Cytoplasmic Domain-mediated Dimerizations of Toll-like Receptor 4 Observed by β-Lactamase Enzyme Fragment Complementation*

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Toll-like receptors (TLRs) detect the presence of microbial challenge and initiate innate immune defensive responses. In this work we have explored the mechanism and role of TLR dimerization in signal transduction using the newly developed technique of β-lactamase protein fragment complementation, among others. We observed that TLR4 interactions with itself, with MyD88, or with TLR2 were accurately reported by the enzyme complementation technique. That technique, as well as co-immunoprecipitation, transfection-initiated cell activation, and site-directed mutagenesis all suggest an important role for TLR intracellular domains in receptor dimerization. These findings broaden our understanding of TLR self-interactions as well as heterodimer formation.

The recent discovery of Toll-like receptors (TLRs) has provided many insights into innate immune recognition of microbial challenge. To date there are ten different TLRs in humans, and ligands for most of the TLRs have been identified (1). Some TLRs function as homo- or heterodimers. For example, TLR2 activity is enhanced by heterodimerization with TLR1 or TLR6 while TLR4 may form homodimers (2–5). Recent studies also show that dectin-1 plays a synergistic role with TLR2 in macrophage and dendritic cell activation by β-glucan-containing particles (6, 7). Important functional interactions of TLRs with non-TLR molecules are well known, for example, TLR4 with MD-2 and CD14. More of these interactions probably remain to be discovered. TLR activation leads to a cascade of intracellular signal propagation events ultimately leading to production of various cytokines. This signal propagation involves recruitment of cellular adaptor molecules for TLRs such as MyD88, TIRAP/MAL, and TRIF/TICAM-1 as well as kinases including IRAKs and MAPKs (reviewed in Refs. 8 and 9). TLRs are known to bind the intracellular adaptor molecules via the Toll/interleukin-1 receptor (TIR) domains, which are present in both TLRs and adaptors, by homotypic protein-protein interactions. Some of these interactions may also be mediated by JNK-interacting protein 3 (10). Recently, the crystal structure of the TIR domains of human TLR1 and TLR2 was elucidated (11). Interestingly, the Lps2 allele, in which the proline at 712 in TLR4 is substituted with histidine (12), does not clearly disturb the TIR domain structure but was hypothesized to abrogate receptor function by interrupting interactions with signaling adaptor molecules (11).

We have been particularly interested to understand mechanisms of TLR receptor oligomerization because we hypothesize that these interactions are crucial to initiation of intracellular signaling. Previously we showed that TLR2 oligomerization with TLRs 1 or 6 was ligand-independent and that ligand binding altered subunit interaction, thereby leading to transmembrane signal propagation (5). Others have come to similar conclusions (2, 3). Ozinsky et al. (2) concluded that TLR2 and TLR6 dimerization was controlled by their extracellular domains. In this work, we utilize a novel approach to the mechanism of TLR receptor oligomerization and conclude that intracellular domains are important for receptor oligomerization as well.

The protein-protein interactions between TLRs and their adaptor molecules have generally been tested by in vitro immunoprecipitation analysis, whereas the protein complementation assay (PCA) is a novel, recently developed method to detect protein-protein interactions in vitro (13). One of the PCAs, which is based on the use of TEM-1 β-lactamase from Escherichia coli, has been successfully used for studying the rapamycin-inducible protein interaction between FK506-binding protein 12 and FKBP-rapamycin binding domain of FKBP-rapamycin-associated protein as well as constitutive protein-protein interactions (14, 15). The strategy of this TEM-1 β-lactamase-utilizing PCA is to mediate the complementation of two partial β-lactamase fragments by two proteins conjugated respectively with the enzyme fragments. The complementation is then detected by an intracellular reporter substrate (CCF-2) whose fluorescence properties change after hydrolysis by the β-lactamase (14, 15).

