IN SILICO STUDY OF HETERODIMERIZATION OF TLR2 AND TLR6

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Accepted on: 13-01-2011; Finalized on: 04-03-2011.

ABSTRACT
Toll-like Receptors (TLRs); a novel class of receptors can recognize conserved motifs so called pathogen associated molecular patterns (PAMPs) predominantly found in microorganisms and initiate a cascade of cellular signaling that direct the subsequent immune responses thus act as a bridge between the innate and acquired immunity. Under the condition of co expression of TLR2 and TLR6 with the help of their agonist molecule could induce maximum NF-kB through MyD-88 dependent pathway. Heterodimerization of TLR2 with TLR6 is an evolutionary process which enhances the ligand recognition capacity to enable the innate immune system. In the present study, a heterodimer of TLR2/TLR6 and a TIR-TIR platform formed by heterodimerization of TLR2/TLR6 has been produced using the computer assisted homology modelling and protein-protein docking method. Detail analysis of selected TLR2 and TLR6 docked complex and TIR-TIR docked complex helped us to find out the interacting amino acid residues between both the molecules. TLR2 and TLR6 interact with each other at three points while a Toll- interleukin 1 receptor (TIR) domain of both TLR2 and TLR6 interacts at single point only. These interactions are very crucial for activation of pro- and anti-inflammatory cytokine cascade and T cell proliferation to stimulate immune responses.

Keywords: Toll-like receptors, Toll- interleukin 1 receptor, heterodimerization, Docking, Modelling.

INTRODUCTION
Recognition of pathogen-associated molecular signatures is critically important in proper activation of the immune system. Toll-Like Receptor (TLR) signaling network is responsible for innate immune response. In human, 10 TLRs recognize a variety of ligands from pathogens to trigger immunological responses1-3. TLRs activate NF-kB and other signaling pathways, which results in the secretion of various inflammatory cytokines4-6.

The TIR-TIR platform formed by the dimerization of TLR2 and TLR6 promotes homotypic protein-protein interactions with additional cytoplasmic adapter molecules to form an active signaling complex resulting in the expression of pro- and anti-inflammatory cytokine genes. It has been reported that heterodimer of TLR-2/6 recognizes some components of Zymosan of yeast, diacylated lipoprotein, and GPI anchors of T. cruzi which result in production of cytokines and chemokines. Recent studies have demonstrated a crucial involvement of TLRs in the recognition of fungal pathogens such as Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans2. Through the study of fungal infection in knock-out mice deficient in either TLRs or TLR-associated adaptor molecules, it became apparent that specific TLRs such as TLR2 and TLR6 play differential roles in the activation of the various arms of the innate immune response. Recent data also suggest that TLRs offer escape mechanisms to certain pathogenic microorganisms, especially through TLR2-driven induction of anti-inflammatory cytokines.

The first member of the TLR family identified was a Drosophila protein implicated in dorsoventral patterning during embryonal development7. Different human homologues of Drosophila Toll were identified and shown to induce activation of the transcription factor nuclear factor-κB (NF-κB) upon overexpression, revealing that TLRs and IL-1 receptors trigger similar signal transduction cascades1,3.

TLRs SIGNAL TRANSDUCTION
The TLR signaling through different intracellular molecules, such as MAP kinases and IkB kinases which are conserved signaling elements for many receptors, leads to a distinct set of proinflammatory gene expressions9 (Figure 1).

TLR mediated myd88-dependent and independent cellular signaling
The signaling pathways activated by TLRs are broadly classified into MyD88-dependent and independent pathways as MyD88 is the universal adapter protein recruited by all TLRs except TLR310. The major pathways activated by TLR engagement are passed through IkB kinase (IKK), MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways regulate the balance between cell viability and inflammation11. TLR2/6 signaling involves four adapter proteins, MyD88, TIRAP, TRIF, and TRAM12.

MyD88 is the primary adapter for microbial signaling
Every TLR member differentially utilizes adapters, but MyD88 (296 amino acid protein) seems to be the widely
used adapter molecule. MyD88 harbors a TIR domain as well as a death domain. The carboxy terminal of TIR domain interacts with the cognate domains in the cytoplasmic tails of the TLRs, and the amino terminal death domain mediates the interaction with the corresponding domain of IRAK4\textsuperscript{13,14}.

