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The conformation of the extracellular binding domain of Death Receptor 5 in the presence and absence of the activating ligand TRAIL: A molecular dynamics study

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ABSTRACT

The Death Receptor 5 (DR5), a member of tumor necrosis factor receptor (TNFR) superfamily of receptors, triggers apoptosis (programmed cell death) when stimulated by its tridentate ligand TRAIL. Until recently it was generally assumed that the activation of DR5 resulted from the recruitment of three independent receptor units, leading to the trimerization of intracellular domains. However, there is mounting evidence to suggest that, in the absence of ligand, such cytokine receptors primarily reside as preformed complexes. In this work, molecular dynamics simulations of the TRAIL-DR5 complex, the unbound receptor trimer and individual receptor monomers are compared to gain insight in the mechanism of activation. The results suggest that, in the absence of TRAIL, DR5 has a strong propensity to self-associate and that this is primarily mediated through interactions of the membrane proximal domains. The association of the free receptors leads to a loss of the threefold symmetry found within the receptor-ligand complex. The simulations suggest that the primary role of TRAIL is to induce threefold-symmetry within the DR5 complex and to constrain the receptor to a specific conformation. The implications of this in terms of the mechanism by which the receptor switches from an inactive to an active state are discussed.

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***Key words:** TNF receptor superfamily; cytokine; self-association; pre-ligand association domain (PLAD); molecular dynamics; simulation.*

INTRODUCTION

TRAIL (Tumor necrosis factor Related Apoptosis Inducing Ligand)[1,2] is an important effector cytokine from the TNF superfamily. It has been shown to selectively induce apoptosis in certain malignant cell lines[1–4] and HIV infected T-cells,[5] while healthy cells remained unaffected.[6] TRAIL exerts its effect through interaction with receptors belonging to the TNF receptor (TNF-R) superfamily. The interaction sites are formed by two death domain containing receptors, Death Receptor 4 (DR4)[7] and Death Receptor 5 (DR5, also known as Apo2, TRAIL-R2, TRICK-2, and KILLER),[8–15] which trigger apoptosis through a caspase-dependent pathway. TRAIL mediated apoptosis is regulated by the presence of three antagonistic decoy receptors. The membrane bound decoy receptors DcR1 (TRAIL-R3 or TRID)[8,9,12,16] and DcR2 (TRAIL-R4 or TRUNDD)[17–19] are present on normal cells while their expression is reduced in certain malignant cell types. The third decoy receptor is the soluble member of the TNF-R superfamily osteoprotegerin (OPG), which is involved in osteoclastogenesis.[20,21]

The members of the TNF-R superfamily typically are trimeric type I membrane proteins. Their activation was generally believed to be triggered by the attraction and interfacing of three receptor monomers mediated by interaction with the trimeric ligand. This was assumed to lead to the alignment and subsequent interaction of the intracellular domains in turn giving rise to the formation of the intracellular signaling complex by recruitment of downstream effector proteins. In the case of the death receptors the signaling complex is termed the Death Inducing Signaling Complex or DISC, which activates caspases, leading to apoptosis.[22,23]

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Although the mechanism outlined above was widely accepted, it is now clear that several members of the TNF-R superfamily have a propensity to self-associate in the absence of ligand. These receptors may instead reside on the cell surface as preformed complexes, activated by a conformational change or the reorientation of the receptor units rather than merely by crosslinking. They include both TNF receptors (TNF-R1 and TNF-R2),[24] CD40,[24] CD95 (Fas),[25,26] and Death Receptor 4.[24] In addition, recent results obtained using surface plasmon resonance (SPR) showed that all four receptors for TRAIL can form homodimers and may even form heterodimers, with the exception of the combinations DR5-DcR1 and DR5-DcR2.[27] For TNF-R1, TNF-R2, and CD95, a domain was identified, which mediates the self-association. This domain was termed the preligand association domain[24] and is formed by the membrane distal domain (CRD1). For both TNF receptors this domain consists of two modules, termed A1 and B2,[28,29] which share a high homology between these receptors. Since CD40 has a homologous domain at the N-terminal, it is expected that this region will also be responsible for self-association of that receptor. The N-terminal cysteine rich domain (CRD) of CD95 only consists of a single B2 module, which apparently is sufficient for self-association.[25] However, in the case of the TRAIL receptors the N-terminal region is formed by a small truncated module, designated N, which is unlikely to mediate self-association.

To gain insight into the self-association of the TRAIL receptors and the mechanism of activation of the Death Receptors, we have examined the case of DR5 binding to TRAIL in detail. TRAIL, together with its receptor DR5, was chosen for these studies, both because of the availability of structures of the complex and because of possible therapeutic interest. The structure of DR5 in complex with TRAIL has been solved by three different groups.[30–32] The structure of the TRAIL-DR5 complex is shown in Figure 1(A). The available structures are in close agreement with regards to the orientation of the receptor and its interactions with TRAIL. However, the structure solved by Cha *et al.* suggests a tighter interaction between the receptor and the AA″-loop of the TRAIL,[32] than the structures of Hymowitz *et al.* and Mongkolsapaya *et al.* The C-termini of the receptor, which are attached to the cell surface, are separated by 5.4 nm in all three structures. It is interesting to note that a similar distance of 5.2 nm separates the binding sites on TRAF2 (TNF Receptor Associated Factor),[33] which is a trimeric adaptor protein involved in the initiation of signaling in certain members of the TNF-R superfamily, albeit not for DR4 or DR5.

