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Molecular Dynamics Simulations and Functional Characterization of the Interactions of the PAR2 Ectodomain with Factor VIIa

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Abstract

Signaling of the tissue factor-FVIIa complex regulates angiogenesis, tumor growth and inflammation. TF-FVIIa triggers cell signaling events by cleavage of protease activated receptor (PAR2) at the Arg36-Ser37 scissile bond. The recognition of PAR2 by the FVIIa protease domain is poorly understood. We perform molecular modeling and dynamics simulations to derive the PAR2-FVIIa interactions. Docking of the PAR2 Arg³⁶-Ser³⁷ scissile bond to the S1 site and subsequent molecular dynamics leads to interactions of the PAR2 ectodomain with P and P' sites of the FVIIa catalytic cleft as well as to electrostatic interactions between a stably folded region of PAR2 and a cluster of basic residues remote from the catalytic cleft of FVIIa. To address the functional significance of this interaction for PAR2 cleavage, we employed two antibodies with epitopes previously mapped to this cluster of basic residues. Although these antibodies do not block the catalytic cleft, both antibodies completely abrogated PAR2 activation by TF-FVIIa. Our simulations indicate a conformation of the PAR2 ectodomain that limits the cleavage site to no more than 33 Å from its membrane proximal residue. Since the active site of FVIIa in the TF-FVIIa complex is ~75 Å above the membrane, cleavage of the folded conformation of PAR2 would require tilting of the TF-FVIIa complex toward the membrane, indicating that additional cellular factors may be required to properly align the scissile bond of PAR2 with TF-FVIIa.

Keywords

TF-FVIIa signaling; PAR2 cleavage; serine protease; molecular dynamics simulation

INTRODUCTION

Tissue factor (TF) is a membrane-bound cell-surface protein and the primary initiator in the extrinsic pathway of the coagulation cascade (reviewed in References¹⁻³). This pathway starts with TF's exposure to the blood stream upon vessel damage, binding to its natural ligand the blood-borne factor VII (FVII), and activation of FVII to FVIIa. The TF-FVIIa binary complex activates the factor X (FX) to FXa, which eventually leads to blood clotting and drives downstream signaling by thrombin. Thus, the complex has been well known for its role in the initiation of coagulation. Besides this hemostatic role, the TF-FVIIa complex has also been found in recent years to regulate a broad range of non-hemostatic cellular responses including gene transcription, protein translation, apoptosis, and cytoskeletal reorganization (reviewed in References^{4,5}). These cellular responses in turn affect several

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cellular processes including inflammation, angiogenesis, and the pathophysiology of cancer and atherosclerosis (reviewed in reference⁴). The TF-FVIIa complex elicits calcium transients in various cell types, and activates the three major MAP kinase family members p42/p44, p38, and JNK⁵.

The primary signaling pathway of the TF-FVIIa complex involves activation of protease-activated receptors (PARs)⁵. PARs are G-protein-coupled receptors that have seven trans-membrane helices and are activated through proteolytic cleavage of their extracellular N-terminal domains by proteases (reviewed in Reference⁶). The cleaved PAR N-terminus functions as a tethered ligand that folds back into PAR's binding pocket and activates PAR, which leads to initiation of intracellular signaling cascades (reviewed in Reference⁶). Among the four PARs discovered so far (PAR-1, PAR2, PAR-3, PAR-4), only PAR2 is directly cleaved by the TF-bound FVIIa, but both PAR-1 and PAR2 can be cleaved by FXa (reviewed in Reference⁵). The TF-FVIIa signaling through PAR2 regulates gene transcription and protein translation, cell proliferation and survival, or cell motility and integrin activation (reviewed in Reference⁵). In tumor cells, this signaling pathway regulates pro-angiogenic growth factor expression as well as integrins involving crosstalk with the TF cytoplasmic domain (reviewed in Reference⁷).

The importance of TF-FVIIa signaling through PAR2 drives us to investigate how the serine protease domain of FVIIa cleaves the extracellular N-terminal ectodomain of PAR2.