Adapters mediating MyD88-independent signaling

MyD88-independent signaling events are controlled by TRIF/TRAM (for TLR4 and TLR 2, 6) and induce IRF3-dependent type I interferon production\textsuperscript{15-18}.

Figure 1: Signaling pathway of TLR 2 and TLR 6

KINASES INVOLVED IN SIGNALING FROM ADAPTERS TO TRANSCRIPTION FACTORS

Downstream of TLR signaling by adapters are mediated by IRAK family

IRAKs are important mediators in the signal transduction of the TLR family as they may act to potentiate the downstream signaling. IRAK1 and IRAK4 possess intrinsic serine/threonine protein kinase activities. IRAK1 has three TRAF6 (tumor necrosis factor receptor associated factor 6) binding motifs to mediate the interaction with TRAF6 and undergoes autophosphorylation. IRAK4 and IRAK1 are sequentially phosphorylated and dissociated from MyD88, which results in activation of TRAF6\textsuperscript{19}.

TRAF6 is the central activator of MAPK during microbial infection

TRAF6 is the activator of canonical NF-κB pathway. TRAF6 is ubiquitinated at K63 chains and this K63 polyubiquitinated TRAF6 mediates activation of the next component in the pathway, which is most likely to be TGF-β activated kinase-1 (TAK1)\textsuperscript{20}. In fact, the TAK1
associated proteins, TAB2 and TAB3, contain a domain that interacts specifically with K63-ubiquitin chains. The TAK1-TAB complex associates with K63-ubiquitin chains. The TAK1-TAB complex associates with K63-ubiquitinated TRAF6 activate TAK1 kinase, which then activates the IKK complex as well as the JNK kinases21.

Transcription factors activated by TLR engagement

PAMPs stimulation through TLR-dependent and independent pathways converges at the activation of transcription factors NF-κB, IRF3/7/5, and/or AP-1. These transcription factors collaborate with each other to produce a large number of cytokines, which are barely detectable in resting cells. The multi-transcription factor binding sites in the promoter of a given gene lead to this highly specific activation9.

NF-κB as double edged sword

The continued research on TLRs has led to the delineation of specificity in the regulation and interaction of transcription factors upon stimulation leading to a highly specific gene expression. NF-κB is the major transcription factor, which functions on TLR signaling to control/elicit inflammation. NF-κB was first described as a B cell specific transcription factor that binds the κB site in the Ig κ light chain enhancer22. Viral promoters contain NF-κB binding sites making it advantageous for its replication. So it is not exaggerating to say that cells which have NF-κB as a sword against the viral infection turn back against to them. NF-κB has often been called a ‘central mediator of the immune response’. MAL-MyD88 and TRAM-TRIF pathways stimulate NF-κB activation albeit with different kinetics23. NF-κB activity was found to be inducible in all cell types and it is now known that members of the NF-κB/Rel family regulate many genes involved in immune and inflammatory responses24,25,26.

Activating protein-1 (AP1)

The JNK and p38 cascades are activated first and foremost in response to inflammatory cytokines, bacterial products, and various stress factors. Activation of TAK1 during TLR signaling results in the activation of MAPKs, including JNK/p38, leading to the activation of AP-125,11,21, which together with NF-κB governs the production of inflammatory cytokines and chemokines26. Activation of these JNK/p38 cascades is associated with selective activation of different AP-1 subunits and transcription factors interacting with AP-1. This activation via p38 is necessary for the full induction of TNF-α and IL-12 as inhibition of p38 abrogates this biological response. All these studies together indicate that it is the differential activation and binding of AP-1 subunits, which contribute to the inflammation27.

The aim of present work was to study about the process of heterodimerization between TLR2 and TLR6 as well as between the TIR domain of TLR2 and TLR6 in humans.

MATERIALS AND METHODS

TOOLS USED

Swiss Model

Swiss model is a fully automated protein structure homology modelling server accessible via the ExPASy web server (http://swissmodel.expasy.org/) accessible to all biochemists and molecules biologists worldwide.

Swiss PDB viewer 3.7

Deep view (formerly called Swiss-PDB Viewer) is a friendly but powerful molecular graphics program. It is designed for use with computing tools available from the Expert Protein Analysis System (ExPasy). Deep view allows building models from scratch, simply by giving an amino acid sequence. It allows viewing several proteins simultaneously and superimposing them to compare their structures and sequences. It supports surface rendering, homology modeling, structure quality (threading) evaluation, energy minimization, site-directed mutagenesis, loop rebuilding, electrostatic field calculation, structure superposition, Ramachandran plot generation, and sequence-structure viewing28.