In this study, we describe a successful use of the β-lactamase PCA with TLRs, showing type I transmembrane protein interactions by this method for the first time. Using this assay, we detected in vivo TLR interactions mediated by the cytoplasmic domains of TLRs.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Lipopolysaccharide (LPS) from E. coli serotype O111:B4 was purchased from List Biological Laboratories (Campbell, CA) and re-purified as previously described (16, 17). Macrophage-activating lipopeptide-2 (MALP-2) used in this study was previously described (17, 18). Anti-FLAG M2 antibody was obtained from Sigma-Aldrich and anti-HA.11 antibody was obtained from Covance-Berkeley Antibody Company (Richmond, CA). Anti-human TLR9 monoclonal antibody (clone 26C593) was obtained from Imgenex (San Diego, CA).

DNA Constructs and Site-directed Mutagenesis—According to previous studies (14, 15), two β-lactamase gene fragments, Bla(a) and Bla(b), were amplified by PCR using the vector pQE32 as a template. The
primers used were the forward primers 5'-GAA GGA CAA CAA-3' for Bla(a) and the reverse primers 5'-GAA TTC GAT ATC TAT-3' for Bla(b), which consist of amino acids 26–196 and 198–290, respectively, and were fused with TLR or MyD88 by a (Gly4Ser)3 linker. 

**FIG. 1.** The *in vivo* β-lactamase complementation assay strategy. A, β-lactamase fragment fusion constructs. Two β-lactamase enzyme fragments, Bla(a) and Bla(b), which consist of amino acids 26–196 and 198–290, respectively, were fused with TLR or MyD88 by a (Gly4Ser)3 linker. B, schematic representation of the *in vivo* β-lactamase PCA. HEK293 cells transfected with a pair of TLR-Bla(a)/TLR-Bla(b) or MyD88-Bla(a)/TLR-Bla(b) are loaded with CCF2/AM. CCF2/AM diffuses across the cell membrane, and the cytoplasmic esterases hydrolyze its ester functionalities (Ac, acetyl; Bt, butyryl; AM, acetoxymethyl), releasing the β-lactamase substrate CCF2. Excitation of the coumarin donor in CCF2 at 409 nm leads to FRET to the fluorescein acceptor generating emission of green fluorescence at 520 nm. The TLR/TLR or TLR-MyD88 interactions bring two β-lactamase fragments into proximity resulting in a complete form of β-lactamase. This β-lactamase further hydrolyzes CCF2 and separates donor and acceptor leading to FRET disruption. Instead, the isolated coumarin donor emits blue fluorescence at 447 nm.
followed by a flexible linker, (Gly-Ser), on the 5’-end and an EcoRI site on the 3’-end. Human TLR2 or TLR4 cDNA without a stop codon was cloned into the vector pFLAG-CMV1 (Sigma-Aldrich) linearized with KpnI/BamHI (TLR4) or NotI/BamHI (TLR2). The Bla(a) or Bla(b) gene fragment was then subcloned into the pFLAG-CMV1-TLR plasmids linearized with BamHI/SmaI. This led to the formation of the TLR-linker-Bla(a) or Bla(b) constructs. Similarly, MyD88 and the death domain (amino acids 19–110) of MyD88 were PCR-amplified and cloned into the pR5K(FA), vector (a gift from Dr. E. Li, The Scripps Research Institute) followed by subcloning with the Bla(a) or Bla(b) gene fragment. The chimeric constructs, T2N-T4C and T4N-T2C, were made by a combination of PCR amplification and conjugation, and cloned into the pFLAG-CMV1 vector. The TLR-PH mutants, in which the proline residue at position 714 of hTLR4, corresponding to the proline at 712 in the mouse TLR4 or 618 in hTLR2, was modified Eagle’s medium (Invitrogen) with 0.05% serum, and 1% nonidet P40, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing protease inhibitor cocktails (Roche Applied Science). The supernatants were incubated with anti-FLAG or anti-HA antibodies for 2 h at 4°C. Protein A-agarose beads were then added to the mixtures and further incubated for 6 h at 4°C. The mixtures were washed five times with lysis buffer, separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to nitrocellulose membrane. Western blotting was performed by probing the membrane with either anti-FLAG or anti-HA antibodies according to standard protocols.

