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Cutting Edge: Differential Inhibition of TLR Signaling Pathways by Cell-Permeable Peptides Representing BB Loops of TLRs

Vladimir Y. Toshchakov,* Matthew J. Fenton,2† and Stefanie N. Vogel3*

We designed cell-penetrating peptides comprised of the translocating segment of Drosophila antennapedia homeodomain fused with BB loop sequences of TLR2, TLR4, and TLR1/6. TLR2- and TLR4-BB peptides (BBPs) inhibited NF-κB translocation and early IL-1β mRNA expression induced by LPS, and the lipopeptides S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lysx4-OH (P3C) and S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-Cys-Ser-Lysx4-OH (P2C). TLR4- and TLR2-BBPs also strongly inhibited LPS-induced activation of ERK. Only TLR2-BBP significantly inhibited ERK activation induced by P3C, which acts via TLR2/1 heterodimers. BBPs did not inhibit activation of ERK induced by P2C, a TLR2/6 agonist. The TLR2-BBP induced weak activation of p38, but not ERK or cytokine mRNA. The TLR1/6-BBP failed to inhibit NF-κB or MAPK activation induced by any agonist. Our results suggest that the receptor BBPs selectively affect different TLR signaling pathways, and that the BB loops of TLR1/6 and TLR2 play distinct roles in formation of receptor heterodimers and recruitment of adaptor proteins. The Journal of Immunology, 2007, 178: 2655–2660.

Toll-like receptors elicit primary immune responses to a variety of microbial molecules. All TLRs are comprised of leucine-rich repeats on one side of the plasma membrane, a membrane-spanning α-helix, and a cytosolic region that contains a Toll/IL-1 resistance (TIR)4 domain (1, 2). TIR domains are also found in proteins of the IL-1R family and in TLR adaptors. TIR domains are composed of five β-strands alternating with 5 α-helices (2, 3). This pattern of secondary structure folds as a repeating αβ structure (2); the core is formed by a parallel β-sheet surrounded by five helices, with connecting loops, i.e., sequences between strands and helices that do not form regular H-bonds. An important physical feature of the TIR loops is that they exhibit the highest surface exposure among the elements of the structure.

The BB loop is a highly conserved sequence in the TIR that is situated between the second β-strand and the second helix (3). The functional importance of this region derives from the observation that mutations within it severely affect the function of TIR domain-containing proteins (reviewed in 4). In particular, a mutation within the TLR4 BB loop at proline 712 to histidine that renders C3H/HeJ mice unresponsive to LPS is situated in this region (5, 6). Importantly, it has been shown that mutations in this region severely affect function, but do not affect the overall structure of the TIR domains (3). These facts, taken together with the localization of the BB loop on the protein surface, imply that this region is an important interaction surface.

It has been postulated that TLR4 initiates signaling by forming homodimers (7), followed by recruitment of four TIR domain-containing adaptor proteins, MyD88, Toll/IL-1R domain-containing adaptor-activating IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and TIR domain-containing adaptor molecule (TIRAP/Mal) (reviewed in Ref. 4). In contrast, TLR2 forms heterodimers with TLR1 or TLR6 (7–10) and recruits MyD88 and TIRAP only (reviewed in Ref. 4). The composition and stoichiometry of the multimeric complexes assembled after stimulation of a TLR by agonist is a matter of much discussion. Dunne et al. (11) modeled the docking complexes between the TIR domains of TIRAP/Mal and MyD88 and their complexes with TLR2 or TLR4. The authors predicted that the receptors use different surfaces to bind the adaptors. They proposed that the TLR2 BB loop forms a point of contact with both adaptors, and that the adaptors may compete for the same binding site. The TLR4 BB loop was predicted to be partially required for binding TIRAP/Mal, but not MyD88; the latter is predicted to bind optimally to the side of TLR4 opposite the BB loop (11). These models also suggested that BB loops of the adaptors may not be important for direct binding to either receptor. Computational modeling of

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3 Abbreviations used in this paper: TIR, Toll/IL-1 resistance; TRAM, TRIF-related adaptor molecule; TIRAP/Mal, TIR domain-containing adaptor molecule; BBP, BB loop peptide; TAK1, TGF-β-activated kinase 1; IRAK, IL-1R-associated kinase; CP, control peptide; poly(I:C), polyinosinic:polycytidylic acid.

