Toll-like receptors (TLRs) are pattern-recognition receptors that detect highly conserved molecular structures of microorganisms or viruses and regulate both innate and adaptive immune responses against pathogens (1). More than 10 TLRs have been identified in human and mouse (2). TLR1, TLR2, TLR4, TLR5, and TLR6 recognize bacterial cell wall and cell surface components, such as lipoproteins, lipopolysaccharide, and flagellin. On the other hand, TLR3, TLR7, and TLR9 recognize pathogen nucleic acids, such as viral RNAs and bacterial DNA (2, 3). All TLRs have a cytoplasmic domain, which associates with intracellular signaling domain called the Toll/interleukin 1 receptor resistance (TIR) domain. In contrast, the intracellular localization of TLR7 is achieved by its transmembrane domain. These elements also targeted a heterologous type I transmembrane protein CD25 to the intracellular compartment that contained TLR3 and TLR7. Despite their using distinct regulatory elements for intracellular localization, TLR3 was found to co-localize with TLR7. In addition, TLR3 and TLR7 were preferentially localized near phagosomes containing apoptotic cell particles. These findings reveal that TLR3 and TLR7 contain unique targeting sequences, which differentially lead them to the same intracellular compartments and adjacent to phagosomes containing apoptotic cell particles, where these receptors may access their ligands for the induction of immune responses against viral infection.

Toll-like receptor 3 and TLR7 are indispensable for host defense against viral infection by recognizing virus-derived RNAs and are localized to intracellular membranes via an unknown mechanism. We recently reported experiments with chimeric Toll-like receptors that suggested that the subcellular distribution of TLRs may be defined by their transmembrane and/or cytoplasmic domains. Here we demonstrate that the intracellular localization of TLR3 is achieved by a 23-amino acid sequence (Glu727 to Asp749) present in the linker region between the transmembrane domain and Toll-interleukin 1 receptor resistance (TIR) domain. In contrast, the intracellular localization of TLR7 is achieved by its transmembrane domain. These elements also targeted a heterologous type I transmembrane protein CD25 to the intracellular compartment that contained TLR3 and TLR7. Despite their using distinct regulatory elements for intracellular localization, TLR3 was found to co-localize with TLR7. In addition, TLR3 and TLR7 were preferentially localized near phagosomes containing apoptotic cell particles. These findings reveal that TLR3 and TLR7 contain unique targeting sequences, which differentially lead them to the same intracellular compartments and adjacent to phagosomes containing apoptotic cell particles, where these receptors may access their ligands for the induction of immune responses against viral infection.

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EXPERIMENTAL PROCEDURES

Mice and Cell Culture—C57BL/10ScN mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our colony. Bone marrow-derived macrophages (BMDMs) were prepared and maintained as described previously (4). BMDMs at day 4 or 5 after isolation from the bone marrow were used for retroviral infection. The infection...
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Efficiency was generally 35–40%. HEK293T cells and mouse 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