The extracellular domain of DR5 is characterized by the presence of two cysteine rich domains, which have a characteristic cysteine knot topology. Differences in the contact area residues of these CRDs allow for very specific ligand recognition. Superposition of DR5 onto the crystal structure of TNF-R1[34] indicates the presence of a hinge bending region,[35] which suggests domain adaptation as another mechanism to account for the high specificity of these receptors.

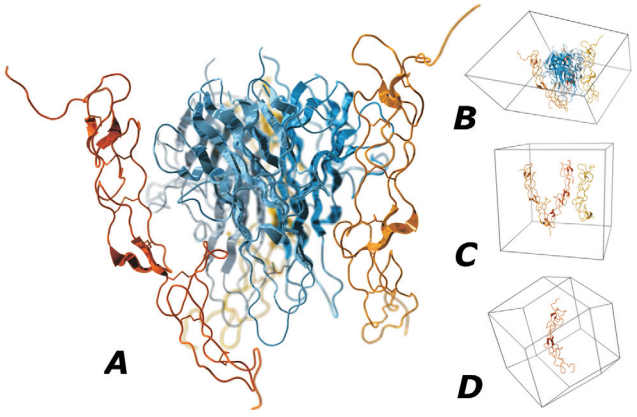

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Figure 1

*Crystal structure of the TRAIL-DR5 complex and simulation systems. (**A**) The crystal structure of the TRAIL-DR5 complex (1D4V[31]). TRAIL is displayed in blue, the Death Receptor in orange. The receptor-ligand complex was simulated in a NDLP box, which is represented by the corresponding triclinic unit cell (**B**). The free receptor trimers were simulated in a rectangular box (**C**) and the free receptor monomers were simulated in a rhombic dodecahedron box (**D**).*

The aim of the present study was to investigate changes in the structure of DR5 induced by the interaction with TRAIL and thereby shed light on possible mechanisms of activation. To achieve this, a series of molecular dynamics simulations of the TRAIL-DR5 complex, the free receptor monomer, and the unbound receptor trimer have been performed. The study provides evidence for the self-association of the unliganded DR5 through the C-terminal cysteine-rich domain of the extracellular region of the receptor, with implications for the mechanism of activation.

METHODS

Starting structures

Initial structures were based on the available crystal structures (PDB codes 1D4V,[31] 1D0G,[30] and 1DU3[32]). For the simulations of the TRAIL-DR5 complex, the structure given in 1D4V was used as a starting configuration, because this is the only structure with a completely resolved AA″-loop in TRAIL. It is worth noting that the residues flanking the AA″-loop in structure 1D0G indicate a similar orientation of this loop, whereas in the structure 1DU3 these residues indicate a more extended conformation, lying tighter around TRAIL and having stronger interactions with the receptor units. However,

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attempts to model the loop in this configuration, e.g. by application of distance restraints, failed, resulting in the distortion of the whole complex (results not shown). This provided further support that the orientation of the AA″-loop as found in 1D4V and indicated by 1D0G is appropriate.

The structures 1D0G and 1DU3 contain a zinc atom coordinated by the three Cys230 residues in TRAIL. The structure 1D4V does not contain a zinc atom at this position. However, it has been shown that the presence of a zinc ion in the core of TRAIL is vital for the activity and stability of the protein.[36] For this reason a zinc ion was added at the equivalent position as found in the structure 1D0G. Furthermore, as Zn(II) is most commonly 4-coordinated, the chloride atom, resolved in 1D0G, but not found in 1DU3, was also added to the system. To describe the interaction between the Zn(II) and the protein, force field parameters for the so-called zinc finger domain, were taken from the literature.[37] The topology and parameters of the zinc finger domain are available as supplementary material.

The TRAIL-DR5 complex was simulated in a Near-Densest Lattice Packing (NDLP; molecular shaped) box.[38] This box, in conjunction with an algorithm to remove overall rotation,[39] allows one to minimize the number of solvent molecules. The volume of the simulation box was 630 nm^3. The system [Fig. 1(B)] contains the protein together with ~17 k water molecules giving ~60 k atoms in total. A rhombic dodecahedron box with the same minimal distance between periodic images would have a volume of 1530 nm^3 (~150 k atoms). We note that it has been previously shown that the use of the NDLP box does not significantly affect the dynamics of the system.[40] We also note that compared with the other systems simulated the complex is relatively rigid making it well suited to the use of the NDLP and that at no time did the periodic image approach closer than 1.0 nm. The production run of the TRAIL-DR5 complex had a length of 35 ns.

Simulations of the unbound receptor trimer were started from the configuration as given in 1D0G with TRAIL removed from the complex. Where possible, missing residues were modeled according to the configuration in the other units. Otherwise, they were modeled according to the configuration in 1DU3. The crystal structures only contain the extracellular domains of the receptor. To mimic the attachment to the membrane in the simulations of the unbound receptor, the C-termini of the units were constrained to the *xy*-plane. The system [Fig. 1(C)] was simulated in a rectangular box with a volume of 920 nm^3 (9.6 × 9.8 × 9.8 nm^3), containing ~29 k water molecules, giving a total of ~90 k atoms. Three replicate simulations were performed, each 20 ns in length.