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the TLR2 TIR domain docking to the MyD88 TIR domain performed by Jiang et al. (12) suggested two modes of interaction of these molecules. The first, “face-to-face,” predicts that the BB loops of TLR2 and MyD88 interact with each other in an antiparallel mode. In the second model, “back-to-back,” the TIR domains interact via fifth α-helices situated on the opposite surface of the domains. Interestingly, docking of MyD88 onto IL-1R accessory protein (IL-1RαcP) modeled by Li et al. (13) suggested that the BB loop of MyD88 docks in the vicinity of the EE loop and the fifth α-helix of IL-1RαcP. This architecture is fundamentally different from those proposed by Jiang et al. (12) and, extrapolating from Jiang et al.’s terminology, would be “back-to-face.” These examples show that this theoretical approach reveals no unified model for TIR-TIR interactions. Moreover, the relevance of such modeling is compromised by the arbitrary choice of protein pairs, the inability of the method to compare the relative affinity of proteins in pairs, and the common assumption that the interactions occur between one TLR molecule and one adaptor, rather than within the context of multimeric structures.

Cell-penetrating decoy peptides represent an emerging tool that capitalizes on the ability of relatively short peptide sequences to carry diverse cargoes across membranes (reviewed in Ref. 14). Such penetrating peptides, joined with a short peptide sequence that represents an important interaction surface of a signaling protein, can act as a decoy that binds the docking site of the prototype protein and inhibits signal transduction (15). Recently, we used this approach to compare the effects of four adaptor BB loop peptides (BBPs), each joined to the translocating segment of antennapedia homeodomain, on TLR4- and TLR2-mediated signaling (16). We found that TRAM and MyD88 BBPs were highly effective inhibitors of TLR4; however, neither exerted significant inhibitory activity on TLR2-induced signaling, suggesting that the manner in which TLR2 and TLR4 interact with MyD88 differs.

In this study, we extend our earlier findings by designing cell-penetrating decoy peptides that contain the BB loops of different TLRs and assess their effects on TLR2- and TLR4-mediated signaling. We observed that TLR2- and TLR4-BBPs are effective inhibitors of LPS-induced NF-κB and a NF-κB-dependent gene, IL-1β, whereas ERK and p38 MAPK are affected differently. TLR1 and TLR6, TLRs that function as coreceptors for TLR2, have identical BB loop sequences. Despite this sequence conservation, the BBP based on this common sequence failed to inhibit TLR2- or TLR4-mediated signaling. Our experiments delineate important differences in the interaction surfaces of these TLRs and illustrate the potential of this approach for the selective inhibition of various branches of TLR signaling.

Table I. The physical-chemical properties of BBPs a

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Length</th>
<th>Number of Positively Charged Residues</th>
<th>Number of Negatively Charged Residues</th>
<th>AI b</th>
<th>Hydropathicity</th>
</tr>
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<tbody>
<tr>
<td>TLR1/6-BBP</td>
<td>RQIKIWFQNRRMKWKKLH*FVPGRSIVE</td>
<td>30</td>
<td>9</td>
<td>2</td>
<td>71.33</td>
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<tr>
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<td>RQIKIWFQNRRMKWKKLH*FVPGRK11D</td>
<td>30</td>
<td>10</td>
<td>2</td>
<td>74.67</td>
<td>−1.110</td>
</tr>
<tr>
<td>TLR4-BBP</td>
<td>RQIKIWFQNRRMKWKKLH*HYRDFPFPGVIAAAA</td>
<td>30</td>
<td>8</td>
<td>1</td>
<td>84.67</td>
<td>−0.567</td>
</tr>
</tbody>
</table>

a The physical-chemical properties of peptides were calculated using Expasy-ProtParam Tool (Swiss Institute of Bioinformatics). The properties of CP, MyD88-, and TRAM-BBPs can be found in Ref. 16.

b Al, Aliphatic index, relative volume occupied by aliphatic chains of A, V, I, L.

c The site of substitution in N/D and D/N mutants.