**DNA Constructs and Stable Cell Lines**—The murine TLR4/TLR chimeras were described previously (4). Truncated forms of TLR4/TLR3 and TLR4/TLR7 chimeras were amplified by PCR and then subcloned into the pMXpie bicistronic retroviral vector, which encodes the gene of interest followed by an internal ribosomal entry site element and the enhanced green fluorescent protein (GFP) gene (12). GFP fluorescence is therefore an indication of infection efficiency. The transmembrane domain swap mutants of TLR4/TLR chimera were prepared by multiple PCRs using overlapping sequences and then subcloned into pMXpie. Murine CD25 cDNA was amplified by PCR from mouse spleen cDNA. cDNAs encoding CD25-TRL3 linker fusions and CD25-TRL7 chimeras were prepared by multiple PCRs using overlapping sequences and then subcloned into pMXpie. The COOH-terminal yellow fluorescent protein (YFP)- or cyan fluorescent protein (CFP)-tagged TLR3 (TLR3YFP), TLR4 (TLR4YFP), TLR7 (TRL4CFP and TLR7YFP), TLR4/TLR3 chimera (TLR4/TLR3YFP), TLR7 (CD25), CD25 (CD25CFP), CD25-EG-ID fusion (EG-IDCFP), or CD25-TRL7 chimera 1 (chimera 1CFP) were constructed by fusing cDNA encoding the full-length TLR3, TLR4, TLR7, and then subcloned into the pMXpie. Murine CD25 cDNA was kindly provided by Dr. K. Miyake (University of Tokyo). Murine CD14 cDNA was amplified by PCR from mouse liver cDNA. cDNAs encoding CD14 and MD-2 were cloned into the pMXpie. Murine MD-2 cDNA was kindly provided by Carpinteria, CA) using PE-conjugated anti-mouse TLR4/MD-2 antibody. Cells obtained from the second sort were then cloned by limiting dilution. Single clones were isolated, and the cell surface level of TLR4/TLR5 chimera in each clone was determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody. Total RNA was prepared from GFP+TLR4/MD-2+ clones and was reverse-transcribed to prepare cDNAs as described previously (4). cDNAs encoding the transmembrane and cytoplasmic domains of TLR4/TLR5 chimera were amplified by PCR, and the mutation in each clone was determined by DNA sequencing.

**Microscopy**—BMDMs expressing YFP or CFP fusion proteins were split onto glass coverslips. The next day, the cells were treated with Alexa Fluor 594-conjugated cholera toxin subunit b (CTXb; Molecular Probes, Eugene, OR) for 20 min on ice. After washes, the images were acquired using a Deltavision deconvolution microscope (Applied Precision, Issaquah, WA) or a Carl Zeiss LSM510 META laser-scanning confocal microscope. The computational deconvolution was carried out with the SoftWoRX software.

To test the co-localization of phagocytic particles with TLRs in macrophages, apoptotic T cells were prepared and added to BMDMs.

**RESULTS**

**TLR3 and TLR7 Are Targeted to an Intracellular Compartment by Their Transmembrane and Cytoplasmic Domains**—Recent data have suggested that TLR3 and TLR7 are localized in intracellular acidic compartments, such as phagosomes, and recognize viral nucleic acids, such as double- and single-stranded RNA derived from virus-infected apoptotic cells or virus particles (4, 6, 11, 14, 15). Consistent with these reports, we found that TLR3 and TLR7 molecules containing a YFP moiety fused to their COOH termini were localized intracellularly but not on the cell surface (Fig. 1A). In contrast, a fusion of TLR4 with YFP (TLR4YFP) was expressed both in internal membranes and on the cell surface, which was visualized by staining ganglioside GM1 with fluorescein.
Distinct Intracellular Targeting Elements for TLR3 and TLR7

We had previously observed that chimeric receptors, which are composed of the extracellular region of TLR4 fused to the transmembrane and cytoplasmic regions of TLR3 or TLR7, were not detected on the cell surface of BMDMs (Fig. 1B and Ref. 4). To test whether the TLR4/TLR chimeras behave like full-length TLRs, both TLR3YFP and TLR4/TLR3YFP were expressed in the same BMDMs, following staining with CTXb, and their subcellular distribution was determined microscopically. As shown in Fig. 1C, the TLR4/TLR3 chimera was clearly co-localized with TLR3 in cytoplasmic vesicles but not co-localized with CTXb at the cell surface, indicating that the TLR4/TLR chimeras may properly reflect the localization of TLRs and therefore that the transmembrane and/or cytoplasmic domains of TLR3 are responsible for this intracellular localization.