RESULTS

Expression and Function of TLR4- and TLR2-β-Lactamase Fusion Constructs—The β-lactamase fusion constructs were made with TLR4 and TLR2 as well as the cytoplasmic proline-histidine (PH) mutants of TLR4 and TLR2. Each construct was expressed in HEK293 cells, and the proteins expressed were immunoprecipitated. The expression level of each construct was similar (Fig. 3A), all the PH mutants exhibited lower activity than that of intact TLR4 and TLR2. Each construct was used to test the β-lactamase activity by FACS analysis (Fig. 2B) and the proportion of β-lactamase-positive cells was quantified by FACS analysis (Fig. 2C). These interactions were further tested by in vitro immunoprecipitation. As expected, TLR4 was co-immunoprecipitated only with wild-type MyD88 but not with DD[MyD88]-Blas(a) or DD[MyD88]-Blas(b) were transiently cotransfected in HEK293 cells, the constructs interacted productively as shown by the fluorescence of blue fluorescin. Conversely, only TLR4-Blas(b) interacted productively with MyD88-Blas(a) but not with DD[MyD88]-Blas(a) (Fig. 2A). The β-lactamase activity was quantified by FACS analysis (Fig. 2B) and the proportion of β-lactamase-positive cells was similar (Fig. 2C). These interactions were further tested by in vitro immunoprecipitation. As expected, TLR4 was co-immunoprecipitated only with wild-type MyD88 but not with DD[MyD88] and MyD88 was co-immunoprecipitated with both full-length MyD88 and DD[MyD88] (Fig. 2D).

In Vivo Detection of TLR4-TLR4 and TLR4-TLR2 Dimerization by β-Lactamase PCA—When TLR4-Blas(a) and TLR4-Blas(b) were transiently cotransfected in HEK293 cells, the overexpressed TLR4 spontaneously formed homodimers and resulted in β-lactamase complementation as analyzed by FACS (Fig. 4A). Interestingly, when TLR4 constructs were cotransfected with TLR2 construct, TLR4 also formed heterodimers (Fig. 4A). The ratio of β-lactamase-positive cells analyzed by FACS was compared (Fig. 4B). Surprisingly, the dimerization of PH mutants was significantly affected by the point mutation. The homodimerization of the TLR4-PH-4PH pair was almost completely abolished while the homodimerization of either pair of wild-type TLR4 and TLR4-PH was significantly affected by the point mutation.
mutant was partially diminished. Similarly, the heterodimerization between TLR4PH and TLR2PH was also significantly diminished while the heterodimerization of either pair of TLR2-TLR4PH or TLR2PH-TLR4 was partially diminished (Fig. 4). The co-immunoprecipitation was further performed to test the \textit{in vitro} TLR-2LR interactions using the cotransfected

Fig. 2. \textit{The in vivo constitutive protein-protein interaction of the overexpressed TLR4 and MyD88 tested by $\beta$-lactamase PCA.} \textit{A, in vivo} $\beta$-lactamase complementation assay. HEK293 cells were transiently cotransfected with different combinations of the $\beta$-lactamase fusion constructs (0.5 $\mu$g/ml) as noted and loaded with CCF2/AM. The cells were then analyzed by fluorescence microscopy. \textit{B and C,} FACS analysis of $\beta$-lactamase activity. The $\beta$-lactamase-positive cells were observed in the upper left quadrant (B) and the percent population of $\beta$-lactamase-positive cells was compared (C). \textit{D,} co-immunoprecipitation of TLR4 and MyD88. HEK293 cells were transiently cotransfected with different combinations of the $\beta$-lactamase fusion constructs (0.5 $\mu$g/ml) as noted. After 48 h, cells were lysed and immunoprecipitated with anti-FLAG antibody. Immunoblotting was performed with anti-FLAG or anti-HA antibody.
HEK293 cells with wild-type or mutant constructs of TLR2 and TLR4 (Fig. 5A). Interestingly, only the mutant pairs of TLR4PH-TLR4PH or TLR4PH-TLR2PH were not co-immunoprecipitated each other (Fig. 5A, middle) while the expression level of all the proteins was similar (Fig. 5A, top and bottom). Furthermore, in a comparative co-immunoprecipitation of TLR4 versus TLR2 or TLR9, TLR4 was co-immunoprecipitated only with TLR2 but not with TLR9, implying that TLR4 specifically interacts with TLR2 (Fig. 5B). Because the Pro → His mutation is in the intracellular domains and blocks dimerization, these results imply an important role for the intracellular domain in TLR4 association with itself or TLR2.

