Kremen is beyond a subsidiary co-receptor of Wnt signaling: an in silico validation

Hemn MOHAMMADPOUR1, Saeed KHALILI2*, Zahra Sadat HASHEMI3

1Department of Medical Immunology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran
2Department of Medical Biotechnology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran
3Department of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Science, Tehran, Iran

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Abstract: Kremen-1 is a co-receptor of Wnt signaling, interacting with the Dkk-3 protein, which is an antagonist of the Wnt/β catenin pathway. In the present study we attempted to shed some light on the possible orientations of Kremen/Dkk-3 interactions, explaining the mechanisms of Kremen and Dkk-3 functions. Employing state-of-the-art software, a Kremen model was built and subsequently refined. The quality of the final model was evaluated using RMSD calculations. Ultimately, we used the Kremen model for docking analysis between Kremen and Dkk-3 molecules. A model built by the Robetta server showed the best quality scores. Near native coordination of the final model was verified getting < 2 Å RMSD values between our model and an experimentally resolved structure. Docking analysis indicates that one low energy orientation for Kremen/Dkk-3 involves all extracellular domains of Kremen, while another only involves the CUB and WSC domains. Kremen receptors may determine either antitumor or protumor effects of the Dkk protein. Based on existing reports and our findings, we hypothesized that there could be different cellular fate outcomes due to the orientation of Kremen and Dkk-3 interactions. One orientation of the Kremen/Dkk complex could lead to Kringle mediated antitumor effects, while another could end with CUB mediated protumor effects.

Key words: Kremen-1, Dkk-3, protein modeling, molecular docking

1. Introduction

The Wnt family signaling proteins and their receptors play pivotal roles in different developmental and physiological processes such as differentiation, proliferation, disease, and tumorigenesis (Clevers, 2006). The canonical and noncanonical/Wnt-β catenin signaling pathways are regulated via a number of transmembrane and extracellular proteins (Nakamura and Matsumoto, 2008).

The low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6), as Wnt co-receptors, are essential for signal transmission via the β-catenin pathway. Binding of the Wnt ligands to low-density lipoprotein receptor-related proteins 5 and 6 leads to inhibition of β-catenin degradation, which in turn allows β-catenin to translocate into the nucleus. Once inside the nucleus, it forms a transcriptional complex with the T-cell factor (TCF)/lymphoid enhancer factor family proteins. Other Wnt co-receptors are Kremen 1 and Kremen 2 proteins, which are single spanning membrane proteins that are high-affinity receptors for the Dickkopf (Dkk) family (Boudin et al., 2013).

The Dkk family are soluble proteins, acting as extracellular Wnt antagonists. This family consists of four main members, including Dkk-3 to 4 and the Dkk-3-related protein Dkkl1. Dkk-1, Dkk-2, and Dkk-4 bind to low-density lipoprotein receptor-related protein (Lrp) 5 and 6 receptors and inhibit the Wnt signaling. They can further form a ternary complex with Kremen 1 and Kremen 2 (Kremen1 and Kremen2). The ternary LRP6/Dkk-3/Kremen complex is rapidly endocytosed from the cell surface, leading to the inhibition of Wnt/β-catenin signaling. Dkk-3 is the least characterized member of the Dkk family (Mao et al., 2001; Zhang and Mao, 2010). Recent reports have demonstrated that Dkk-3 has distinct roles in regulating the Wnt pathway, depending on the cell (Mao et al., 2002; Nakamura and Hackam, 2010). However, its emerging role in carcinogenesis has led to increased interest in how this protein functions to inhibit the Wnt pathway.

Kremen was originally discovered as a novel transmembrane receptor-like protein (Nakamura et al., 2001). Later, Kremen was shown to be the Dkk protein receptor, which is the inhibitor of the Wnt signaling pathway (Mao et al., 2002). Kremen is a type-I transmembrane protein composed of an extracellular region of 389 amino acids, a transmembrane domain, and
a cytoplasmic region of 64 amino acids. The extracellular region of Kremen has a Kringle domain, a WSC domain, and a CUB domain. Together with Dkk, Kremen forms a machinery that functions as a cell surface gatekeeper for the entry of Wnt signaling (Nakamura and Matsumoto, 2008).