Random Mutagenesis of the Transmembrane and Cytoplasmic Domains of TLR4/TLR Chimeras—We hypothesized that TLR3 and TLR7 are targeted to intracellular compartments by sequences present in their transmembrane and/or cytoplasmic domains. This hypothesis predicts that mutation of the targeting sequence would allow the intracellular TLR to come to the cell surface. Since the subcellular distribution of the TLR4/TLR chimeras was easily determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody, we decided to use these chimeric receptors to investigate the putative targeting signals. cDNAs encoding the transmembrane and cytoplasmic domains of the TLR4/TLR chimeras were randomly mutated by error-prone PCR and put back into the context of the TLR4 chimera (Fig. 2A). Random sequencing of plasmids encoding the mutated TLR chimeras confirmed that the mutations were randomly generated throughout the transmembrane and cytoplasmic domains of the TLR4/TLR chimeras (data not shown). The mutation rates of the TLR4/TLR3 and TLR4/TLR7 chimeras were 1.7 ± 0.3 and 2.9 ± 0.4 per clone at the nucleotide level and 1.1 ± 0.4 and 1.6 ± 0.3 per clone at the amino acid level, respectively. The pool of mutated chimeras was introduced into HEK293T cells stably expressing mouse CD14 and MD-2 (293TCM cells), because 1) transfected mouse TLR4 was detected on the cell surface in greater amounts in 293TCM cells than in 293T cells (data not shown), 2) the TLR4/TLR3 and TLR4/TLR7 chimeras were detected intracellularly in 293TCM cells similarly to BMDMs (data not shown), and 3) the high susceptibility of these cells to retrovirus infection enabled us to screen a large number of TLR4/TLR mutant chimeras. Retroviral vectors producing different TLR4/TLR mutant chimeras were prepared from about 40,000 different transformed Escherichia coli colonies. The 293TCM cells were infected with retroviruses containing the mutated TLR4/TLR3 or TLR4/TLR7 chimeras, and the cell surface expression level of the chimeras was determined by flow cytometric analysis using color), and CTXb is shown in red. Regions with co-localization appear yellow. Higher magnification panels to the right show the red and green channels separately for the boxed area. B, both TLR4/TLR3 and TLR4/TLR7 chimeras are not detected on the cell surface. The expression of TLR chimeras in BMDMs was determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody as described under “Experimental Procedures.” The GFP-positive cells were gated and displayed for the TLR chimera staining. Intact, cells without fixation/permeabilization; Fixed/Perm, cells with fixation/permeabilization. Gray, isotype control; line, anti-TLR4/MD-2 antibody. C, the TLR4/TLR3 chimera co-localizes with TLR3. BMDMs expressing both TLR3YFP and TLR4/TLR3YFP were stained with Alexa Fluor 594-CTXb, and images were acquired as described for A. TLR3, TLR4/TLR3 chimera, and CTXb are shown in green (pseudocolor), and red (pseudocolor), respectively. The boxed area is enlarged in the right panels, which show green, red, and merged versions of the image. Merged images with red and green co-localization result in yellow color.
FIGURE 2. Random mutagenesis of the transmembrane and cytoplasmic domains of TLR4/TLR chimeras. A, random mutagenesis of the transmembrane and cytoplasmic domains of TLR4/TLR3 and TLR4/TLR7 chimeras by error-prone PCR. The transmembrane and cytoplasmic domains of TLR3 or TLR7 were amplified by error-prone PCR using primers indicated by the arrows, and then the PCR products were cloned into the pMXpie-TLR4(EX) vector as described under “Experimental Procedures.” The mutation rates of the TLR4/TLR3 and TLR4/TLR7 chimeras were 1.7 ± 0.3 and 2.9 ± 0.4 per clone at the nucleotide level and 1.1 ± 0.4 and 1.6 ± 0.3 per clone at the amino acid level, respectively. The stars show random
antimouse TLR4/MD-2 antibody. Interestingly, a small population of GFP+TLR4/MD-2+ cells was detected in 293TCM cells infected with the TLR4/TLR3 mutant chimera-producing retroviruses, but not wild-type chimera-producing retroviruses (Fig. 2B), suggesting that some TLR3 mutations had interfered with the normal intracellular targeting. In contrast, a GFP+TLR4/MD-2+ population was not detected in 293TCM cells expressing TLR4/TLR7 mutant chimeras, although we tried to obtain a positive population by two rounds of cell sorting (Fig. 2C and data not shown).