**Cytoplasmic Domain-dependent TLR4 Constitutive Activity**—The overexpression of TLR4 highly activates HEK293 cells and this ligand-independent constitutive activation leads to NF-κB induction. Since the cytoplasmic domain of TLR4 seems to be important in its spontaneous homodimerization, we hypothesized that the cytoplasmic domain of TLR4 is responsible for its constitutive activity in HEK293 cells. Thus, we made chimeric constructs using TLR4 and TLR2 (Fig. 6A). Each construct was efficiently expressed at a similar level (Fig. 6B). Interestingly, only the TLR4 cytoplasmic domain-containing...
FIG. 4. *In vivo* homo- and heterodimerization of TLR4 determined by the β-lactamase PCA. A and B, *in vivo* β-lactamase complementation analyzed by FACS. HEK293 cells were transiently cotransfected with different combinations of the TLR-Bla fusion constructs (0.5 μg/ml) as noted and loaded with CCF2/AM. The cells were then analyzed by FACS. The β-lactamase-positive cells were observed in the upper left quadrant (A) and the percent population of β-lactamase-positive cells was compared (B).
constructs, T2N-T4C and T4C, exhibited constitutive activity as did wild-type TLR4. In contrast, wild-type TLR2 and the construct T4N-T2C did not show constitutive activity (Fig. 6C).

The cytoplasmic domain of TLR4 was co-immunoprecipitated with wild-type TLR4 (Fig. 7A). In the in vivo β-lactamase complementation assay, the TLR4 cytoplasmic domain formed dimers with the cytoplasmic domain itself as well as with wild-type TLR4 (Fig. 7B). Not surprisingly, the cytoplasmic domain of TLR4 interacted with wild-type MyD88 but not with the death domain of MyD88 (Fig. 7B). These results correspond with the results observed in the in vivo homodimerization of TLR4 (Fig. 4) and indicate that the cytoplasmic domain-mediated spontaneous dimerization of TLR4 is responsible for the ligand-independent TLR4 constitutive activation.

β-Lactamase Reconstitution in HeLa cells—To ensure that the associations shown in Fig. 7B were generalizable to other cellular systems and to ensure that these events were not dependent on HEK293 cell activation in response to TLR4 transfection as in Fig. 6, we repeated the PCA experiments of Fig. 7B in HeLa cells. In these cells, transfected TLR4 is quiescent. As shown in Fig. 7C, the same pattern of association events seen in HEK293 cells was observed in HeLa cells.