In the present study we tried to predict Kremen's 3D model and its ternary complex with Kremen1 and LRP5/6. We also explored various possible functions of Kremen in different physiological and non-physiological processes, and particularly in carcinogenesis.

2. Methods

2.1. Sequences and homology analyses

UniProt (Universal Protein Resource) knowledgebase at http://www.uniprot.org/ was employed to obtain the protein sequence for the Kremen protein. To find a suitable template structure for homology modeling predictions, the NCBI protein BLAST tool at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used. The BLAST was done against the Protein Data Bank proteins; the search was restricted for Homo sapiens only and all other parameters were set at default.

2.2. Protein modelling

To predict the three dimensional (3D) structure of the Kremen protein, we used both fold recognition and ab initio modeling approaches. The I-TASSER server at http://zhanglab.ccmb.med.umich.edu/ITASSER/, ranked as the No 1 server for protein structure predictions in recent CASP7, CASP8, CASP9, and CASP10 experiments, was used for the Kremen structure prediction. This server builds its 3D models based on multiple-threading alignments by LOMETS and iterative template fragment assembly simulations. Rosetta de novo protocol was used to model domains without a detectable PDB homolog; locally installed versions of HHSEARCH/HHpred, RaportX, and Sparks-X were used to build comparative models.

2.3. Model quality assessment

As a vital step in the protein structure prediction process, the obtained models were fed as input files into the QMEAN model quality assessment server at http://swissmodel.expasy.org/qmean/cgi/index.cgi. QMEAN provides access to a composite scoring function capable of deriving both global and local error estimates on the basis of one single model. The Prosa server at https://prosa.services.came.sbg.ac.at/prosa.php was used for further quality assessment. Moreover, the packing quality of the model was assessed by the atomic empirical mean force potential ANOLEA at http://swissmodel.expasy.org/ along with its QMEAN quality plot.

2.4. Model refinement analyses

To arrive at models associated with higher quality scores, a model refinement process was executed on the selected best model. Two loops spanning 1–10 and 181–200 regions of the model indicating high residue error peaks (according to QMEAN residue error plot) were remodeled using UCSF Chimera, production version 1.9 software and ModLoop server at http://modbase.compbio.ucsf.edu/modloop/. The resultant modified model was further refined by two consecutive runs of the 3Drefine server at http://sysbio.rnet.missouri.edu/3Drefine/. This server modifies protein structures by optimizing the hydrogen-bonding network and atomic-level energy minimization. Finally, the obtained 3D models were subjected to the steepest descent minimization in chimera for 10,000 steps with 0.02 step sizes, without fixing any atoms, followed by 100 steps of conjugate gradient minimization. The first minimization phase is supposed to relieve highly unfavorable clashes, while the second phase is more effective at reaching an energy minimum after severe clashes have been relieved. The stereochemical quality of the predicted models was assessed using Procheck software at http://swissmodel.expasy.org/ to evaluate geometry of the residues in a given protein structure. The SolvX server at http://ekhidna.biocenter.helsinki.fi/solvx/start was harnessed to compute solvation preference of the refined model over a sliding window of 11 residues. The ResProx server at http://www.resprox.ca/ was used to predict the atomic resolution of the predicted structure.

2.5. Data validation

The refined structure was superimposed onto a 3D structure of a crystallographically resolved Kringle domain under PDB ID 1PK2 to check the accuracy of the predicted structure. Click server at http://mspc.bii.a-star.edu.sg/minhn/pairwise.html was employed to make the superimposition and subsequent RMSD calculation.

2.6. Protein-protein interaction study

2.6.1. Shape complementarity principle based docking

Three docking servers listed in the international protein–protein docking experiment Critical Assessment of Predicted Interactions (CAPRI) were employed to predict the best interacting conformation between Kremen and Dkk-3 proteins (data under consideration for publication). The Cluspro server at http://cluspro.bu.edu/ uses an automated rigid-body docking and discrimination algorithm to filter docked conformations with good surface complementarity and rank them based on their clustering properties. Patchdock at http://bioinfo3d.cs.tau.ac.il/PatchDock/ applies a geometry-based molecular docking algorithm to arrive at docking transformations with good molecular shape complementarity. ZDOCK at
http://zdock.umassmed.edu/ aims at finding an efficient global docking search on a 3D grid, utilizes a fast Fourier transform algorithm, and the scoring is done based on a combination of shape complementarity, electrostatics, and statistical potential terms.