Although it is known that a serine protease cleaves PAR2 at the site between Arg36 and Ser37 to yield the tethered ligand SLIGRL⁸, there is no structure or model to demonstrate what PAR2-FVIIa interactions other than those at the catalytic site are required to facilitate the cleavage. Knowing these interactions is essential not only to understand the cleavage mechanism but could also be useful in the development of inhibitors that selectively block this cleavage and the related signaling pathways. For example, to suppress tumor growth⁷, it may be possible to develop a FVIIa inhibitor that selectively prevents PAR2 cleavage while still allowing coagulation initiation.

In this study, we perform molecular dynamics simulations and antibody epitope analysis to derive the PAR2-FVIIa interactions. First we built an extended PAR2 extracellular n-terminal polypeptide model with its Arg36 and Ser37 engineered into the catalytic site of FVIIa protease domain. We then simulated the complex of PAR2 and FVIIa protease domain for 4 ns in implicit solvent (to drive initial condensation of the PAR2 ectodomain and its approach to FVIIa) followed by 54 ns in explicit solvent (for simulating binding and sampling equilibrium configurations). The last 18-ns equilibrium simulation of the explicit solvent molecular dynamics has been used for analyzing PAR2-FVIIa interactions. Finally we report the resulting model interactions and an antibody study reporting on interactions distal to the active site.

METHODS

1. Starting Structure

The starting structure was built based on the crystal structure (PDB ID: 1DAN⁹) of the TF-FVIIa complex inhibited by a partial-peptide compound Dpn-Phe-Arg-Ch2 (D-Phe-Phe-Arg-chloromethylketone with chloromethylketone replaced by a methylene group). As the six human PAR2 amino acids at the cleavage site are Lys34-Gly35-Arg36-Ser37-Leu38-Ile39 (whole sequence in References^{10,11}), we altered the compound's Dpn residue to Lys34, mutated Phe to Gly35, kept Arg as Arg36, and changed Ch2 to Ser37. We adjusted the configuration of Ser37 to satisfy the required interactions with the catalytic site of a serine protease (see Figure 11–27 of ¹²); the FVIIa catalytic site was monitored through the simulations see – Figure 1). Then we appended the ends of this four-amino acid peptide with

the n-terminal Met1 and c-terminal Gly71 peptides respectively (Figure 2(A)). Gly71 is the last residue of PAR2 extracellular ectodomain prior to the first transmembrane helix in our human PAR2 homology model (Zhang Q, Ruf W, & Olson AJ, unpublished). (In the UniProtKB/Swiss-Prot database, human PAR2 residues 1–75 are marked as extracellular (<http://ca.expasy.org/uniprot/P55085>); thus residues 72–75 are still extracellular although they are part of the first transmembrane helix.) The PAR2 n-terminal polypeptide was elongated on both sides of the Arg36-Ser37 scissile bond, leaving this area rigid and adjusting certain torsions of adjacent PAR2 polypeptide residues to reduce steric clashes where the chain enters and exits the FVIIa protease active site.

FVIIa has four domains: Gla, EGF1, EGF2, and protease^{9,13}. As PAR2 is cleaved by the protease domain and there is no evidence that the PAR2 ectodomain interacts with other FVIIa domains, we removed FVIIa's Gla, EGF1, and EGF2 domains as well as TF from the TF-FVIIa complex in order to simulate the PAR2-FVIIa complex more efficiently. In the simulations, the FVIIa protease domain residues that interact with TF and FVIIa's other domains are kept rigid to mimic the bound state. Water molecules that are more than 3 Å from the FVIIa protease domain were removed as well as the cacodylate ion that is part of the crystallization buffer. The only ions remaining in the structure are one Ca²⁺ ion and one Cl[−] ion. The Ca²⁺ ion is essential for activation of FVII by TF¹⁴ and is located on the loop from Gly69 to Glu80⁹. The Cl[−] ion is not documented to be of significance and may come from the CaCl₂ solution. We kept this ion at the beginning of the simulation but eventually removed it during explicit-solvent molecular dynamics. PAR2 residue Asn30 is modified by an N-Acetyl-D-Glucosamine (NAG) because glycosylation on this residues regulates human PAR2 expression and/or signaling¹⁵. The NAG moiety was taken from the crystal structure of endothelial protein C receptor (EPCR) complexed with Gla domain of protein C (PC Gla) (PDB ID: 1LQV¹⁶) in which there are 6 NAG-modified asparagines. The modeling was done in InsightII (Accelrys), and the resulting starting structure is shown in Figure 2(A).