Materials and Methods

A detailed description of mice, cell culture of peritoneal macrophages, TLR agonists, the design, synthesis, and handling of BBPs, and other experimental details can be found in our previous report (16). Table I provides the sequences of TLR1/6, TLR2, and TLR4 BBPs and their physical-chemical properties. Phosphorylation of MEK1/2, MKK3/6, and TGF-β-activated kinase 1 (TAK1) were detected with Abs from Cell Signaling Technology. Total IL-1R-associated kinase (IRAK)1 was measured using Abs from Santa Cruz Biotechnology. Nuclear extracts were prepared using the kit from Active Motif, according to the manufacturer’s instructions. Conditions for EMSA were previously described (17). Polyinosinic: polycytidylic acid (poly(I:C)) was obtained from Amersham.

Results and Discussion

TLR2- and TLR4-BBPs, not TLR1/6-BBP, inhibit NF-κB activation by TLR4 and TLR2 agonists

We previously reported that the inhibitory action of adaptor BBPs is much stronger toward TLR4- vs TLR2-induced responses (16). Therefore, we sought to examine functional properties of BBPs that correspond to homologous regions of TIR domains of TLR4, TLR2, TLR1, and TLR6. TLR1 and TLR6 were included because both heterodimerize with TLR2 in an agonist-dependent manner. Because the BB loop sequences of TLR1 and TLR6 are identical, only one BBP was synthesized (TLR1/6-BBP).

We examined the effects of BBPs on NF-κB translocation induced by LPS, as well as by the TLR2 agonist S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys2-OH (P3C) (a TLR2/1 agonist) and S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-Cys-Ser-Lys2-OH (P2C) (a TLR2/6 agonist) (Fig. 1A). EMSA revealed that stimulation of primary murine macrophages with each agonist induced NF-κB nuclear translocation. NF-κB translocation was not affected by the control peptide (CP), a random amino acid sequence joined to the translocating segment of the antennapedia homeodomain (Ref. 16). Inhibitory BBPs, comprised of the homologous BB loop of the adaptor proteins TRAM and MyD88, blocked LPS-induced NF-κB activation but were ineffective in blocking P3C-induced activation. This observation is consistent with the failure of these BBPs to inhibit P3C-induced cytokine gene expression or MAPK phosphorylation, as reported previously (16). The TLR1/6-BBP did not inhibit activation of NF-κB induced by any agonist. In contrast, the TLR2-BBP-inhibited activation of NF-κB induced by all three agonists (Fig. 1A). Inhibition of LPS-induced translocation by the TLR4 BBP was stronger than that achieved by the TLR2-BBP, but weaker than the inhibitory effect of TLR2-BBP on TLR2-stimulated NF-κB translocation (Fig. 1A).

We next sought to confirm the effect of TLR BBPs on TLR-inducible cytokine gene expression. Fig. 1, B and C, demonstrate the effect of the TLR BBPs on induction of IL-1β and...
IFN-β mRNA by LPS, respectively. The effect of BBPs on cytokine mRNA agrees well with its effect on NF-κB translocation. Both TLR2- and TLR4-BBPs inhibited activation of IL-1β mRNA by LPS, with the TLR4-BBP being slightly more effective than the TLR2-BBP (Fig. 1B). The greater inhibitory effect of the TLR4-BBP is most obvious when the BBPs are compared at 10 μM (Fig. 1, B and C). Interestingly, induction of IFN-β, a cytokine activated by the MyD88-independent pathway (18, 19), was similarly inhibited (Fig. 1C), indicating that the TLR BBPs block an interaction upstream of this bifurcation in the signaling cascade. When cells were stimulated with P3C (TLR2/6) or P2C (TLR2/1), the TLR2-BBP also inhibited induction of IL-1β gene expression, whereas the effect of the TLR4-BBP was minimal (Fig. 1, D and E). In agreement with the inability of the TLR1/6-BBP to block NF-κB translocation, it did not inhibit cytokine gene expression induced by either P3C or P2C (Fig. 1, D and E). We also examined the effects of TLR2- and TLR4-BBPs on cytokine induction mediated by TLR3. TLR3 is the only TLR that does not use MyD88 for signaling (reviewed in Ref. 4). Induction of IFN-β mRNA by poly(I:C), a TLR3 agonist, is delayed relative to the LPS-induced mRNA due to endosomal localization of TLR3. Poly(I:C)-induced IFN-β mRNA was not measurable earlier than 2 h after stimulation, and was further increased by 3 h (Fig. 1, F and G). LPS-induced transcription peaks at 1–2 h after stimulation, then decreases dramatically (19). Poly(I:C) induced no IL-1β mRNA during the first 3 h after stimulation (data not shown). Inhibition of LPS-induced IFN-β mRNA by TLR2- and TLR4-BBPs was complete at both 2 and 3 h poststimulation (Fig. 1, F and G). The BBPs did not exert statistically significant effects on poly(I:C)-induced IFN-β mRNA (Fig. 1, F and G).