2.6.2. Interaction refinement
FireDock at http://bioinfo3d.cs.tau.ac.il/FireDock/ is a fast, flexible, induced-fit backbone and side chain refinement server utilizing a coarse refinement method to optimize the interaction in molecular docking studies. The initially docked protein complex files were subjected to restricted side-chain optimization, followed by soft rigid-body minimization using FireDock.

2.6.3. Identifying low-energy conformations
As the last step of the protein interaction prediction, FireDock results were subjected to rigid-body orientation and side-chain conformations optimization using the RosettaDock server at http://rosettadock.graylab.jhu.edu/.

2.6.4. 2D interaction diagrams
LigPlus software was harnessed for automatic generation of 2D interaction diagrams. Finally, the refined protein complex was used to calculate intermolecular energies within the predicted complexes.

3. Results

3.1 Sequence and homology analyses
The Kremen protein 1 sequence under UniProtKB ID Q96MU8 was retrieved for in silico analyses. It is 492 amino acids in length, containing one CUB, one Kringle, and one WSC domain. The protein contains a 19 amino acid signal peptide that would be further processed into a mature form. The BLAST search against PDB using this sequence as a query returned similar sequences, the best of which had 17% query coverage and 43% sequence identity.

3.2. Obtaining the protein’s 3D model
Both I-TASSER and Robetta successfully modeled the Kremen protein 3D structure. Both servers provided 5 top predicted models based on their scoring algorithms. Table 1 lists the quality assessment z-scores calculated for the best models predicted by both, the QMEAN and Prosa servers, along with their Ramachandran total G-factor. Figure 1 shows ANOLEA results, indicating most of the amino acids are in their favorable energy environment with acceptable QMEAN scores.

3.3. Model refinement
Remodeling of the two loops spanning 1–10 and 181–200 regions resulted in resolving two major residue error peaks. Loop modeling and refinements performed by the 3Drefine server improved the quality z-scores for both the QMEAN and Prosa servers (Table 1). The final energy minimization performed on the refined model resulted in a final model (Figure 2). The quality scores of the final model are -0.4, -1.6, and 1.86 for Procheck total G-factor, Solvex, and ResProx respectively. Figure 3 presents the Ramachandran plot for the finally achieved structure, revealing that more than 90% of residues are in the allowed regions.

3.4. Data validation
As depicted in Figure 4, the structures of the predicted Kremen model and the crystallographically resolved Kringle domain are significantly equivalent. The two structures superimposed on each other with RMSD of 1.93 Å and a topology score of 1.

3.5. Protein–protein interaction results
Feeding the results of each docking step to the following one resulted in three finally refined complexes with lowest binding energies and highest docking scores. As illustrated in Figure 5, initial docking using Patchdock, Zdock, and Cluspro and refinement by Firdock and Rossetadock (Rosetta total scores are −578.669, −578.724, and −579.237, respectively) results in complexes with different possible orientations of the Dkk-3 and Kremen interaction.

3.6. 2D interaction diagrams
A 2D interaction diagram for each predicted orientation indicates that the main Dkk-3 and Kremen interaction sites are the cytosolic, Kringle, WSC, and CUB domains of the Kremen protein. A sample of a 2D interaction diagram for the Dkk-3 and Kremen interaction is depicted in Figure 6. Table 2 lists the main amino acids involved in the interaction sites along with intermolecular energies calculated by Ascalaph Designer.

Table 1. Quality assessment scores. Improvements in Prosa and QMEAN quality scores are revealed for the initial and refined model. The quality scores for all predicted models were calculated and the best models were selected accordingly. To verify the quality improvements, quality scores for the refined model were reassessed.