2. Force Field Assignments

Simulations were performed in AMBER 9¹⁷ with the ff99 force field¹⁸. This force field contains standard amino acids but no NAG-modified asparagines (NAG-Asn). Here we describe the procedure to build the topology and partial charges for NAG-Asn. The topology can be derived from any existing NAG-Asn residue, such as those in the EPCR-PC Gla complex (PDB ID: 1LQV¹⁶). The partial charges, however, should be derived from all possible conformations of this residue since the conformation of this NAG-Asn30 residue is unknown. The NAG-Asn conformations were generated as combinations of three parts: (1) Asn conformations, (2) Asn-NAG torsions, and (3) NAG torsions. For the first part, AMBER gives two conformations C₅ and α_R with different torsions¹⁹. For the second part, we sampled the torsions from the six NAG-Asn residues in the EPCR-PC Gla complex found two conformation classes named as L1 and L2 here. For the third part, the torsions were sampled from the same six NAG-Asn residues and were found to have two conformation classes as well, named as N1 and N2 here. Thus, we sampled a total of eight NAG-Asn conformations. While ideally the Asn-NAG torsions and NAG torsions should be sampled more extensively, the NAG-Asn30 residue was not a critical element of this study.

Asn:	ϕ	ψ	χ_1	χ_2
C ₅	217	160	297 (g ⁺)	263
α_R	−60	40	180 (t)	269

Asn:	ϕ	ψ	χ_1	χ_2
Asn-NAG:	Asn:CB—Asn:CG— Asn:ND2—NAG:C1		Asn:CG—Asn:ND2— NAG:C1—NAG:C2	
L1	170±5		153±7	
L2	-180±27		-143±13	
NAG:	NAG:C1—NAG:C2—NAG:N2—NAG:C7			
N1	138±17			
N2	89±2			

These eight conformations combined were used to compute the NAG-Asn atomic partial charges following the multi-conformational RESP procedure for amino acids¹⁹. The procedure was partially automated by the R.E.D. II program²⁰, but the geometric optimization option or the molecular re-orientation option in the program was not applied. R.E.D. II in this case was used to sequentially execute (i) the Gaussian 03 program²¹ to compute the molecular electrostatic potential for each NAG-Asn conformation and then (ii) AMBER's RESP program¹⁹ to fit the atom-centered partial charges to the grid determined in the previous step. In the second step, molecular equivalences between the eight conformations were applied in addition to the required atomic equivalences among each conformation¹⁹, thus all the conformations have the same partial charges. The derived NAG-Asn topology and partial charges in the AMBER PREPI file format¹⁷ are provided in the Supporting Information S1.

3. Simulations in Implicit Solvent

The starting structure has the PAR2 n-terminal polypeptide in an extended conformation from residue Met1 to the cleavage site and from the cleavage site to residue Gly71 (Figure 2(A)). Simulations of this starting structure in explicit solvent would have been extremely expensive since solvent has to fill a large box that encloses the entire extended complex. Thus, implicit solvent was used at the beginning to allow PAR2 to quickly condense and approach the FVIIa protease domain; then explicit solvent was used to simulate the binding and equilibrium configurations. All simulations were performed using AMBER 9¹⁷ with the ff99 force field¹⁸. The Ca²⁺ ion, which did not interact with PAR2 at any point of the implicit-solvent simulations, was distance-restrained to the oxygen atoms in the Ca²⁺ coordinated system.