Region of the Cytoplasmic Domain Responsible for Intracellular Localization of TLR3—To isolate the cells expressing mutated TLR4/TLR3 molecules that were expressed on the cell surface, the GFP+TLR4/MD-2+ population was enriched by two rounds of cell sorting, and then about 200 single clones were isolated by limiting dilution. Sixty-five clones were confirmed to express TLR4/TLR3 chimera on the cell surface by flow cytometric analysis, and the mutation in each clone was determined by DNA sequencing (Fig. 3A). Interestingly, all mutant chimeras contained either point mutations giving rise to premature stop codons or short deletions or insertions leading to frameshifts. These mutations had the result of deleting the whole TIR domain and a part of the linker region between the transmembrane segment and the TIR domain. No full-length chimeras with point mutations were obtained in this screen.

To confirm the results of random mutagenesis, several truncation mutants of the TLR4/TLR3 chimera receptor were created by site-directed mutagenesis, and their subcellular distributions were examined in BMDMs from TLR4-deficient mice. As shown in Fig. 3C, the chimeras lacking the region from Ile748 to the COOH termini were detected on the cell surface. In contrast, the chimeras that are slightly longer than the Ile748→STOP mutant were localized intracellularly, suggesting that a short region in the cytoplasmic domain prior to the TIR domain is sufficient for the intracellular localization of TLR3.

Intracellular Localization of the TLR4/TLR7 Chimera Lacking Its Entire Cytoplasmic Domain—in contrast to the TLR4/TLR3 chimera, a GFP+TLR4/MD-2+ population was not detected in the pool of randomly mutated chimeras (Fig. 2C) even after two rounds of sorting for potentially positive cells (data not shown). To determine whether either the linker region and/or TIR domain is necessary for the intracellular localization of TLR4/TLR7 chimera, several truncated mutants of the TLR4/TLR7 chimera were created by site-directed mutagenesis as shown in Fig. 4A, and the subcellular distributions of these mutated chimeras in BMDMs were determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody. All mutants were still localized intracellularly (Fig. 4B), suggesting that the transmembrane region of TLR7 was sufficient to cause the intracellular localization of the TLR4/TLR7 chimera.

The Cytoplasmic Linker Region of TLR3 Is Sufficient to Mediate Intracellular Localization of a Heterologous Transmembrane Protein—To determine whether the cytoplasmic linker region of TLR3 might be sufficient for targeting this receptor to an intracellular compartment, the Glu727 to Asp499 sequence from TLR3 and various truncated versions of it were fused to the COOH terminus of CD25 (interleukin 2 receptor α chain), which is a type I transmembrane receptor with a short cytoplasmic region (14 amino acids) that is normally expressed on the cell surface (16). The resulting fusion proteins were expressed in BMDMs, and their subcellular distributions were determined by flow cytometric analysis using antimouse CD25 antibody. The cell surface expression of CD25 was significantly attenuated by fusing it to the TLR3 link region (CD25-EG-ID, containing Glu727 to Asp499 of TLR3). Expression of CD25 on the cell surface was reduced about 4-fold by the presence of the linker region of TLR3. Removal of additional residues from either end of the TLR3 linker region abolished this partial intracellular retention (Fig. 5, B and C). Similar results with the CD25-TLR3 fusion proteins were seen in 293TCM cells and 3T3CM cells (data not shown).