Individual receptor monomers were simulated in order to characterize the extent of the conformational space accessible to the monomer and to determine the relative importance of rigid body motions in this system. The starting structures for these simulations were taken from each of the available crystal structures, giving 10 systems in total. Again, where necessary, missing residues were added according to the configuration of these residues as found in one of the other monomers. Each of the monomers was simulated in a rhombic dodecahedron box of 800 nm^3, solvated with ~26 k water molecules, giving a total system size of ~80 k atoms [Fig. 1(D)]. The free receptor monomers were simulated for 20 ns.

Simulations

Simulations were performed using the Gromacs package for molecular simulations[41–43] in conjunction with the GROMOS96 43a2 united atom force field.[44,45] Water was treated explicitly using the Simple Point Charge (SPC) model.[46] The protonation state of ionizable groups was chosen to correspond to pH 7.0. Counterions were added to compensate the net charge of the system. Nonbonded interactions were evaluated using a twin range cut-off of 0.9 and 1.4 nm. Interactions within the shorter range cut-off were evaluated at every step whereas interactions within the longer range cut-off were evaluated every 10 steps. To correct for the neglect of electrostatic interactions beyond the longer range cut-off, a Reaction Field (RF) correction[47] was used with $\varepsilon_{RF} = 78.0$. In all simulations the system was kept at a constant temperature of 300 K by applying a Berendsen thermostat.[48] Protein and solvent were independently coupled to the heat bath with a coupling time of 0.1 ps. For the simulations of the receptor trimer the pressure was weakly coupled to a reference pressure of 1 bar using a Berendsen barostat,[48] with a coupling time of 1.0 ps and a compressibility of 4.6×10^{-5} bar^{-1}. Simulations of the receptor monomers and of the TRAIL-DR5 complex were performed at constant volume. The time step used for integration of the equations of motion was 0.002 ps. The bond lengths and angle of the water molecules were constrained using the SETTLE algorithm.[49] Bond lengths within the protein were constrained using the LINCS algorithm[50] except for the simulation of the receptor ligand complex, where SHAKE[51] was used together with an algorithm to remove overall rotational and translational motions.[39]

Analysis

Solvent accessible surface

The solvent accessible surface area (SASA) was determined according to the method of Eisenhaber *et al.*[52] using a solvent probe radius of 0.14 nm. The classification of hydrophobic atoms was based on an absolute charge less than or equal to −0.2 *e*. The interfacial surface area (ISA), that is the area buried by the interaction of two components A and B in the complex, was determined from the difference between the sum of the

surface area of the separate components and the surface area of the complex:

$$\mathrm{ISA(AB)} = \mathrm{SASA(A)} + \mathrm{SASA(B)} - \mathrm{SASA(AB)}$$

Decomposition of the mean square displacement

The mean square displacement (MSD) for each frame with respect to the starting structure was determined, after performing a least-squares fit of all atoms to the starting structure, according to

$$\mathrm{MSD_{Total}} = \frac{1}{M}\sum_{i=1}^{N} m_i(\mathbf{x}_i - \mathbf{x}_{\mathrm{ref}})'(\mathbf{x}_i - \mathbf{x}_{\mathrm{ref}})$$

where m_i denotes the mass and $\mathbf{x}_i$ the coordinates of atom i. N is the number of atoms and M is the total mass. To determine the extent to which the dynamics of the systems could be expressed in terms of rigid body motions, the total MSD was decomposed into two components: a rigid body term and a residual term. The non-rigid body term or the residual component was defined as the MSD obtained after separately fitting each predefined domain to the corresponding domain in the reference structure, again using an all-atom least-squares fit:

$$MSD_{\mathrm{Residual}} = \frac{1}{M}\sum_{i=1}^{N} m_i(\mathbf{y}_i - \mathbf{x}_{\mathrm{ref}})'(\mathbf{y}_i - \mathbf{x}_{\mathrm{ref}}).$$

Here $\mathbf{y}$ denotes the coordinates of atom i after the fitting of the domains. The contribution of rigid body motions to the total MSD was taken to be the difference between $\mathrm{MSD_{Total}}$ and $\mathrm{MSD_{Residual}}$.

Principal components analysis (PCA)

PCA was used to analyze collective motions in the system. In PCA the fluctuations of the individual particles are described in terms of a set of mutually linearly independent collective fluctuations. To perform the PCA, a positional covariance matrix was constructed,

$$\mathbf{S} = \frac{1}{N}\sum_{k=1}^{N}(\mathbf{x}_k - \overline{\mathbf{x}})(\mathbf{x}_k - \overline{\mathbf{x}})^{\mathrm{T}} = \frac{1}{N}\sum_{k=1}^{N}\mathbf{x}_k\mathbf{x}_k^{\mathrm{T}} - \overline{\mathbf{x}}\overline{\mathbf{x}}^{\mathrm{T}}$$

where $\mathbf{x}_k$ is the positional vector representing the kth frame from the trajectory and N is the total number of frames. This matrix was subsequently decomposed into a diagonal matrix $\mathbf{D}$ of eigenvalues and a matrix $\mathbf{P}$ of associated eigenvectors:

$$\mathbf{S} = \mathbf{PDP}^{\mathrm{T}}$$

Typically, rotational and translational degrees of freedom are removed from the trajectory before construction of the covariance matrix by performing a least squares fit onto a reference structure. In addition to this standard approach, in this study PCA was also performed in which the least squares fit was performed using one specific domain. This exaggerates the fluctuations of the domains relative to the reference domain and was used to highlight hinge-bending motions between the domains of the extracellular part of the DR5.