The implicit solvent simulation used a Generalized Born model²² (igb=5) with a distance cutoff of 16 Å. It began with a four-step energy minimization: (1) 500 steps of steepest descent (SD) and 500 steps of conjugate gradient (CG) with a positional restraint of 100 kcal/mol/Å² on all heavy atoms including waters in order to equilibrate the hydrogen atoms; (2) 200 SD and 300 CG with a positional restraint of 100 kcal/mol/Å² on heavy atoms of PAR2's Arg36-Ser37 and FVIIa in order to partially remove steric collisions between PAR2 and FVIIa; (3) 200 SD and 300 CG with a positional restraint of 100 kcal/mol/Å² on heavy atoms of FVIIa only to further remove the steric collisions; (4) 50 SD and 300 CG with a positional restraint of 10 kcal/mol/Å² on heavy atoms of the rigid FVIIa residues. The rigid residues are those in FVIIa protease domain that are within 5 Å of TF and other FVIIa domains. These residues do not interact with PAR2, and the restraint keeps the FVIIa protease domain in the form with which it binds to TF and the other FVIIa domains. In the energy minimizations, SHAKE (bond length constraints)²³ was not used.

After energy minimization, the molecular dynamics simulation was initiated with a 10 ps equilibration phase to increase the system temperature from 0 to 310 K using Langevin

dynamics (ntt=3) with a collision frequency of 5.0/ps. A restraint of 1 kcal/mol/A² was applied to all residues to keep them stable during heating. The equilibration was followed by 4 ns of production dynamics with a constant 310-K temperature weakly coupled at every 2 ps (ntt=1²⁴). A restraint of 1 kcal/mol/A² was applied to the heavy atoms of PAR2's Arg36-Ser37 residues, all FVIIa residues, ions, and waters in order to focus the computation on the PAR2 n-terminal peptide folding and approach to FVIIa. Other settings in molecular dynamics included the use of SHAKE (ntc=2; on bonds involving hydrogen), a time step of 2 fs, a salt concentration of 0.15 M, a solvent dielectric constant of 78.5, and the option to carry out GB/SA (generalized Born/surface area) simulation with surface area computed using the LCPO model²⁵ (gbsa=1). The resulting structure is shown in Figure 2(B).

4. Simulations in Explicit Solvent

The explicit solvent simulation was initiated from the last snapshot of the implicit-solvent simulation (Figure 2(B)). This structure was first axis-aligned and then solvated in a TIP3 water²⁶ box with buffer sizes of 10, 8, and 15 Å in the X, Y, and Z directions, respectively (a larger buffer size in Z direction was specified to prevent the solute from approaching the box edges in that direction). The system was neutralized with 44 Na⁺ and 50 Cl⁻ counter ions, resulting in a physiological ionic strength of 0.15 M given the solvent volume in the water box. The system was then energy minimized without SHAKE in three steps: (1) 500 SD and 5500 CG with a restraint of 100 kcal/mol/A² on the solute heavy atoms to relax hydrogen atoms; (2) 200 SD and 800 CG with a restraint of 10 kcal/mol/A² on the solute heavy atoms to further relax hydrogen atoms and allow slight movement of the heavy atoms; (3) 200 SD and 800 CG with no restraint to relax all atoms.

The energy minimization was followed by a four-step equilibration with SHAKE and a time step of 2 fs: (1) Heat up – 100,000 steps to increase temperature from 1 to 300 K, coupled at every 0.5 ps, at constant volume with a 10 kcal/mol/A² restraint on the solute; (2) Constant pressure – 100,000 steps at 300 K, coupled at every 1 ps, with a 5 kcal/mol/A² restraint on the solute; (3) More constant pressure – 100,000 steps at 300 K, coupled at every 2 ps, with a 3 kcal/mol/A² restraint on the solute; (4) Final equilibration – 200,000 steps at constant pressure and 300 K, coupled at every 4 ps, with a 3 kcal/mol/A² restraint on the rigid FVIIa residues.