HEX 4.5

Hex is an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein and DNA molecules. Hex can also calculate small-ligand/protein docking (provided the ligand is rigid), and it can superpose pairs of molecules using only knowledge of their 3D shapes. The main thing which distinguishes Hex from other macromolecular docking programs and molecular graphics packages is its use of spherical polar Fourier correlations to accelerate the docking and superposition calculations. In Hex’s docking calculations, each molecule is modeled using 3D parametric functions which are used to encode both surface shape and electrostatic charge and potential distributions. By writing an expression for the overlap of pairs of parametric functions, one can derive an expression for a docking score as a function of the six degrees of freedom in a rigid body docking search29.

METHODOLOGY

The primary sequences of TLR2/TLR6 and Toll-interleukin 1 receptor (TIR) domains of TLR2 & TLR6 were retrieved from the NCBI site (http://www.ncbi.nlm.nih.gov/). These protein sequences were saved in FASTA file format. In order to perform homology modeling, the homologous protein structures were required. For that purpose database searching was performed using the BLAST (blastp) program which is available on the website of NCBI (http://www.ncbi.nlm.nih.gov/blast). The results showed a number of homologous proteins, which were similar in their protein sequence with the target protein. The extent of similarity was judged on the basis of certain parameters like E-value, % Identities and % Positives. The cut-off of E-value was set 10\(^{-10}\). The homologous sequences which were having more the 30% identity...
were selected as templates during homology modeling. The homologous sequences of TLR2 (PDB ID: 2Z7X and 1FYW) were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb). The target and templates were submitted to the automated SWISS MODEL server for homology modeling. Similarly modeling for TLR 6 and TIR domain of TLR6 were also carried out respectively using the same server. All 3D models generated by the swiss model server were then uploaded to SAVES Server (http://nihserver.mbi.ucla.edu/SAVES/) to evaluate the predicted protein structures. On the above server, PROCHECK module was used to evaluate the stereochemical properties of all models. PROCHECK summary of all predicted models shows the allowed & disallowed regions according to the RAMACHANDRAN Plot and the bad contacts of the model. Then Verify-3D module was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D). To rectify the bad contacts and to improve the overall quality of all predicted 3D protein structures, the energy minimization was done using the Deep View (spdbv) program. The 3D structures of all proteins were visualized by the above program.

Protein –protein docking

The HEX program was used for rigid body protein-protein docking between TLR2 and TLR6. The docking was also carried out between TIR domains of both TLR2 and TLR6 respectively. For the docking of TLR2/TLR6 and TIR domains of TLR2 and TLR6, we employed grid dimension of 0.6, twist range of 15Å, distance range of 15Å, scan step of 1Å, sub steps 2Å, steric scan 16Å, and final search 25Å and 500 solutions. The correlation type used was the ‘shape and electrostatic’. The program generated 500 lowest energy matches. Post processing involved bumps removal and NEWTON like energy minimization. Automated selection of docked structure was based upon threshold of RMS within 2Å. Selection from subsequent output was done on the basis that only those docked solutions, which had their TIR domains in the same plane and their respective N-terminals facing the cell membrane. Out of these, the docked complex having the least energy was selected.

Analysis of region of interaction

Selected docked complex of TLR2 and TLR6 and TIR domains of both TLR2 and TLR6 were analyzed to find out the regions of interaction among them. Swiss PDB viewer was used for the visualization of interacting amino acid residues between both receptors.

RESULTS

Docking Result of TLR2 and TLR6

TLR2 and TLR6 were submitted for docking to Hex program which generated 500 models. The following model was selected on the basis of minimum energy score.

Amino acid residues involved in the interaction between TLR2 and TLR6

The amino acid residues which were involved in the process of heterodimerization of TLR 2 and TLR 6 have been shown in Figures 5, 6 & 7.

Interaction between TIR domains of TLR2 and TLR6

The docked complex of TIR domains of both TLR2 and TIR6 were selected while both proteins were lying in the same plane and their N-terminals were lying towards the cell membrane (Figure 8 and 9).