To visualize the effect of ligand binding on the extent of motion within the system the trajectories were projected onto a selected set of eigenvectors, revealing the sampling along these vectors. The projection of a frame $\mathbf{x}(t)$ from a trajectory on a set of eigenvectors is given by

$$\mathbf{p}(t) = \mathbf{P}^{\mathrm{T}}(\mathbf{x}(t) - \overline{\mathbf{x}})$$

In addition, the loadings of individual atoms with a selected set of eigenvectors were also determined. The loadings indicate the extent to which the positional fluctuations of a given particle are described by a specific eigenvector. For each atom the loadings of the x, y, and z coordinates were calculated and summed to reveal the direction of the fluctuation along that eigenvector in Cartesian space. A higher value of the loading, represented as the length of the arrow, means the motion of the particle is more strongly correlated to the component specified. The matrix of loadings was calculated according to

$$\mathbf{A} = \mathbf{PD}^{1/2}$$

The nature of the collective motions was further analyzed by taking the extreme projections of the trajectories along a selected set of eigenvectors and processing these using the program DynDom.[53] DynDom determines the relative motions of domains, calculates hinge axes, and determines hinge-bending residues and rotation angles.

Principal components analysis was performed using the available tools within the Gromacs package and using Matlab.[54] The visualization of the results from the principal components analysis was performed using Pymol[55] and POV-Ray.[56]

RESULTS AND DISCUSSION

Self association of the unbound receptors

To investigate the fate of the individual receptors in the absence of TRAIL, a series of simulations of the three receptor units within the 1D0G complex from which TRAIL was removed were performed. A summary of the events observed in the simulations is presented in Figure 2(A), F2
which shows a sequence of stereo images covering the first 5 ns of a simulation in which the three receptor units are constrained to the XY plane to mimic the effect of attachment to the membrane. A similar sequence of events was also observed in two replicate simulations performed with different starting velocities. As can be seen in Figure 2(A),

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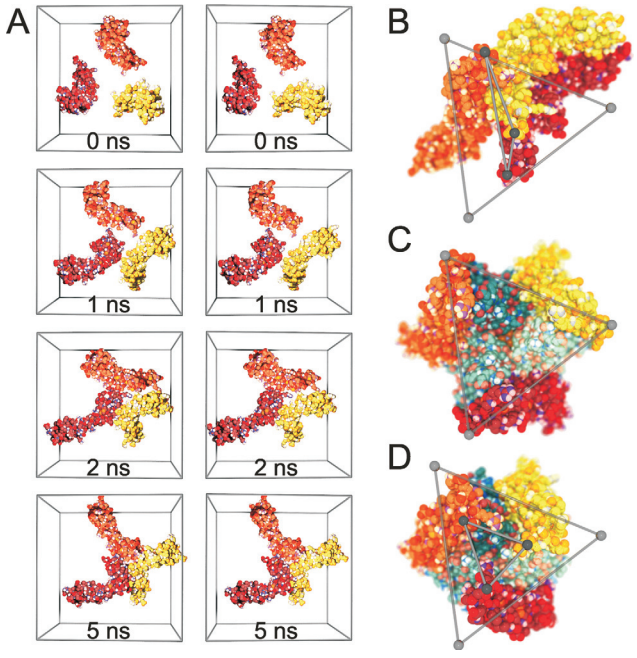

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Figure 2

*Self-association and C-terminal positions of Death Receptor 5 and interface surface areas (ISA). (**A**) Cross-eyed stereo images summarizing events observed in the simulations of the free receptor trimer. The sequence of images shows the evolution of the system over the first five nanoseconds and demonstrates the rapid aggregation of the receptor units. The images provide a top view of the Death Receptor, looking down from the N-termini. (**B**) Bottom view of the final structure of a simulation of the free receptor trimer. The outer triangle connects the C-terminal positions as present in the crystal structure, whereas the inner triangle connects the C-terminal positions as observed after 20 ns of simulation. Colors are as above. (**C**) Bottom view, looking up from the C-termini, of the crystal structure of the TRAIL-DR5 complex, with the C-terminal C-alpha atoms shown as corners of a triangle. Colors are as indicated above. (**D**) Bottom view of the final structure obtained from the TRAIL-DR5 complex simulation. The outer triangle connects the C-terminal positions as present in the crystal structure, whereas the inner triangle connects the C-terminal positions as observed after 35 ns of simulation. The TRAIL subunits are colored in different shades of green, the DR5 units are shown in yellow, orange and red.*

following the removal of TRAIL from the complex, the three receptor monomers rapidly self-associate. To confirm that this association is driven by interactions between the receptors as shown in Figure 2(A) and is not an artefact due to interactions between periodic images the minimum distance between receptors in different periodic images was determined. The distance was found to increase from 2.4 to 2.9 nm during the simulations showing that, despite the large changes in the relative orientation of the individual receptors evident in Figure 2(A), there are no interactions between periodic images. The complex obtained in the absence of TRAIL is not well ordered. This may reflect the limited time scale of the simulation. While there is a strong tendency for the receptors to aggregate, 20 ns is not sufficient to allow a system of this size to fully equilibrate. The final structures from the three independent simulations show significant differences in terms of the packing between the receptors and while indicative the final structure shown in Figure 2(A) is expected to rearrange further with time. In fact, as discussed below, in each of the simulations it was seen that initially two of the receptor units interacted with each other with the third unit following slightly later. This leads to an asymmetric arrangement of the receptor units in the aggregate. This is reflected in the distances between the Val^{138} C_α atoms (an indicator of the relative positions of the C-terminal positions) and is illustrated in Figure 2(B) which gives a bottom view of the aggregate formed in one of the simulations of the unliganded DR5 trimer, showing the relative orientations of the membrane proximal (CRD2) domains.