We further examined whether the linker region of TLR3 targets CD25 to an intracellular compartment where TLR3 is located. Both TLR3YFP and CD25CFP or CD25-EG-IDCFP were expressed in the same BMDMs, following staining with CTXb, and their subcellular distribution was determined microscopically. As shown in Fig. 5D, the CD25CFP was clearly co-localized with CTXb, while CD25-EG-IDCFP was less co-localized with CTXb, but was strongly co-localized with TLR3YFP in the cytoplasm. Taken together, these results suggest that a part of the linker region (Glu727 to Asp499) contains an important motif responsible for targeting not only TLR3 but also a heterologous type 1 transmembrane protein to an intracellular compartment.

The Transmembrane Domain of TLR7 Is Sufficient to Mediate Intracellular Localization of a Heterologous Transmembrane Protein—To test whether the transmembrane domain of TLR7 is capable of targeting a heterologous transmembrane protein to an intracellular compartment, the transmembrane region of CD25 was replaced with that of TLR7 as shown in Fig. 6A, and the localization of the resulting chimeric CD25 molecules containing or lacking the CD25 cytoplasmic domain in BMDMs was determined. The cell surface expression of CD25 was nearly completely prevented when the transmembrane domain of TLR7 was introduced into CD25 (Fig. 6, B and C). Similar results were seen in 293TCM cells and 3T3CM cells (data not shown).

We further examined whether the transmembrane domain of TLR7 targets CD25 to an intracellular compartment where TLR7 is located. Both TLR7YFP and CD25CFP or chimera 1CFP were expressed in the same BMDMs, following staining with CTXb, and their subcellular distributions were determined microscopically. As shown in Fig. 6D, chimera 1CFP was strongly co-localized with TLR7YFP in the cytoplasm, but not co-localized with CTXb on the cell surface, suggesting that the transmembrane domain, but not the linker region or TIR domain, is responsible for targeting not only TLR7 but also other type 1 transmembrane proteins to an intracellular compartment.

Region 2 of the Transmembrane Domain of TLR7 Is the Most Important Region for Intracellular Localization of TLR7—Since no mutants of TLR4/TLR7 chimera were detected on the cell surface in a random mutagenesis approach, in which the mutation rate was about 1.6 amino acid change per sequence (Fig. 2C), the intracellular localization of...
A short sequence in the linker region between the transmembrane and TIR domains targets the TLR4/TLR3 chimera to an intracellular compartment. A, isolation of mutations in TLR4/TLR3Mu chimeras that express on the cell surface. The GFP+ TLR4/MD-2+ population gated by the oval shown was sorted twice, and then individual cells were cloned by limiting dilution. About 200 clones were isolated, and the cell surface level of the TLR4/TLR3Mu chimera in each clone was determined by flow cytometry using anti-mouse TLR4/MD-2 antibody. Sixty-five clones were selected for further analysis. The flow cytometric data of clone no. 89 is shown as a representative profile of an individual clone. Total RNA was isolated from each clone and reverse transcribed. The cDNA encoding the transmembrane and cytoplasmic domains of mutant TLR3 was amplified by PCR, and the mutations were determined by DNA sequencing. B, mutations and amino acid sequences of the cell surface TLR4/TLR3Mu chimeras. The nucleotide positions of the mutations are based on starting with the 5′-open reading frame of the full-length TLR3. STOP means that the mutation resulted in a novel termination codon. DL and INS stand for “deletion” and “insertion” of nucleotides, respectively. The stars show introduced termination codons of the mutant chimeras. C, analysis of TLR4/TLR3 truncation mutant chimeras lacking the TIR domain and variable parts of the juxtamembrane linker region. Several truncated forms of the TLR4/TLR3 chimera were created by site-directed mutagenesis and expressed in BMDMs from C57BL/10ScN mice. The subcellular distribution of those chimeras was determined as described in the legend to Fig. 1. B. Gray, isotype control; line, anti-TLR4/MD-2 antibody.
TLR7 is probably not dependent on a single amino acid residue in its transmembrane domain. Therefore, we examined whether a subregion within the transmembrane domain is responsible for the intracellular localization of TLR7. The TLR4/TLR7 chimera in which most of the cytoplasmic region had been removed (TLR4/TLR7TM) was used, and subregions within the TLR7 transmembrane domain were replaced by corresponding regions of TLR3 (Fig. 7A). The resulting swap mutants were expressed in BMDMs, and their subcellular distributions were determined by flow cytometric analysis using anti-mouse TLR4/MD-2 antibody. Consistent with the data in Figs. 3 and 4, the TLR4/TLR3TM chimera, which lacked a linker region and TIR domain, was detected on the cell surface, while the TLR4/TLR7TM chimera was completely localized intracellularly (Fig. 7B). Interestingly, the replacement of Region 2 of TLR7 with the corresponding region of TLR3 restored the cell surface expression of TLR4/TLR7TM chimera to about 50% of that seen in the TLR4/TLR3TM chimera (Fig. 7B). The effect of Region 2 on intracellular localization was greater than that of the other regions tested. Similar results were seen in 293TCM cells (data not shown).