**Constitutive TLR4 Dimerization Enhanced by MD-2**—MD-2 physically binds to the extracellular portion of TLR4 (21), and this interaction is also shown in Fig. 8E in which MD-2 only interacts with intact TLR4 but not with the ECD-deleted mutant (T4C) or TLR1 (Fig. 8E). Together with CD14, MD-2 is required for LPS-mediated cell signaling via TLR4. Since the LPS-mediated TLR4 activation occurred only in the presence of MD-2, we wondered if the ligand-independent TLR4 constitutive activation could be enhanced by MD-2. In addition to MD-2, CD14, or MD-1 was cotransfected with TLR4. MD-2 significantly enhanced TLR4 activation whereas CD14 and MD-1 did not help the activation (Fig. 8A). Because MD-2 interacted with the TLR4 extracellular portion (Ref. 21 and Fig. 8E), we questioned if MD-2 could also enhance the consti-
The constitutive activities of the chimeric constructs, T2N-T4C and T4C, which are free of the extracellular domain of TLR4. Not surprisingly, MD-2 did not enhance either T2N-T4C or T4C activity (Fig. 8B). The addition of LPS further increased TLR4 activation in the presence of MD-2 and CD14 (Fig. 8C). The MD-2-enhanced TLR4 dimerization was further tested using in vivo lactamase complementation. Note that the amounts of TLR4-Bla constructs used in Fig. 8D was 10-fold less than that used in Figs. 2, A and B and 4A. In this experiment, the in vivo TLR4 dimerization was significantly enhanced by MD-2 in a dose-dependent manner (Fig. 8D). Taken together, these observations demonstrate that TLR4 dimerization occurs via its cytoplasmic domain and the dimerization is enhanced by binding of MD-2 to the extracellular domain of TLR4.

DISCUSSION

These data definitively show the utility of the β-lactamase PCA for studies of TLR and TLR-adaptor molecule interactions. They also show that interactions between TLRs may be mediated by intracellular domains. The latter finding suggests that published work ascribing TLR heterodimer formation to extracellular domain interactions (2) is not a fully general finding.

The β-lactamase PCA is a newly developed method. Its utility has been tested for intracellular proteins such as apoptotic proteins Bcl2 and Bad as well as homodimerization of Smad3, producing successful in vivo protein-protein interactions (15). In our study, the utility of the β-lactamase PCA was focused on the interactions between type 1 transmembrane proteins and cellular proteins as well as dimerization of transmembrane proteins, which was not previously shown by this PCA. This first was validated by testing interactions of MyD88 with TLR. Burns et al. (19) first showed homodimerization of MyD88 and the involvement of the MyD88 death domain (in addition to the TIR domain) in this dimerization. Our data in Fig. 2 again show MyD88 dimerization through the death domain by immunoprecipitation as shown by Burns et al. and confirm it newly by the use of the β-lactamase PCA. The PCA also demonstrates the well known association of TLR4 with MyD88 and that this is not mediated by the MyD88 death domain. To our knowledge, self-association of TLR4, without the mediation of CD4 or integrin interacting domains (2, 4), has not previously been shown. Co-immunoprecipitation demonstrates the self-association of TLR4 (Fig. 5) and the β-lactamase PCA also demonstrates it (Fig. 4). To our sur-
prise, the PCA also demonstrated an association between TLR4 and TLR2 (Fig. 4), verified by co-immunoprecipitation (Fig. 5). While autoactivation of TLR4 transfected HEK293 cells suggests that TLR4 might self-associate, this self-association is not dependent on autoactivation, since it is seen also in quiescent HeLa cells (Fig. 7). The association of TLR4 with TLR2 is unprecedented since there are no ligands known which require the co-presence of TLR4 and TLR2 for cellular activation. The hypothesis of Ozinsky et al. (2) that novel specificities can be formed by heterologous dimerization of TLRs suggests that some novel ligand may yet be found for such a TLR pair. While TLR4 self-association and TLR4-TLR2 association could be due to extracellular domain interactions as suggested for TLR2-TLR6 (2), blocking these associations by the Pro\textsuperscript{3}His mutation of the Lpsd allele clearly points to an important role for intracellular domain interactions. While the PCA data show only that the mutation blocks the associative reactions of the lactamase fragments, the immunoprecipitation data clearly show that the Pro → His mutation actually does block the TLR interactions. The correlation between loss of activity (Fig. 3, B and C) and loss of association (Figs. 4 and 5) caused by the Pro → His mutation suggests that dimerization is required for signaling.