The aggregation of the receptors is associated with the burial of approximately 5.2 nm^2 of solvent exposed surface area per interaction interface. The surface buried is predominantly hydrophobic with the ratio of hydrophobic to hydrophilic surface area buried being between 1.2 and 1.5 depending on the precise interface formed in a given simulation. It is expected that the burial of this hydrophobic surface area provides the change in free energy that drives the association.[57–60] The surface area per residue buried in the interfaces in the simulations of

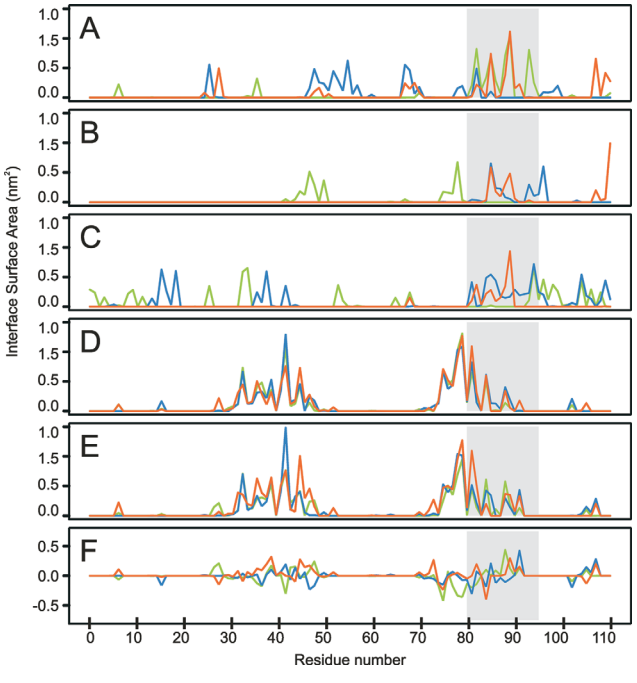

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Figure 3

*(**A–C**) Residue contributions to interface surface areas involving Death Receptor 5 in three simulations, time averaged over the last nanosecond. (**D**) DR5 residue contributions to the TRAIL-DR5 interface surface area time averaged over the first nanosecond of the simulation. (**E**) DR5 residue contributions to the TRAIL-DR5 interface surface area time averaged over the last nanosecond (34–35 ns). (**F**) Changes in residue contributions to the TRAIL-DR5 interface surface, given as the difference between the time averaged values from 34–35 and 0–1 ns. The different colors indicate the different surfaces in each system.*

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the unliganded receptor trimer, averaged over the last nanosecond of the simulation, is given in Figure 3(A–C). As noted above, different sets of residues are buried in interfaces formed in the different simulations. This reflects the fact that the systems have not fully equilibrated. However, one set of residues, indicated by the shaded area in Figure 3, was involved in the interactions between two out of three units in each of the simulations of the unbound trimer. This supports the suggestion that a specific interaction is formed between two receptors, with the third being less tightly bound.

The rapid association of the receptor units observed in the simulations after the removal of TRAIL is in agreement with recent SPR studies by Lee *et al.*, suggesting that the Death Receptor 5 has a propensity to homo-oligomerize.[27] Interestingly Lee *et al.*[27] propose that the dominant species is a dimer. The simulations, in contrast, suggest the formation of an asymmetric trimer, with the binding of two of the receptor units being stronger than that of the third. Note it is not possible from the current simulations to determine whether the dimer or the trimer is the most important species physiologically. For one, the effective concentration in the receptor within the simulation cell is much higher than that on the surface of the cell (assuming a homogenous distribution). Second, as the simulation cell contains only three receptor units the formation of a trimer is favored. Finally, the tighter interaction of two out of the three receptors could reflect the fact that the system might not be fully equilibrated. However, if, as suggested in the simulations, the third receptor unit binds much more weakly, this interaction could be easily overlooked in the SPR experiments leading to the conclusion that dimers were the predominant species.

The possibility that the receptors are pre-associated has major implications for the mechanism of activation. If the receptors are pre-associated, then activity must depend on the precise orientation or the relative positions of the extracellular binding domains and not merely on the bringing together of the intracellular death domains. For example, if dimers are the dominant species on the surface of the cell, the activation of DR5 by TRAIL could involve the crosslinking of receptor dimers, according to the expanding network hypothesis,[61] or the rearrangement of receptor dimers to form trimers. Alternatively, TRAIL may simply enforce symmetry onto a preformed but asymmetric trimer. In either case, the rearrangement of the extracellular domains leading to the repositioning of the cytosolic domains would be the critical step in the activation of the associated effector proteins (FADD).