These results suggest that Region 2 may be the most important for targeting TLR7 to an intracellular compartment, although other regions contribute to some degree.

**TLR7 Co-localizes with TLR7 in Cytoplasmic Membranes**—Since TLR3 and TLR7 appeared to be targeted to intracellular compartments by distinct regulatory elements, e.g. the linker region of the former and transmembrane region of the latter, we next examined whether TLR3 and TLR7 reside in the same intracellular compartments or different compartments. Both TLR3YFP and TLR7CFF were expressed in BMDMs, and their subcellular localization was examined by deconvolution microscopy. As shown in Fig. 8, TLR3 strongly co-localized with TLR7, suggesting that the distinct regulatory elements of these two proteins lead TLR3 and TLR7 to the same intracellular compartment.

**Both TLR3 and TLR7 Were Preferentially Localized Near Phagosomes Containing Apoptotic Cell Particles**—TLR3 and TLR7 have been shown to recognize viral nucleic acids, such as double- and single-stranded RNA, respectively (11, 14, 15). These nucleic acids may become available for recognition in phagocytes, such as macrophages and dendritic cells, when they take up virus-infected apoptotic cells and after initial digestion of the apoptotic cells by lysosomal hydrolases in intracellular acidic compartments, such as phagosomes. Therefore, we next examined whether TLR3 and TLR7 were recruited to phagosomes containing apoptotic cells. 7-AAD-labeled apoptotic T cells were added to BMDMs expressing TLR3YFP or TLR7YFP, and the localization of TLRs and apoptotic cell particles was examined microscopically. TLR3 was preferentially localized at the periphery of phagosomes containing apoptotic cell-derived particles (Fig. 9A). The particles with weaker signal of 7-AAD and stronger signal of TLR3YFP may have been taken up by macrophages earlier. Similarly to TLR3, TLR7 was localized near phagosomes containing cell particles in most cells (Fig. 9B, the left cell). These data suggest that intracellular compartments containing TLR3 and TLR7 may fuse to phagosomes.

**Figure 4.** Neither the linker region nor the TIR domain is necessary for targeting the TLR4/TLR7 chimera to an intracellular compartment. A, constructs of various truncated mutants of the TLR4/TLR7 chimera created by site-directed mutagenesis. B, intracellular localization of mutants of TLR4/TLR7 chimeras. Truncated forms of the TLR4/TLR7 chimera were created by site-directed mutagenesis, as shown in A, and the subcellular distribution of those chimeras was determined as described in the legend to Fig. 18.
DISCUSSION

All known mammalian TLRs have primary structures indicating that they are typical type I transmembrane proteins containing an NH₂-terminal signal peptide, an extracellular region consisting mostly of leucine-rich repeats, a single transmembrane region, and a cytoplasmic region largely made up of the TIR signaling domain. Whereas some TLRs are expressed on the cell surface, surprisingly, data obtained with chimeric receptor approaches (4, 9), fluorescently labeled TLRs (8), and some anti-TLR antibodies (6, 8) have indicated that TLR3, TLR7, and TLR9 are localized intracellularly, and are not detected on the cell surface. Here we present studies aimed at characterizing the regions of TLR3 and TLR7 that mediate the intracellular localization of these two TLRs: the cytoplasmic linker region for TLR3 and the transmembrane domain for TLR7. Interestingly, these distinctive localization signals target TLR3 and TLR7 to the same intracellular compartments and possibly also direct these TLRs adjacent to phagosomes containing apoptotic cells, where these TLRs may access their ligands.