Region of interaction between TIR domains of TLR2 and TLR6

The TIR domains of TLR2 and TLR6 were interacting at only single point. Amino acid residues which were involved in the heterodimerization are shown in Figure 10.

DISCUSSION

As of now, it is understood that complex TLR2 signaling functions via forming a heterodimer either with TLR1 to recognize Tri-acylated lipoprotein, or with TLR6 to recognize Di-acylated lipoprotein, which triggers the production of cytokines and chemokines. Previous in vitro studies as well as computerized docking studies suggest that MyD88 and TIR domain of TLR2, which is activated by the formation of heterodimer with either TLR1 or TLR6 (Gautam et al., 2006). For the fungal infection to occur, it is essential to form a heterodimer between TLR2 and TLR6 as well as TIR domains of both TLR2 and TIR6 to activate the signaling. In the consequence of an ability to form such a heterodimer, a decreased activity is observed in innate immune response. The molecular modeling studies were carried out for the interaction between TLR2 and TIR6 molecules, and TIR domains of both TLR2 and TIR6 respectively. The three dimensional structures of TLR2, TLR6, and TIR domain of TLR2 and TLR6 were modeled using the homology modeling technique. All modeled 3D protein structures were structurally refined by energy minimization followed by evaluation by SAVES server. In the present work, studies of heterodimerization of the TLR2 and TIR6 were corroborated with computer assisted docking methodology. Hex program was used for rigid protein- protein docking between TLR2 and TLR6. The docking was also performed to study the interaction between TIR domains of both TLR2 and TLR6. The energy score (Etotal) of docked complex of TLR2 and TLR6 was calculated by summing the value of Eshape (KJ/Mol) and Eforce (KJ/Mol). We employed grid dimension of 0.6Å, twist range of 15Å, distance range of 15Å, scan step of 1Å, sub steps 2Å, steric scan 16Å, and final search 25Å and 500 solutions. The program had generated 500 lowest energy matches. The Hex program selected the docked structures, which had threshold RMS within 2Å. The docked complex, which had least energy score was selected for further analysis. The energy score of TLR2- TLR6 docked complex was found to be -343.56 KJ/Mol (Table 1). The lower energy score refers to the higher
binding affinity towards the receptor. Among different docked orientations only those complexes were selected which had their N-terminal facing towards cell membrane and were in same plane. This complex represented the functionally meaningful orientations between TLR2 and TLR6 (Figure 2, 3).

Table 1: Docking correlation summary of TLR2/TLR6

<table>
<thead>
<tr>
<th>Etotal (KJ/mol)</th>
<th>Eshape (KJ/mol)</th>
<th>Eforce (KJ/mol)</th>
<th>Vshape (KJ/mol)</th>
<th>Vclash (KJ/mol)</th>
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Table 1 depicts the energy score (Etotal) of docked complex of TLR 2 and TLR 6 which calculated during the process of docking.

Figure 2: Backbone structure of docked complex

Figure 3: Interaction between TLR2 and TLR6

Detail analysis of selected TLR2 and TLR6 docked complex was carried out to find the interacting amino acid residues between both molecules. From the figure 4, we can see that TLR2 and TLR6 interact with each other at three points.

Figure 4: Regions of interaction between TLR2 and TLR6

It was found that in the first interacting region ASN248 of TLR2 interacts with ASP233 of TLR6 with an average distance of 1.45 Å, in the second interacting region LEU250 of TLR2 interacts with ASN231 of TLR6 with average distance of 1.74 Å while in the third interacting region LYS338 of TLR2 interacts with LEU318 of TLR6 with an average distance of 2.51 Å (Figure 5, 6, 7; Table 2).