<table>
<thead>
<tr>
<th>Quality assessment</th>
<th>Prosa Zscore</th>
<th>QMEAN Zscore</th>
<th>Total Ramachandran G-factor</th>
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<tr>
<td>ITASSER best model</td>
<td>−2.11</td>
<td>−7.26</td>
<td></td>
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<tr>
<td>Robetta best model</td>
<td>−6.14</td>
<td>−2.93</td>
<td></td>
</tr>
<tr>
<td>Refined Robetta model</td>
<td>−6.1</td>
<td>−2.64</td>
<td>−0.40</td>
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4. Discussion

Due to the costly and laborious nature of crystallographic studies, bioinformatics tools are increasingly seen as standard and promising alternatives for determining protein tertiary (3D) structures. In silico studies in clinical trials could provide new insights into various biologically challenging problems, including the design of therapeutic molecules that inhibit or improve protein binding, the design of theoretically proper ligands, the discovery of accurate receptors and ligands folding, and the prediction of protein–protein interactions (Sefid et al., 2013; Khalili et al., 2014). Providing the 3D structure of a protein would shed light on the tough challenges that lie ahead for its functional analysis. Here, we invoked an in silico approach to predict the 3D structure of the Kremen protein and its interaction with the Dkk-3 molecule. Using state-of-the-art software, a Kremen 3D model was developed and refined. Following the verification of a native like coordination of the structure, a thorough docking analysis was conducted to reveal the intricacies of its interacting amino acids with the Dkk-3 protein. Ultimately, we developed two most probable orientations for the Kremen/
**Figure 2.** Final Kremen protein 3D model. Each domain of the Kremen molecule is colored in a unique color: the Kringle domain—blue; the WSC domain—red; the CUB domain—green; and the cytoplasmic domain—yellow.

**Figure 3.** Ramachandran plot for the refined structure. Only one residue is situated in the unfavorable region, indicating the high quality of the final model.
Dkk-3 complex, each suggesting a plausible mechanism for Wnt antagonism.

Since the homology modelling approach is the only technique capable of predicting reliable models of high quality over a wide range of sizes, we performed a BLAST search to find a reliable template molecule necessary for homology modelling (Moult, 2005). The accuracy scale of models predicted by the homology modeling method depends remarkably on the quarry and template sequence identity. Bearing more than 50% sequence share, the predictions are of significantly high accuracy, comparable in quality to low-resolution X-ray predictions or medium resolution NMR structures. Models of ~30%–50% sequence identity between quarry and template are expected to have more than 80% of their Ca atoms within 3.5 Å of the correct position. However, sharing less than 30% identity makes the prediction more likely to fail in accurate modeling due to alignment errors (Kopp and Schwede, 2004). According to the BLAST search results, there are no amenable templates for the Kremen molecule to employ homology modeling convincingly enough to harness threading and ab initio modeling approaches. The QMEAN Z-score is a quality score that is independent of protein size. It could be used to assess individual protein structure models by relating the model’s structural features to experimental structures of a similar size. Considering the significantly more appropriate Z-scores ascribed to a model built by Robetta, we chose the Robetta model for the model refinement process.
**Figure 6.** A sample of a 2D interaction diagram between 167–177 amino acids of the Dkk-3 and Kremen protein. The outer residues are interacting Kremen residues, and the inner strand (mainly blue and violet ball and stick presentation) of residues are Dkk3 167–177 region.

**Table 2.** Protein–protein interaction. The main residues involved in Dkk-3 and Kremen interaction interfaces are listed for the four main Kremen domains. Predictions executed by three leading docking servers indicate that Kremen can have two main interaction orientations with Dkk-3 (the interaction with the cytoplasmic domain is not possible due to the unviability to Dkk-3). As listed in the table, one orientation (predicted by Cluspro) includes the Kringle domain contribution and the other (predicted by Zdock) does not. Intermolecular energies for each complex are also calculated, revealing the stability of the interactions.