The equilibration was followed by 53-ns of production dynamics (1 to 54 ns) at constant pressure with the use of SHAKE and a time step of 2 fs. In this long simulation, we carefully monitored the stability of the FVIIa catalytic site, the stability of the Ca²⁺ coordination system, the stability of the crystal Cl⁻ ion, the interactions between PAR2 and FVIIa, and whether the water box was still large enough for the PAR2 ectodomain undergoing conformational changes. This simulation was initiated from the last snapshot of the equilibration phase (at 1 ns) with a restraint of 3 kcal/mol/A² on the rigid FVIIa residues, FVIIa catalytic site residues, and PAR2's Arg36-Ser37. The simulation subsequently underwent four parameters-changing events: (1) at 11 ns, the restraint on FVIIa catalytic site and PAR2's Arg36-Ser37 was removed in order to allow all interacting residues to move freely (the restraint on the rigid FVIIa residues was retained throughout); (2) at 15 ns, the atomic charges of the Gly71 carboxyl group were zeroed out to mimic Gly71 linked to the next amino acid; (3) at 19 ns, the atomic charge of the non-essential crystal Cl⁻ ion was changed to zero to allow it to leave the PAR2-FVIIa interface (which it did); (4) at 33 ns, the system temperature was increased from room temperature of 300 K to a physiological temperature of 310 K. The PAR2-FVIIa complex structure averaged over the last 18 ns (36 to 54) is shown in Figure 2(C).

In the explicit solvent simulations, the generalized Born model was not used (igb=0). The particle mesh Ewald method was used to calculate the long-range electrostatic interactions.

The non-bonded distance cutoff was reduced to 8 Å, and the Ca²⁺ ion was not distance-restrained to its binding oxygen atoms in case the ion interacted with PAR2. The Ca²⁺ ion is stable throughout the simulations and was not found to interact with PAR2. The van der Waals parameter for Ca²⁺ ($R=1.3263$ Å, $\epsilon=0.4497$ kcal/mol) was derived from Aqvist's work²⁷ instead of using the one in AMBER's default ff99 force field parm99.dat ($R=1.7131$ Å, $\epsilon=0.459789$ kcal/mol for "C0"). The Aqvist's parameter has the radius R close to those used in the force fields of CHARMM (1.3670 Å²⁸) and OPLSAA²⁹ (1.3537 Å).

5. Analysis of Simulations

Root mean squared deviations (RMSD), residue distances, and average structures were computed by AMBER's ptraj program¹⁷. Hydrogen bonds were computed by the CARNAL program in AMBER 8 (documented in AMBER 7 or earlier versions). Plots of RMSD and residue distances are drawn in Python using the matplotlib module (matplotlib.sourceforge.net). Molecular structures are visualized in either InsightII (Accelrys) or PMV (mgltools.scripps.edu).

6. Antibody Study

TF-FVIIa signaling was studied in human umbilical vein endothelial cells that were transduced with adenovirus to express high levels of TF and PAR2³⁰. Cells were serum starved for 5 h, before stimulation with 10 nM FVIIa in the presence of 50 µg/ml anti-FVII antibody 12D10 or 12C7. TF-FVIIa signaling was quantified by TagMan analysis measuring TR3 nuclear orphan receptor gene induction after 90 minutes of stimulation. For TagMan (Applied biosystems) 2 µg total cellular RNA was reversed transcribed using oligo-dT primers (Superscript II reverse transcriptase, Invitrogen). All samples were normalized with human glyceraldehyde phosphate dehydrogenase (GAPDH). The epitopes of these antibodies have previously been mapped in detail with Ala exchange mutants in the FVIIa protease domain³¹. In control experiments, the inhibitory effect of these antibodies on factor Xa (FXa) generation was confirmed using a parallel reaction, where the cells were incubated in the presence of 100 nM FX. FXa generation was measured using a chromogenic assay, as previously described³⁰.