The TRAIL-DR5 complex

Comparing the simulations of the TRAIL-DR5 complex to the simulations of the free receptors, two features are immediately obvious. The first is that, as observed in the case of the free receptors, in the simulation of the TRAIL-DR5 complex, the C-termini come closer together. This is illustrated in Figure 2(C,D), which show the relative orientations of the membrane proximal (CRD2) domains in the crystal structure of the TRAIL-DR5 complex and the TRAIL-DR5 complex after 35 ns of simulation, respectively. The second notable feature is that, unlike the simulations of the unliganded DR5, the symmetry between the three receptor units is retained. The high degree of symmetry within the structure at the end of the simulation is clearly evident from Figure 2(D). This can be contrasted with Figure 2(B), which shows the same view of the complex obtained in one of the simulations of the unliganded DR5 trimer.

The decrease in the distances between the C-termini observed in the simulations of the TRAIL-DR5 complex is the result of a change in the packing of the receptor around TRAIL. This is evident in Figure 3(D–F). Figure 3(D,E) shows the contributions of residues of DR5 to the interface with TRAIL in the simulation of the TRAIL-DR5 complex averaged over 1 ns at the beginning and end of the simulation (0–1 ns and 34–35 ns respectively). The changes in the interface of the DR5 in complex with TRAIL per residue between the start and end of the simulation are shown in Figure 3(F). As can be seen, there are significant differences in the residues that form the interface between the beginning and end of the simulation [Fig. 3(F)]. These changes are accompanied by a decrease in the solvent accessible surface of both the receptor units and TRAIL. It is difficult to determine to what extent these changes are physiologically relevant although it is expected that crystal packing forces, not present in the simulations, heavily influence the extended conformation of the C-terminal region in the initial structure.

Comparing Figure 3(A–C) with Figure 3(D,E) it can also be seen that many of the main residues consistently buried during the association of the free receptors (residues 80–95) are also those involved in the binding of TRAIL. This suggests that this region in particular may play an important role in switching between the active and inactive states.

Collective motions

Besides affecting structural properties, such as the conformation of the receptor units or their relative orientation, the binding of TRAIL will also affect the dynamic properties of the receptor units. To investigate possible changes in the collective motions within the system associated with the binding of TRAIL to DR5, principal component analysis (PCA) was performed on the receptor in the TRAIL-DR5 complex, the unliganded receptor trimer and the individual receptor monomers. It was found that the CRD1 and CRD2 domains are in practice quite rigid. This is illustrated in Figure 4(D), which shows the contribution of the interdomain motions to the total mean

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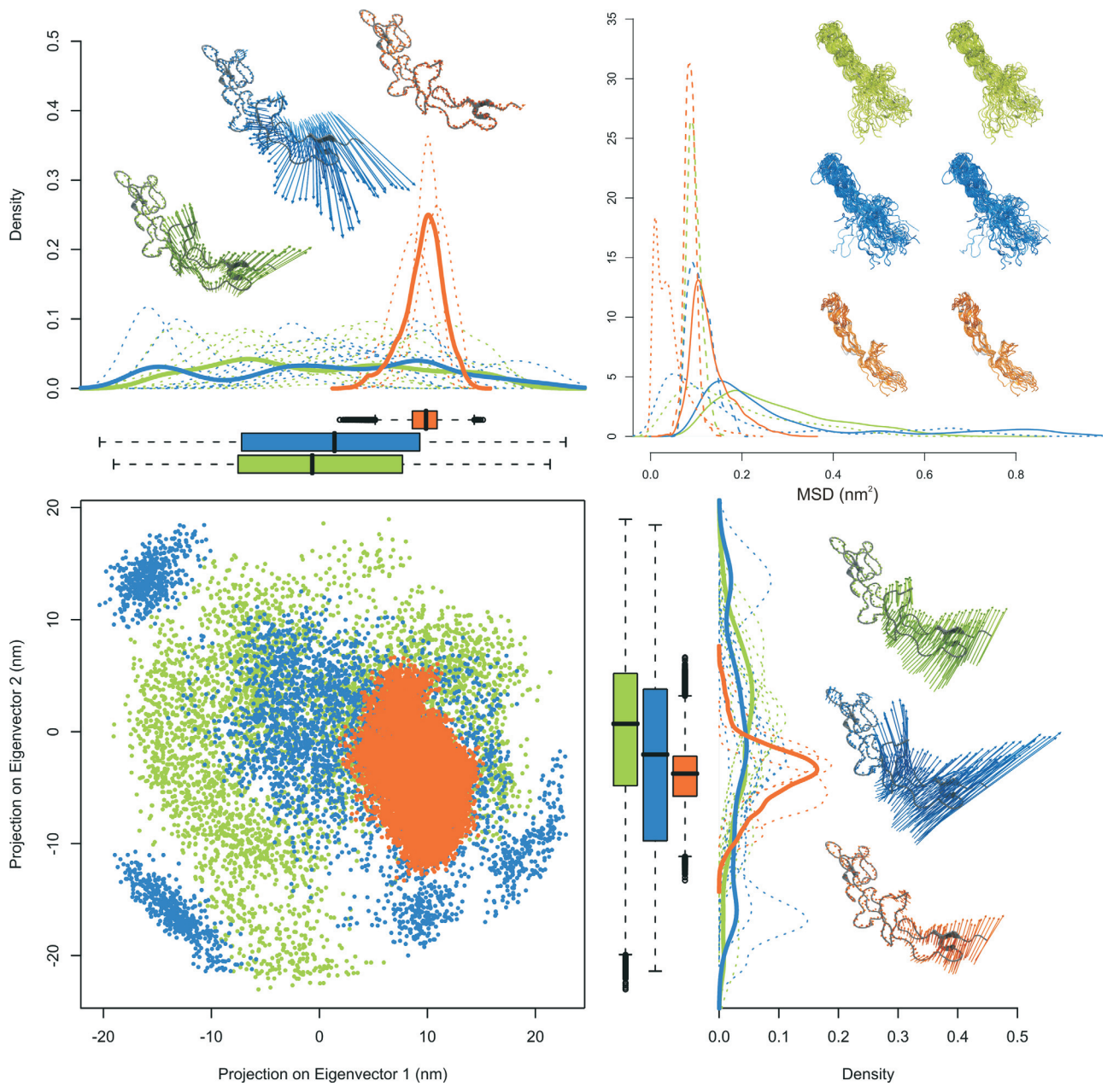