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<tr>
<td>Cluspro</td>
<td>17,18, 31,33, 74,90,91</td>
<td>137,140</td>
<td>215,216,217,218,219,220,222,224,225, 228,243,246,248,249,250,251,266,288, 289,290,291,292,293,294,295</td>
<td>–</td>
<td>–54.346</td>
<td></td>
</tr>
<tr>
<td>Patchdock</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–429,458,461,463, 465,471,472,</td>
<td>–75.6858</td>
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Predicted models usually show some discrepancy from their native coordinates even when the best prediction criteria are taken into consideration. The necessity of detecting and correcting plausible errors of coordinate information, and therefore bolstering the obtained model closer to the native structure, makes the model refinement process an inevitable protein structure prediction step. Accurate loop modeling could be a major factor for improving model quality. Therefore, existing high residue error picks according to the QMEAN energy profile were the initial targets of loop remodeling. A further full-atomic refinement simulation would determine the global topology and local details of the predicted model. Moreover, steepest descent energy minimization as the first minimization step would relieve highly unfavorable clashes, while conjugate gradient minimization as the following energy minimization step is more effective at reaching an energy minimum after severe clashes have been relieved. ANOLA plot indicates a favorable energy environment for most of the amino acids, buttressing the predicted model's high quality.

A protein structure comparison using RMSD values between predicted and experimentally resolved structures is commonly used to weigh the effectiveness of the prediction strategy. RMSD is the measure of the average distance between the atoms of superimposed proteins. Smaller RMSD values, in the range of closely homologous protein values (<3 Å), verify the spatial equivalency of two structures (Chothia and Lesk, 1986; Reva et al., 1998). Less than 2 Å RMSD between our model and the crystallographically resolved Kringle domain indicates fairly well the accuracy of the built model. Protein topology, as the relative orientation of the regular protein's secondary structural features (Rawlings et al., 1985), could be used to assess structural similarity. Topological similarity between the matched structures could be determined based on the topology score. This score is calculated based on the directionality of the matched sequence fragments (Nguyen et al., 2011). Our structure got a topology score of 1, indicating a topological similarity between the two superimposed structures.

Although a high resolution structure of proteins and their structures in complexes with other proteins could be resolved using X-ray crystallography or NMR methods, experimental determination of protein structures, especially membrane proteins, remains a challenge in the field of structural biology (Persson and Argos, 1996). This could apparently be deduced from the existence of a wide sequence-structure gap. While experimental methods to resolve the correct orientation of protein complexes are still costly and time consuming, protein docking can be a viable alternative to discover the correct association of interacting molecules within complexes. Revealing the atomic details of residue–residue contacts within protein–protein interactions that play pivotal roles in a wide range of key biological processes such as cell signaling, enzyme inhibition, and immune recognition, would elucidate the intricacies of their binding and hopefully would bring about a better understanding of their roles in biological pathways. Our docking results suggest that Kremen can interact with the Dkk-3 molecule mainly in two orientations with comparable binding energies. One orientation involves the Kringle, WSC, and CUB domains, while the other involves the WSC and CUB domains.

Kremen is a type 1 transmembrane protein with three extracellular domains: the Kringle domain, the CUB domain, and the WSC domain (Nakamura and Matsumoto, 2008). Due to specific structural and functional properties associated with each domain, engagement of each domain supposedly would lead to a different cellular fate. The Kringle domain is a unique structural motif composed of triple disulfide linked peptides. It is a conserved domain in serine proteases, usually involved in blood clotting and fibrinolysis such as thrombin, plasminogen, and plasmin (Nakamura et al., 1989; Oishi et al., 1997). The Kringle domain is a potent inhibitor of tumor induced angiogenesis in vitro and in vivo experiments (Kim et al., 2003). The anti-angiogenic functions of the Kringle domain have been extensively studied using recombinant Kringle fragments, especially plasminogen Kringles. Previous research has shown that the recombinant Kringle domain inhibits angiogenesis through blocked VEGF-induced signal transduction associated with proliferation, survival, and migration (Kim et al., 2003). Likewise, it suppresses VEGF165-induced activation of MMP-2 and VEGFR2. Ahn et al. demonstrated that truncated Kringle domain, termed rhkkl68, hinders endothelial cell migration by interfering with extracellular signal regulated kinase 1/2 (ERK1/2) activation (Ahn et al., 2004). ERK and phosphosotide 3 kinase (PI3K)/Akt signaling pathways appear to play a crucial role in endothelial migration (Kim et al., 2012).