Our previous results using a TLR chimeric receptor approach suggested that the transmembrane and/or cytoplasmic domain of TLRs may define both their subcellular distribution and their signaling properties (4). Although type I transmembrane proteins usually enter the secretory pathway in the ER, and are eventually translocated to the plasma membrane, some type I transmembrane proteins are retained in intracellular compartments, such as the ER or the Golgi apparatus (17). These intracellular type I transmembrane proteins typically possess intracellular retention signals such as the H/KEDL and di-lysine motifs.
which are located at the COOH terminus of the protein (17). Our studies indicate that neither TLR3 nor TLR7 are targeted to intracellular membranes by any of these well characterized targeting motifs and moreover that these two molecules have distinct targeting motifs that nonetheless target these TLRs to the same intracellular compartment. In the case of TLR3, we were able to narrow the targeting signal down to a 23-amino acid region from E727 to D749 in the linker region between the transmembrane domain and the TIR domain. This 23-amino acid region was able to confer a substantial degree of intracellular localization to the heterologous type I transmembrane protein CD25, with cell surface expression reduced by \( \frac{1}{4} \) fold. Furthermore, this region targeted CD25 to an intracellular compartment where TLR3 was located. Shortening either end of this motif substantially abolished its function in intracellular retention of CD25, and, in the case of COOH-terminal truncations, in the retention of the TLR4/TLR3 chimera. Thus, this 23-amino acid sequence may be the minimum requirement for targeting TLR3 to an intracellular compartment. This 23-amino acid sequence contains residues recently identified as important for TLR3 intracellular localization by Funami et al. (5). They mutated in pairs Arg740-Ile741, Phe745-Lys746, or Glu747-Ile748 in human TLR3 lacking the TIR domain (TLR3ΔTIR) to alanines and found that the resulting mutated TLR3 molecules were mislocalized to the cell surface in HEK293 cells and Ba/F3 cells. Surprisingly, they found that critical residues for intracellular localization of TLR3ΔTIR in 293T cells were different from those in Ba/F3 cells, suggesting the existence of different sorting mechanisms in those cells. We did not observe any differences in cell surface expression of TLR4/TLR3 mutant chimeras between 293T/CD14/MD-2 cells, 3T3/CD14/MD-2 cells, and BMDMs, indicating that the same sequence is important in these different types of cells.