Figure 4

*Changes in collective motions in Death Receptor 5 due to self-association and TRAIL-binding. (**A**) Projection of the second half of each trajectory onto the first two eigenvectors obtained from the combined simulations of the free receptor monomer in solution. Projections of the free receptor monomer are shown in green, projections of the receptor trimer are shown in blue and projections of the DR5 in complex with TRAIL are shown in orange. Taking the free receptor monomer as a reference, it is clear that the DR5 is likely to sample more extreme regions, whereas the extent of sampling is strongly inhibited in the case of the TRAIL bound DR5. (**B**) Box plots and solid lines show the density of the combined projections of each set of simulations on the first eigenvector obtained from the combined simulations of the free receptor monomer in solution. Dotted lines show the densities for individual simulations. The colors correspond to those in panel A. (**C**) Box plots and solid lines show the density of the combined projections of each set of simulations on the second eigenvector obtained from the combined simulations of the free receptor monomer in solution. Dotted lines show the densities for individual simulations. The colors correspond to those in panel A. (**D**) Decomposition of the mean square deviations (MSD) for the free receptor monomer in solution (green) and the TRAIL bound receptor (orange). The distribution of the total MSD is shown with solid lines, whereas the rigid body and residual fluctuation MSD components are given with dotted lines and dashed lines, respectively. The stereo views on the right give an impression of the range of conformations sampled in the simulations.*

square displacement. As can be seen, most of the fluctuations involve the relative motion of one domain with respect to the other. For this reason, it was chosen to perform the PCA using only one of the domains to align the molecules in order to highlight the relative motions between the domains. Such a linear transformation of

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Table I
Eigenvalues and Their Cumulative Sums for the First 12 Eigenvectors

	Free monomer		Free trimer		Bound trimer	
Index	Eigenvalue (nm^2)	Cumulative (%)	Eigenvalue (nm^2)	Cumulative (%)	Eigenvalue (nm^2)	Cumulative (%)
1	73.73	50.97	145.65	50.00	10.65	54.25
2	43.27	80.88	119.93	91.17	3.22	70.64
3	8.20	86.55	14.56	96.16	1.49	78.22
4	6.74	91.21	2.44	97.00	0.98	83.21
5	2.67	93.05	2.33	97.80	0.47	85.60
6	1.25	93.92	1.18	98.21	0.42	87.76
7	1.21	94.75	0.92	98.52	0.32	89.38
8	1.05	95.48	0.51	98.69	0.20	90.41
9	0.72	95.98	0.37	98.82	0.18	91.31
10	0.50	96.32	0.35	98.94	0.15	92.08
11	0.46	96.64	0.28	99.04	0.13	92.74
12	0.42	96.93	0.24	99.12	0.12	93.35

Eigenvectors and values were obtained per set by decomposition of the covariance matrices of concatenated trajectories of the monomers in solution, the receptor units in the simulations of the free receptor trimer and the receptor units in the simulation of the TRAIL-DR5 complex, respectively. Note that in each case, the eigenvectors are arranged in order according to magnitude. A given index does not necessarily correspond to the same eigenvector in the different systems.

the coordinates results in a quadratic transformation of the positional covariance matrix changing the quantitative, but not the qualitative information. It is important to note, however, that the order of the principal components obtained after PCA is affected with more weight being given to inter-domain motions. Note also that the data presented in Figure 4 represents the combined results for the three receptor units simulated as free monomers (green), as the unliganded-trimer (blue) and as the complex (orange). Results were combined for each set of simulations separately and projected onto the eigenvectors obtained for that set. There was, however, a close correspondence between the eigenvectors of the different sets (data not shown).

The first 12 principal components and their (relative) cumulative sums are given in Table I. As can be seen, the unliganded trimer has the largest range of conformations, followed by the free monomer. As expected, the binding of TRAIL dramatically decreases the conformational freedom of the receptor. Figure 4 summarizes the results of the PCA with regards to the first two eigenvectors, which describe the hinge-bending motions of CRD1 and CRD2. The flexion point identified by comparing the bound DR5 with TNF-R1[35] appears to be part of a very flexible hinge region connecting two otherwise freely moving domains. DynDom[53] analysis of the extreme projections of each set of trajectories on these two eigenvectors was used to determine the total angle of rotation around the hinge axes. In the case of the free receptor monomer and the free receptor trimer the rotations were very similar (49° and 39° compared to 55° and 44° respectively). In the presence of TRAIL, these rotations were reduced to 26° and 20°.