Our findings show that TLR2- and TLR4-BBPs demonstrate cross-reactivity, with each peptide being slightly more selective for its respective TLR. Such cross-reactivity most likely originates from homology between these BB loops; the BB loops of TLR4 and TLR2 share eight identical amino acids. Homology, together with similarity of inhibitory effects, suggests that these sequences may share overlapping targets, yet the higher degree of specificity of these BBPs toward their prototype pathway suggests that their targets may not be identical. The finding that the TLR2- and TLR4-BBPs failed to inhibit TLR3 may suggest that these regions of TLR2 and -4 may be important for binding MyD88 or TIRAP, common adaptors between them, and not involved in TLR3 signaling. However, the possibility that these loops are important for receptor dimerization cannot be excluded. BB loops of TLR4 and TLR3 are less similar than TLR4 and TLR2; they share only five identical amino acids, with proline 712 of TLR4 not conserved in TLR3. This dissimilarity may provide structural bases for the relative inefficiency of the TLR2- and TLR4-BBPs toward TLR3 signaling.

The inability of the TLR1/6-BBP to block activation suggests that the heterodimer of TLR2 with TLR1 or TLR6 is not symmetrical, i.e., BB loops of the coreceptors are not equally important for heterodimer formation and/or recruiting adaptors. Lee et al. (20) previously showed that it is necessary to mutate both BB loops in a pair to eliminate the TIR-TIR binding completely. This finding was true for TLR4 homodimerization and for heterodimerization of TLR4 with TLR2. The fact that receptor dimerization occurs even if only one of two BB loops is intact, implies that such dimers are not formed exclusively via BB-BB interaction. Our data generated using the decoy peptide approach suggest that the TLR2 BB loop only is necessary for assembly of a functional signaling complex comprised of TLR2 and TLR1 or TLR6, and the adaptors. However, it remains to be determined whether the TLR2 BB loop interacts with TLR1/6 or with the adaptors, TIRAP or MyD88. In this regard, Gautam et al. (21) recently used site-directed mutagenesis to show that the DD loop of TLR2, rather than the BB loop, interacts with TLR1.

Effect of TLR2-, TLR4-, and TLR1/6-BBPs on activation of MAPKs by TLRs

Activation of MAPKs is one consequence of TLR signaling that precedes induction of cytokine gene expression. Therefore, we examined whether the TLR BBPs were capable of inhibiting
this branch of TLR2- or TLR4-mediated signaling. Fig. 2 shows that both TLR2- and TLR4-BBPs are potent inhibitors of LPS-induced ERK activation and that inhibition persists over a wide time range (Fig. 2, A and B, left panels). For both LPS and P3C, maximal ERK phosphorylation was observed 30–60 min after stimulation (Fig. 2B); it was decreased considerably by 2 h (data not shown). For stimulation with the TLR2/1 agonist, P3C, inhibition of ERK phosphorylation by TLR BBPs was weaker than that seen with LPS, particularly at 30 and 45 min poststimulation (Fig. 2, A and B, right panels), with TLR2-BBP being the strongest inhibitor of P3C-induced phospho-ERK. The inhibitory action of these TLR BBPs on the TLR2-induced response differs from previously described effects of adaptor BBPs that were highly specific for TLR4-induced ERK activation (Ref. 16 and Fig. 2B for TRAM-BBP). Interestingly, the TLR1/6-BBP did not inhibit LPS- or P3C-induced ERK phosphorylation (Fig. 2A). P2C-induced ERK phosphorylation was much faster and more transient than phosphorylation induced by LPS or P3C; ERK phosphorylation peaked 10–15 min after stimulation and, by 30 min, decreased to ~10–20% of the maximum (data not shown). None of the receptor BBPs inhibited activation of phospho-ERK induced by P2C (Fig. 3B and data not shown for TLR4-BBP).