Kremen 1 and 2 (containing a Kringle domain) are high affinity receptors for Dkk proteins, especially Dkk-3. Recent data reveals that the Dkk-3 interaction with Kerem 1 eliminates the Dkk-3 potentiation of Wnt3a mediated signaling. According to our results, Kremen 1 can mainly interact with Dkk-3 in two different low energy orientations. One orientation involves all three domains of Kremen, while the other includes only the CUB and the WSD domain. Our observations sufficiently bridge in silico and in vitro data. Although some data have suggested that Kremen has no functional domains (Nakamura and Matsumoto, 2008), it can be deduced that the Kringle domain of Kremen could most likely be an active participant in tumor growth suppression and metastasis.
inhibition (Nakamura and Hackam, 2010). Regarding the important role of the Kringle domain to inhibit cancer cell growth, and the interaction of Dkk-3 through the Kringle domain of Kremen, we hypothesize that the Dkk-3 protein can interact with the Kringle domain and activate the intracellular signaling pathway via an intra- and intermolecular crosstalk mechanism, leading to inhibition of cell proliferation. As an alternative hypothesis to explain the mechanism of the Kremen domain activation, we suggest that following the Dkk/Kremen complexation, the complex could be an endocytosis into the cell and the Kringle domain could bind to specific effector molecules in the cytoplasm. In the cytoplasm, the Kringle domain suppresses dimerization of VEGFR-2 as a crucial receptor for cell growth and remarkably abolishes cell proliferation (Kim et al., 2012). In parallel to our hypothesis, Nakamura et al. supposed that Dkk-3 and Kremen interact in the membrane portion that is located in the perinuclear region of the cell (Nakamura and Hackam, 2010).

CUB (complement protein subcomponents Clr/Cls, Urchin embryonic growth factor, and Bone morphogenetic protein 1) is another main extracellular domain of Kremen. The CUB domain has been identified in proteins regulating development such as embryogenesis and organogenesis, and recently in cancer invasion and metastasis (Uekita and Sakai, 2011). CUB domains are characterized by their immunoglobulin-like fold and they are involved in diverse protein–protein and protein–carbohydrate interactions. One such protein containing a putative CUB domain is CDCP-1 (CDCP-1). CDCP-1 plays pivotal roles in cellular adhesion, cell migration, and cancer invasion linked to cell signaling via the Src family kinase and protein kinase C δ (Liu et al., 2011). The expression of CDCP-1 is unregulated in many cancers such as lung, breast, and colon tumors (Perry et al., 2007; Uekita et al., 2013). Our findings revealed the involvement of the CUB domain in the Kremen interaction with the Dkk-3 protein. We think this involvement may cause cancer progression in different tumor microenvironments. Interestingly, previous data showed that Kremen could accelerate the Wnt signaling solely through interaction with LRP5/6 (Boudin et al., 2013). We presume that the Dkk protein interacts with the LRP receptor and the following structural changes may alter the Kremen and LRP interaction, leading to a CUB mediated Wnt signaling activation because in this orientation of the Dkk-3/Kremen interaction the Kringle domain is not involved. Therefore, the mechanisms that Kremen involvement is responsible for would not be activated. This could rationalize the two main orientations of Dkk-3/Kremen interactions, leading to two different cellular fates: Kringle mediated tumor suppression and CUB mediated tumor progression.

In conclusion, using in silico tools would pave the way to derive compelling results and hypotheses in various fields of biology (Ali et al., 2014; Liangbing et al., 2014; Poorinmohammad and Mohabatkar, 2014). Kremen receptors in tumor cells may determine either the antitumor or the protumor effect of the DKK protein, depending on which domains are involved. Antitumor effects of the DKK protein are majorly ascribed to involvement of the Kringle domain. Conversely, involvement of the CUB domain in Kremen receptor interactions may lead to active cell migration and cancer invasion. Moreover, different complex interactions among DKK-3, DKK-1, LRP, and Kremen-made various complex formations induce different Wnt signaling.

References