RESULTS

1. FVIIa Catalytic Site

The FVIIa catalytic site was carefully monitored throughout the simulations to ensure that the key interactions between the FVIIa catalytic site and PAR2's Arg36-Ser37 were maintained. *In vivo*, these interactions are directly involved in PAR2's cleavage by FVIIa. In our simulations these known interactions were used to tether the remaining PAR2 polypeptide, helping to accelerate interactions with the FVIIa protease domain. Even though *in vivo* some parts of the PAR2 ectodomain may bind FVIIa before docking of the Arg36 P1 residue, the simulation (started with Arg36-Ser37 in place) serve as an efficient way of discovering the binding modes between the PAR2 ectodomain and the FVIIa protease domain. Simulations to derive the final binding mode without any PAR2 residues in place would be highly challenging with current simulation techniques and computational speeds.

Figure 1 shows the FVIIa catalytic site after implicit-solvent energy minimization (Left) and explicit-solvent molecular dynamics (Right). FVIIa's Asp102, His57, and Ser195 form the catalytic triad³², while the amines of Ser195 and Gly193 form the oxyanion hole³² hosting PAR2's Arg36:O (based on Figure 11–27 of ¹²). FVIIa's His57 should have a hydrogen on the delta nitrogen (ND1) but not on the epsilon nitrogen (NE2), thus it is named as Hid57 (AMBER nomenclature). At the beginning (Left), His57:ND1 donates two hydrogen bonds to Asp102:OD1 & OD2 and His57:NE2 accepts a hydrogen bond from PAR2's Ser37:N.

The side-chain oxygen of Ser37 (OG) forms a hydrogen bond with both of the nitrogens of FVIIa's His57. These two hydrogen bonds are apparently not required for cleavage by a serine protease and thus may have been artificially created in building the starting structure. They are eventually lost during molecular dynamics. The right-hand panel of Figure 1 shows the catalytic site averaged over the last 18 ns of the 54-ns explicit-solvent molecular dynamics simulation. In the last 43 ns, there were no positional restraints on the FVIIa catalytic site or PAR2's Arg36-Ser37. Thus, PAR2's Ser37 rotates away from FVIIa's His57 and towards FVIIa's Gly193. FVIIa's His57:NE2, previous forming a hydrogen bond with PAR2's Ser37:N, loses this hydrogen bond, forms a new hydrogen bonds with PAR2's Arg36:N and with FVIIa's Ser195:OG. This is a step back from cleavage as seen in the transition state diagram (Figure 11-27 of ¹²) where His57:NE2—Ser195:OG hydrogen bond occurs before, not after, the His57:NE2—Ser37:N hydrogen bond. It suggests that the simulations captured both hydrogen bonds at different times, but they did not appear in the right time sequence since bond formation and breaking at Arg36 and Ser37 were not part of the simulation. The four hydrogen bonds on the side-chain of Arg36 however were very stable despite movements of Asp189, Ser190, and Gly219 during the simulations. Overall, the transition state of the FVIIa catalytic site is maintained throughout the simulations thus facilitating meaningful interactions of other parts of the PAR2 extracellular polypeptide with the FVIIa protease domain.