Next, we examined the effects of the receptor BBPs on p38. Surprisingly, TLR2-BBP and, to a lesser extent, TLR4-BBP exerted weak agonist activity with respect to p38 phosphorylation (Fig. 2C). This activation resulted in the apparent absence of inhibition of LPS- or P3C-induced p38 phosphorylation by these BBPs (Fig. 2, A–C). Activation was specific for p38 and pJNK (Fig. 2C and data not shown), whereas ERK and cytokine mRNA (IL-1β or IFN-β) were not up-regulated by these peptides alone. In contrast to TLR2- and TLR4-BBP, the TLR1/6-BBP neither induced activation of p38 (data not shown) nor inhibited its activation by LPS or P3C (Fig. 2A). We also assessed phosphorylation of MEK1/2 and MKK3/6, kinases that mediate phosphorylation of ERKs and p38, respectively (reviewed in Ref. 22). The effect of BBPs on TLR-induced activation of MEK1/2 paralleled their effects on the ERK (Fig. 2B) in that the appearance of p-MEK1/2 species was significantly reduced in cells treated with agonist and TLR2- or TLR4-BBPs. In parallel with their weak agonist activity for p38, the receptor BBPs also induced activation of MKK3/6 (data not shown).

TAK1, a member of MAPK kinase kinase family, was recently reported to have a differential role in the activation of ERK and p38 by TNF-α and IL-1 (23). TAK1 activation is associated with phosphorylation of the enzyme on multiple

![FIGURE 2. Effect of TLR BBPs on activation of MAPKs. Seven to 10 μg of total protein was loaded per lane. One representative blot of three experiments is shown. A, Dose-dependence. Cells were lysed 30 min after stimulation with TLR agonist. B, Time course of phosphorylation of ERK, p38, and MEK1 and -2. C, TLR2-BBP induces phosphorylation of p38. Effects of BBPs on activation of TAK1 (D) and IRAK1 by LPS (E). B–E, Forty micromolars of each BBP was added to wells 30 min before stimulation with TLR agonist. B–E, Numbers indicate time in min between addition of a BBP or LPS and cell lysis.](http://www.jimmunol.org/)

![FIGURE 3. Second amino acid in -RD4zPG motif does not determine blocking properties of BBPs. A, Sequences of specific parts of receptor BBPs. Site of N/D or D/N substitutions is shown in bold italic. B, Effects of BBPs on MAPK activation. Cells were stimulated by LPS and P3C for 30 min (left and middle panels) and for 15 min by P2C (right panel). C, Effects of various BBPs on induction of IL-1β mRNA by LPS and P3C. Cells were preincubated for 30 min in the presence of BBP before stimulation with a TLR agonist for 1 h.](http://www.jimmunol.org/)
sites. Stimulation of macrophages by LPS led to appearance of slower migrating TAK1 species on SDS-PAGE (Fig. 2D). TLR2-, TLR4-, and TRAM-BBPs completely inhibited TAK1 phosphorylation (Fig. 2D). Activation of MAPKs by LPS is diminished in IRAK1-deficient macrophages (24). TLR2-, TLR4-, and TRAM-BBPs inhibited activation of IRAK1 as evidenced by the absence of its degradation after stimulation by LPS (Fig. 2E).