The third principal component describes the degree of deflection and is complementary to the hinge-bending motions described by the first two components. The fourth principal component describes a twisting motion of CRD1 relative to CRD2. Again, DynDom analysis was used to determine the total twist based on the extreme projections of the trajectory. The degree of twist was much larger in the case of the free monomer and trimer (81° and 91° respectively) than for the bound trimer (43°). The higher order principal components capture primarily local motion.

In summary, the motions within the free monomer and the free trimer were found to be very similar. It was observed that in the simulation of the unbound trimer more bent conformations were sampled, giving rise to slightly larger eigenvalues, suggesting a higher degree of motility. The presence of TRAIL strongly inhibits the hinge-bending and twisting motions. This is reflected in the narrow distributions of the projections on the first two eigenvectors seen in Figure 4(B,C). Interestingly, the differences between motions of the receptor units within trimers with and without TRAIL is almost exclusively due to differences in the rigid body motions as measured by the between-domains component of the mean square displacement (MSD) shown in Figure 4(D). Thus, the receptor could be considered to be a mechanical construct in which the interactions are determined by hinge-bending and twisting motions.

Comparison with experiment

In their SPR experiments Lee *et al.*[27] found that the on-rate (association) was slower than the off-rate (dis-

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sociation). On the basis of this they proposed that self-association was coupled to a conformational change within the receptor units. The simulations suggest that an unliganded receptor subunit readily samples a wide range of conformations including those found in the TRAIL bound state. The projections shown in Figure 4 also reveal that, while the unliganded receptor monomers show more or less uniform sampling, the receptor units in the unliganded receptor trimer yield a non-uniform distribution with more sampling of extremely bent conformations. It is possible that this finding corresponds to the conformational change proposed by Lee *et al.*[27] However, the time-scales on which the bending is observed are small compared to the time-scales expected for conformational changes associated with a difference between the on-rate and off-rate in SPR experiments.

The activation of DR5 by TRAIL

The simulations suggest that TRAIL affects three factors presumably necessary for alignment of the intracellular domain and the activation of DR5. First, TRAIL enforces threefold-symmetry onto the receptor complex. In the absence of TRAIL, the receptor units associate to form an asymmetric trimer preventing the alignment of the intracellular domains. Second, TRAIL restricts the conformational freedom of the extracellular binding domains. This would increase the probability that the intracellular domains align and form an active complex. Third, the binding of TRAIL is associated with a large change in the degree of twist within the receptor units with respect to the unbound form. This change in twist angle will affect the relative orientation of the membrane-spanning α-helical domains and in turn alter the alignment of the cytosolic domains. We note that a similar twist mechanism has previously been proposed for the dimeric erythropoietin receptor[62] and more recently for the growth hormone receptor.[63]

Note, the simulations can only provide information on the nature of conformational changes associated with the binding of TRAIL. It is known that other factors are also involved in the process of activation. For example, experiments involving the treatment of resistant carcinogenic cells with resveratrol suggest that there is a requirement for Death Receptors to be localized in lipid rafts.[64] Most likely a combination of factors, including those listed above, is needed to explain the almost binary on/off effect that TRAIL exerts when initiating apoptosis.[65]

The association and structural changes observed in the simulations after the removal of TRAIL from the complex could in principle be verified by experiment. If activation involved the crosslinking of initially independent receptors by a multidentate ligand, then the binding response would be expected to be dependent on the concentration of the receptor. In addition it would be expected to show a distinct maximum as increasing concentration of the ligand will eventually lead to the formation of inactive one-to-one complexes.[66] If the receptor is pre-associated, these effects would not be observed. The importance of the precise spacing or orientation of the receptor could be assessed by introducing specific spacers into the transmembrane domains to vary the relative positions of the intra- and extracellular domains as has been done in related systems.[62,67]

Finally, we note that the work represents one of the few examples in which protein-protein self-association has been seen in simulation. This area has recently attracted attention in regard to the process of dewetting of hydrophobic surfaces.[68,69] While in the current case the interactions between the surfaces are not sufficiently regular to investigate whether similar processes to that observed by Liu *et al.*[69] occur the work does highlight the fact that the simulation of the assembly of large scale regulatory complexes in atomic detail is becoming possible.

CONCLUSIONS

Simulations of the Death Receptor 5, both unbound and in a complex with its ligand TRAIL have been performed. It was found that in the absence of TRAIL, the receptor units rapidly self-associate to form an asymmetric trimeric complex stabilized by interactions between the CRD2 domains. This finding is in line with recent experimental studies, suggesting that a number of cytokine receptors, including the four receptors for TRAIL, exist as preformed complexes. The asymmetric nature of the unbound complex with the interaction to the third receptor unit being relatively weak might also explain the observation of dimers as opposed to trimers in SPR studies.

The simulations suggest that the unbound DR5 receptors initially reside at the cell surface as asymmetric trimers. We propose that in these complexes the distance and relative orientation between the C-termini of the extracellular domains of the receptor prevent the formation of an active trimeric cytoplasmic death domain. The primary role of TRAIL is to enforce threefold symmetry on the complex, which leads to the repositioning of the receptor units. In addition, TRAIL strongly restricts the conformational freedom of the extracellular domains, which might also contribute to stabilizing the activated state of the death complex.

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