Because the data indicate an important role for the intracellular domains in subunit dimerization, we further studied intracellular domain function by activity (Fig. 6), co-immunoprecipitation, and PCA activation (Fig. 7). Fig. 6 shows that the characteristic autoactivation effects of transfection of HEK293 cells with TLR4 as opposed to TLR2 are carried by the intracellular domains of these two TLRs. Fig. 7 shows that the intracellular domain of TLR4 binds to intact TLR4 and that it will carry a lactamase fragment into a productive interaction with the complementary lactamase fragment linked to either TLR4 or MyD88, as long as the MyD88 is intact. Enhanced self-association of TLR4 by coexpression of MD-2, but not of CD14 or MD-1, was shown by both enhanced autoactivation as well as enhanced β-lactamase reconstitution. These data suggest that the presence of MD-2 can enhance the intracellular domain initiated self-association of TLR4 monomers, and, at least in part, confirm the data of da Silva Correia and Ulevitch (22).

We have previously used an alternative PCA based on dihydrofolate reductase (DHFR) to show that TLR2 ligands induce a conformational change in TLR2 heterodimers associated with
signal transduction (5). In those experiments, activation of the PCA required ligand although TLR2 was associated with TLR6 or TLR1 in the absence of ligand (5). In the present instance, with the β-lactamase PCA, complementation occurs independently of ligand although the enzyme complementation induced by TLR4 homodimerization was significantly enhanced by MD-2. Why the two PCA should report different events is not yet clear but may reflect functional differences between TLR2 and TLR4. We are currently testing modifications of the β-lactamase PCA to determine if subtle, and some not so subtle, changes in the linker will make the β-lactamase PCA-sensitive to the presence of TLR ligands.

**FIG. 8.** Constitutive TLR4 dimerization enhanced by MD-2 but not by CD14. A, constitutive TLR4 activity enhanced by MD-2. HEK293 cells were transiently transfected with control vector (0.1 μg/ml), TLR4 plasmid (0.1 μg/ml) or TLR4 plasmid plus increasing amounts (0.01, 0.05, 0.1 and 0.5 μg/ml, respectively) of MD-2, CD14, MD-2/CD14, or MD-1 plasmid. Each sample was cotransfected with pNF-κB-Luc and pSV-β-galactosidase vectors. After 24 h, luciferase activity was measured. *, p < 0.05 versus TLR4 alone (Mann Whitney U test). B, TLR4 ECD-dependent MD-2 enhancement for TLR4 activity. HEK293 cells were transiently transfected and analyzed for luciferase activity as described in A except for using T2N-T4C plasmid (0.05 μg/ml), T4C plasmid (0.1 μg/ml) and increasing amounts (0.01, 0.05, 0.1 and 0.5 μg/ml) of MD-2 plasmid. C, LPS effect on TLR4 activity. HEK293 cells were transiently transfected and analyzed for luciferase activity as described in A except for stimulating cells with LPS (0.1 μg/ml) for 6 h after 24-hour transfection. All the luciferase activity was normalized with β-galactosidase activity. Results are shown as the mean ± S.D., and determinations were made in triplicate. D, in vivo detection of TLR4 dimerization enhanced by MD-2. HEK293 cells were transiently transfected with a pair of TLR4-Bla(a) and TLR4-Bla(b) (0.05 μg/ml, respectively) or plus increasing amounts (0.1, 0.5 and 1.0 μg/ml) of MD-2 plasmids. After 24 h, cells were loaded with CCF2/AM. The percent population of β-lactamase-positive cells was compared. E, co-immunoprecipitation of MD-2 and TLR4. HEK293 cells were transiently cotransfected with MD-2 (0.5 μg/ml) plus TLR construct (0.5 μg/ml) as noted. After 48 h, cells were lysed and immunoprecipitated with anti-FLAG antibody. Immunoblotting was performed with anti-FLAG or anti-HA antibody.
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