Docking study of TIR domains of both TLR2 and TLR6 was performed separately using the Hex program (Figure 8, 9). The energy score (Etotal) of docked complex of TIR-TLR2 and TIR-TLR6 was calculated and it was found to be -135.7 KJ/Mol (Table 3). Analysis of selected docked complex (Figure 8) of TIR domains of TLR2 and TLR6 was also carried out. It was found that TIR domains of both TLR2 and TLR6 interact at only single point during the process of heterodimerization (Figure 9). From the figure 10 we can see that amino acid residue ILE745 of TIR-TLR2 interacts with PHE678 and PRO680 of TIR-TLR6 respectively, while GLN747 residue of TIR-TLR2 interacts with PRO680 residue of TIR-TLR6 domain. The three bonds have been emerged as the prominent factor for the stabilization of this region in the docked complex of TIR domains of TLR2 and TLR6. These bonds are formed between the residues ILE745(CD)--PHE678 (C), ILE745(CA)--PRO680(CD), and GLN747(CA)-- PRO680 (C) which have distances of 2.84Å, 2.55Å and 2.37Å, respectively (Table 4). These interactions are crucial for TLR2/TLR6 mediated signaling responses. This Heterodimerization of TLR2 and TLR6, and TIR domains of both TLR2 and TLR6 activates the NF-κB signaling. This active signaling complex further recruits other intracellular adapter molecules such as MyD88 and TIRAP. This active signaling results in the expression of pro- and anti-inflammatory cytokines IL-1, IL-6, IL-12 and TNF-α. IL-12 plays a pivotal role in Th1 cell differentiation to stimulate immune responses.
Figure 6: Second region of interaction between TLR2 and TLR6

Figure 7: Third region of interaction between TLR2 and TLR6

Table 2: Amino Acid residues involved in the heterodimerization of TLR2 and TLR6

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Distances (Å)</th>
<th>Amino Acid residue of TLR2</th>
<th>Amino Acid residue of TLR6</th>
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<tr>
<td>1</td>
<td>1.43</td>
<td>ASN248 (OD1)</td>
<td>ASP233 (OD1)</td>
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<tr>
<td></td>
<td>1.49</td>
<td>ASN248 (OD1)</td>
<td>ASP233 (OD2)</td>
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<td>ASP233 (OD2)</td>
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<td>LEU318 (CB)</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>2.67</td>
<td>LYS338 (CE)</td>
<td>LEU318 (CG)</td>
</tr>
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Abbreviations: ASN- Asparagine, ASP- Aspartic Acid, LEU- Leucine, LYS- Lysine, B- Beta, C- Carbon, D- Delta, E- Epsilon, G- Gamma, N- Nitrogen, O- Oxygen, Z- Zeta.

Figure 8: Docked complex of TIR-TLR2 and TIR-TLR6 (Ribbon structure)

Figure 9: Docked complex of TIR-TLR2 and TIR-TLR6 (Backbone and molecular surface view)
Table 3: Docking correlation summary of TIR domains of TLR2 and TLR6

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Figure 10: Interacting amino acid residues in TIR domains of TLR2 & TLR6

Table 4: Amino Acid residues involved in the heterodimerization of TIR domains of TLR2 & TLR6

<table>
<thead>
<tr>
<th>S. No.</th>
<th>DISTANCE (Å)</th>
<th>TIR DOMAIN OF TLR2</th>
<th>TIR DOMAIN OF TLR6</th>
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<tbody>
<tr>
<td>1</td>
<td>2.84</td>
<td>ILE745 (CD)</td>
<td>PHE678 (C)</td>
</tr>
<tr>
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<td>2.55</td>
<td>ILE745 (CA)</td>
<td>PRO680 (CD)</td>
</tr>
<tr>
<td>3</td>
<td>2.37</td>
<td>GLN747 (CA)</td>
<td>PRO680 (C)</td>
</tr>
</tbody>
</table>

Abbreviations: A- Alpha, C- Carbon, D- Delta, GLN- Glutamine, ILE- Isoleucine, PHE- Phenylalanine, PRO- Proline.

CONCLUSION

TLRs not only recognize pathogens but also, upon ligand binding, initiate a cascade of cellular signaling that direct the subsequent immune responses. We conclude that heterodimerization of TLR2 with TLR6 is an evolutionary process which enhance the ligand recognition capacity to enable the innate immune system. We found that the amino acid residues ASN248, LEU250 and LYS338 of TLR2 interact with residues ASP233, ASN231 and LEU318 of TLR6 respectively in three regions while ILE745 and GLN747 residues of TIR domain of TLR2 interact with PHE678 and PRO680 residues of TIR domain of TLR6 respectively in single region only. These amino acid residues play an important role in the formation of heterodimer. Subsequent to heterodimerization, TLR2-TLR6 complex can recognize numerous structures of lipoproteins present in various pathogens, thus providing a sort of basic level specificity to the innate immune system in humans. In future this heterodimer can act as a drug target to stimulate immune responses.

Acknowledgement: The authors would like to acknowledge the facilities provided by Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed to-be University), Allahabad (U.P), India.

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