The different effect of TLR2- and TLR4-BBPs on ERK and p38 may suggest that activation of these MAPKs emanates from different surfaces on the TLRs. It also suggests that, similar to activation of the MyD88-independent pathway in MyD88−/− macrophages, partial assembly of receptor complexes remains capable of selective activation of some branches of the signaling cascade. Yet, it remains to be demonstrated whether receptor activation results in the assembly of a number of variant structures of signaling complexes, each with a different functional role.

TLR2-BBP inhibited ERK activation induced by P3C, but was ineffective in blocking P2C-induced phospho-ERK. Interestingly, the recent publication by Jiang et al. (12) describes the “Pac” mutation in MyD88 (I179N) that produces a similar phenotype/specificity. Mice bearing this mutation are unresponsive to TLR1/2, TLR4, TLR7, and TLR9 agonists; however, Pac mice respond normally to the TLR2/6 agonist P2C (12). When the homologous mutation was introduced into the TLRs, a similar unresponsive phenotype was produced; however, the mutated receptor was still responsive to the TLR2/6 agonist. Our findings show that, unlike the adaptor BBPs, the TLR2- and TLR4-BBPs are more selective toward separate MAPK pathways activated by a particular TLR, but are not as highly discriminating with respect to receptor specificity.

**Mutation that confers MyD88-binding activity on TLR1 does not alter activity of corresponding BBPs**

Brown et al. (25) found that, unlike TLR2, TLR1 and TLR6 do not bind MyD88, and also identified a single point mutation, N672D in the BB loop of human TLR1, that conferred MyD88-binding ability on this protein (25). Accordingly, introduction of a point mutation at the homologous residue in TLR2 (D678A) eliminated the MyD88-binding ability of TLR2 (25). The N672D mutation in TLR1 restores the “RDϕϕϕϕϕϕPG” motif that is highly conserved in BB loops of many TIR domains. In particular, this sequence can be found in BB loops of other MyD88 binding partners including TLR2, TLR4, TLR5, MyD88, and TIRAP/Mal. Interestingly, the only residue that distinguishes active inhibitors of TLR4 signaling, the TLR2- and TLR4-BBPs, from the inert TLR1/6-BBP, is amino acid N672 in mouse TLR1, and D679 and D705 in mouse TLR2 and -4, respectively (Fig. 3A). To determine whether this amino acid dictates the inhibitory potential of the BBPs, we synthesized the analog of TLR1/6-BBP in which asparagine-21 was replaced by aspartic acid (TLR1/6-BBP-N/D) and, conversely, exchanged aspartic acid at the 21st position of TLR2-BBP for asparagine (TLR2-BBP-D/N). Surprisingly, these substitutions did not affect the behavior of the BBPs with respect to either activation of MAPKs or induction of IL-1β gene expression (Fig. 3, B and C). One possible explanation for this observation is that, unlike the same sequences incorporated into a three-dimensional protein structure, these relatively short peptide decoys have “free” ends that could allow for additional structural flexibility that may explain why mutations that change the function of a native protein may not eliminate the effect of the same sequence in a BBP.

In summary, we have identified two new sequences that can specifically inhibit TLR signaling. The fact that the sequences of BB loops of TLR2 and TLR4 joined to a cell-penetrating peptide inhibit signaling, whereas a similar sequence in the BB loops of TLR1 or TLR6 is inert, supports the hypothesis that these proteins use different surfaces to form signaling complexes. The TLR2-BBP inhibited activation of NF-κB and ERK induced by the TLR2/TLR1 agonist P3C. This BBP also inhibited activation of NF-κB induced by the TLR2/6 agonist P2C, but was incapable of inhibiting P2C-induced ERK activation. These findings suggest different mechanisms of activation of ERK by P2C and P3C. The TLR2- and TLR4-BBPs are both capable of inhibiting activation of NF-κB and ERK induced by LPS or P3C, but are incapable of inhibiting p38 activation. This selective action is due to its weak agonist activity with respect to p38 and JNK branches of MAPK signaling; such specificity has yet to be observed using mice with targeted mutations in their TLRs or adaptor proteins, or pharmacological agents, and suggests that different receptor/adaptor complexes can activate ERK and p38.

**Disclosures**

The authors have a patent pending (International Patent Application No. PCT/US06/014243).

**References**


