and characterized PDCs from rat spleen and reported the low level expression of TLR10 in these cells. This manuscript does not show the entire sequence of the *tlr10* gene from the rat; however, the primers used and their data suggest that they isolated the same gene as discussed in this report. Although the genetic manipulation of rat models are not as advanced as in mice, we anticipate that this will serve as an immunological tool in identifying the ligand

In humans, TLR7, TLR9, and TLR10 expression is limited to granulocytes (in particular eosinophils), germinal center B cells, and PDCs (8, 30, 31). These expression data may indicate a role for TLR10 in terms of biological function in inducing type I IFN from PDCs as in the case of TLR7 and 9 (32) or in the case of B cell activation, inducing proliferation and cytokine secretion as observed for TLR9 in the presence of CpG motifs (8). We have confirmed and extended this expression to DCs generated in vitro resembling Langerhans cells (33). However, in several ex vivo Langerhans cell skin samples, we were unable to detect the expression of TLR10 (data not shown). This may suggest that the expression of TLR10 is directed by conditions similar to those in our in vitro cultures.

Detection of the endogenous TLR10 protein has not been previously reported. We extended the RT-PCR results shown by Bourke et al. (8) by examining TLR10 protein expression. TLR10 was detected in all samples except Reh-6, an early pre-B cell precursor. However, TLR10 mRNA was detected in all cell lines tested, suggesting that mRNA expression starts early in the B cell lineage, but translation of TLR10 is by cells that are committed to B cell differentiation.

We observed for the first time protein complexes with TLR10 as a homodimer and association with TLR1 or TLR 2. In addition, ImageQuant densitometry analysis, using immunoprecipitation controls as standards, reveals that homodimerization of TLR10 has a binding affinity of 100%, whereas for a TLR1/10 heterodimer 87% was observed and for a TLR1/2 complex 80%. Phylogenetic analysis indicates that among all human TLRs, TLR10 is closely related to TLR1 and TLR6. From the sequence homology, the most plausible hypothesis is that a common TLR1/6/10 ancestor duplicated to produce a TLR1/6 precursor and TLR10 (34). Extensive research on TLR2 has shown that the association with TLR1 and TLR6 is necessary for efficient ligand binding and the discrimination of triacyl and diacyl lipopeptides from bacteria (35). Potentially TLR10 may act as a coreceptor to these TLRs and therefore share the same family of ligands. However, the expression pattern of TLR10 in B cells and PDCs (19, 22) is confined to cells that do not appear to express TLR2. It is thus possible that TLR10 does not associate with TLR2 in vivo. Homo- and heterodimer formation by TLRs and associated non-TLR surface Ags increases the potential for ligand recognition and immune system modulation and contributes to the wide range of recognized PAMPs (28). In assessing the nature of the TLR10 agonist, we tested TLR10 in single and cotransfections with TLR1, TLR2, and TLR6 with a luciferase-driven NF-κB minimal promoter to determine whether the TLR10 ligand is related to PAMPs already determined for this family of proteins. However, we were unable to clearly demonstrate that bacterial-derived components activated TLR10 to induce NF-κB. We did not test extensively potential viral ligands in this system. As mentioned previously, TLR10 is clearly expressed on PDCs which produce high levels of type I IFN in response to viral infection and activation of TLR7 and 9 (36); therefore, we cannot exclude that a potential TLR10 ligand could be viral (37). It is noteworthy that PDCs and B cells isolated from tonsils in this study would have been exposed to a reservoir of infection before isolation since human tonsils can, for example, harbor EBV, respiratory syncytial virus (38), HIV (39), and other herpesviruses (40), as well as a range of bacterial pathogens (41). From these reports, we conclude that TLR10 expression in PDCs from tonsils may be enhanced because cells may already be in a state of pathogen activation. It is also probable that TLR10, which also has an extremely restricted expression profile, responds to molecules derived uniquely from very specific

pathogens. A recent study that implicated polymorphisms in TLR10 in asthma patients (42) suggests that TLR10 may have a particular role in the lung. Thus, it cannot be excluded that TLR10 may also have a role in the recognition of airborne pathogens, such as bacteria or fungal pathogens, or alternatively airborne allergens.

Although lacking a candidate ligand, we continued to evaluate the signaling potential of TLR10 by using a constitutively active form of the molecule. With the exception of TLR3, all TLRs recruit MyD88, and some TLRs (i.e., TLR4, TLR2) can interact with several adaptors such as TIRAP and TRAM to generate diverse effects on gene transcription (5). We previously described the construction and assessed the activation of certain cytokine promoters by CD4TLR10 (18). In this study, we additionally show that MyD88 is also an adapter molecule involved in TLR10 signalization. Following previous studies on TLR4 (4), we also mutated the proline residue found at position 674 on TLR10. Although the reporter activity induced by TLR10 P674H was decreased compared with that of wild-type CD4TLR10, the activity was not completely abolished. This may imply that other sequences in the TIR domain may also be involved in the signaling of TLR10 and we speculate that either MyD88 may not necessarily bind only at this site or that another TIR adapter can partially substitute for MyD88 in the case of TLR10. Interestingly, the TLR10TIR mutant lacking a conserved potential serine phosphorylation site continued to activate the ENA-78 promoter. The necessity for a serine phosphorylation site in the TIR domain of TLRs has not previously been demonstrated and further work will be required to clarify the function of this site. The TLR10 deletion may suppress the formation of secondary structures specifically formed by TLR10 which can be potentially important for signaling, or may indicate that nonconsensual sequences in the TLR10 TIR activate an important part of the signaling pathway in the case of NFκB and IL-4, but not entirely for ENA-78. This suggests that unknown cytosolic factors and/or transcription factors may depend on the presence of these nonconserved TLR10 sequences.

These data, in addition to the discovery of a rodent homologue, should facilitate the identification of the natural ligand and help to further elucidate the function of TLR10.

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Disclosure

The authors have no financial conflict of interest.

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