Some transmembrane proteins have multiple different intracellular targeting motifs. For example, the intracellular localization of the cation-dependent mannose 6-phosphate receptor (CD-M6PR) is controlled by three different motifs in its cytoplasmic domain (18–20). CD-M6PR functions to transport newly synthesized acid hydrolases from the trans-Golgi network to an acidified endosomal compartment. CD-M6PR binds M6P-modified hydrolases in the trans-Golgi network and then moves with them to late endosomes, where it releases its M6P-hydrolase cargo and returns to the Golgi to repeat the process or alternatively moves to the plasma membrane where it is rapidly internalized via clathrin-coated vesicles (21, 22). A di-leucine motif (Leu256-Leu257) near its carboxyl terminus is required for efficient entry into Golgi clathrin-coated pits (19). Two other signals, Phe224-X-X-X-Phe229 and Tyr256-X-Val259, mediate the rapid internalization at the plasma membrane (18). We found that the 23-amino acids sequence (Glu727 to Asp749) in the linker region of TLR3 is sufficient to mediate intracellular retention both of the TLR4/TLR3 chimera and of the heterologous type...
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I transmembrane protein CD25. Since a Y-X-X-V motif is present in this sequence, we tested whether TLR3, like CD-M6PR, is first localized on the plasma membrane and then immediately endocytosed in a clathrin-dependent manner. PE-anti-TLR4/MD-2 antibody was added to BMDMs expressing the TLR4/TLR3 chimera, the cells were incubated for 2 h at 37 °C, and then the level of endocytosed TLR4/TLR3 chimera was assessed by flow cytometry. However, no endocytosis of the TLR4/TLR3 chimera was detected by this method (data not shown), suggesting that TLR3 is not routed to the plasma membrane, even transiently. CD-M6PR has been shown to move between the trans-Golgi network and endosomes, while TLR3 selectively accumulated in multivesicular bodies-like vesicles (6), but was not co-localized with any particular markers for cytoplasmic organelles including endosomes (5). Thus, the mechanism for the intracellular localization of TLR3 appears to be different from that for the CD-M6PR. It should be noted, however, that although we have identified regions of TLR3 and TLR7 that are sufficient for intracellular targeting, we cannot at this time rule out the presence of additional targeting motifs in these molecules.

In contrast to the situation with TLR3, we found that TLR7 is localized to intracellular membranes by its transmembrane domain. Truncations of the TLR4/TLR7 chimera removing almost the entire intracellular domain were still retained intracellularly, and moreover, the TLR7 transmembrane domain was able to target another type I transmembrane protein CD25 to an intracellular compartment, where the targeted CD25 strongly co-localized with TLR7. It has been suggested that some type II transmembrane proteins, such as β-galactoside α2,6-
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Molecular mechanisms of how signal sequences in transmembrane domains are recognized in the lipid bilayer, however, are largely unknown, and further biochemical characterization is needed.

TLR7, TLR8, and TLR9 are highly related in primary sequence and therefore constitute a TLR9 subfamily of Toll-like receptors (29). It was recently reported that TLR9 is localized to the ER (8). In addition, our previous results suggest that TLR8 is also mainly but not completely localized in an intracellular compartment (4). Since TLR8 and TLR9 have transmembrane domains that are similar to that of TLR7, they might also be targeted to an intracellular compartment such as the ER by their transmembrane domains.

When macrophages expressing YFP-tagged TLR3 or TLR7 were exposed to apoptotic cells, there was preferential localization of TLR3 and TLR7 adjacent to the phagocytosed apoptotic cells. During a virus infection, phagocytes take up apoptotic infected cells, and the action of lysosomal hydrolyses on the apoptotic cells presumably causes release of virus-derived RNAs, which are ligands for TLR3 and TLR7. Therefore, cell biological mechanisms for delivering TLR3 and TLR7 to the membranes of phagosomes containing apoptotic cells would likely be important for the function of these innate immune receptors. Our imaging studies are consistent with this hypothesis, as are findings that inhibitors of endosome and phagosome maturation, such as chloroquine and brefeldin A, block cellular responses to known ligands of TLR3 and TLR7 (5, 7). Although the mechanism by which these inhibitors block the signaling initiated by these intracellular TLRs is unknown, they might in some way block the fusion of intracellular vesicles containing these TLRs with endosomes or phagosomes containing the added ligands.

The localization of different TLRs seems to be optimized for the recognition of their ligands. TLRs whose ligands are likely to be present outside host cells, such as those recognizing bacterial cell wall components, are localized on the cell surface, whereas TLRs whose ligands are released inside host cells, such as those recognizing nucleic acids, are localized intracellularly. Here we demonstrate that the localization of intracellular TLRs may be differentially controlled by distinct regulatory elements found either in the linker region between the transmembrane domain and the TIR domain or in the transmembrane region itself. Those elements lead TLRs to functionally useful positions in cells, where TLRs may access their ligands and induce responses for host defense.

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