2. Structural Changes Along Simulation Trajectories

With PAR2's Arg36-Ser37 in place, the other parts of the PAR2 ectodomain fold and approach the FVIIa protease domain as demonstrated in Figure 2, from starting structure (A) to the end of the 4-ns implicit solvent molecular dynamics (B) and to the last 18-ns average of 54-ns explicit-solvent molecular dynamics (C). The implicit solvent molecular dynamics is stopped before other parts of the PAR2 bind to FVIIa. This allows water molecules to be added between PAR2 and FVIIa in order to simulate their binding in a fully solvated environment. The RMSDs of the FVIIa protease domain and PAR2 ectodomain as a function of simulation time in explicit solvent are shown in Figure 3. The four vertical lines mark the four parameter-changing events in the simulation (see METHODS). The FVIIa RMSD increases to 0.8 Å during equilibration but only slightly increases after that. It is not affected by either Event 1 or 2, has a slight drop and then increase again after Event 3 (the crystal Cl⁻ ion is released from the PAR2-FVIIa interface at 19 ns), and is mostly stable after Event 4 (increase of temperature to 310 K at 33 ns). The PAR2 RMSD fluctuations are expectedly much larger. It basically fluctuates between 5 and 9 Å during 0–15 ns, fluctuations are smaller after Event #2 (the atomic charges of the Gly71 carboxyl group are changed to zeros), becomes stable after Event #3, and fluctuates between 8 and 12 Å after Event #4. Thus, at physiological temperature the PAR2 ectodomain is more dynamic than it is at room temperature. Visualizing the trajectory we see that the most of the flexibility in the PAR2 ectodomain occurs before Arg31 and after Glu56, accounting for the fluctuation of the PAR2 RMSD (see also Supporting Information S2). We also note the stability of folded regions of the ectodomain that are distal from the cleavage site (see Figure 8). Considering the RMSD and parameter changes along the simulation, we have used the simulation trajectory between 36 ns (3 ns after the temperature is set to 310 K) and 54 ns (last snapshot) for the analysis of the PAR2-FVIIa interactions.

3. Electrostatic Interactions

The charged PAR2 residues and their nearby charged FVIIa residues are shown in Figure 4 (Bottom). Besides Arg36 that interacts with FVIIa's Asp189, there are three charged PAR2 residues—Arg31, Asp43, and Glu56—that may form electrostatic interactions with FVIIa. The possible electrostatic interactions of these three residues are plotted in Figure 4 (Top) as a function of simulation time. The final, stable PAR2-FVIIa electrostatic interactions are

Arg31—Asp170g and Glu56—Arg62. The Arg31—Asp170g interaction is the most stable throughout the simulation and may have already formed during the implicit-solvent molecular dynamics. PAR2's Glu56 and FVIIa's Arg62 are initially 30 Å apart but they approach and form an interaction within 13 ns. Long-range electrostatic influence of FVIIa's Arg84 may help attract PAR2's Glu56 during this process. PAR2's Asp43 forms a transient electrostatic interaction with either FVIIa's Lys60a or Lys60c within the first 11 ns but it is eventually lost due to the repulsion between Asp43's neighbor Lys41 and these two FVIIa residues. The binding of PAR2's Glu56 on FVIIa's Arg62 at 13 ns apparently causes bending of the PAR2 polypeptide between Ser37 and Glu56, which also prevents PAR2's Asp43 from binding to either FVIIa's Lys60a or Lys60c.

4. Hydrogen Bonds

The hydrogen bonds between the PAR2 ectodomain and FVIIa were computed over the last 18 ns of the explicit solvent molecular dynamics, and those with more than 25% occupancy (percent of time the hydrogen bond is intact) are reported in Table 1. Three sets of the PAR2-FVIIa hydrogen bonds are actually charge interactions, including Arg31—Asp170g, Arg36—Asp189, and Glu56—Arg62. The hydrogen bonds on PAR2's Arg36 have been reported in Figure 2. The remaining six PAR2-FVIIa hydrogen bonds are novel, including three with occupancy larger than 50%: Leu38:N—Leu41:O (60%), Asp43:OD1/OD2—Asn37:ND2 (30.3%+29.4%), Val53:O—Asn60d:ND2 (97.9%). The residues involved in the six hydrogen bonds are mapped in Figure 5.

5. Other Interactions

In addition to electrostatic interactions and hydrogen bonds, there are significant van der Waals interactions between FVIIa and the PAR2 residues between Arg31 and Asp43 as shown in Figure 5. In the region where these PAR2 residues bind, FVIIa forms a relatively hydrophobic trapezoidal binding pocket surrounded by several polar residues. The PAR2 ectodomain enters the binding pocket along the one edge gated by FVIIa's K170d and K192, makes a turn at Lys34, goes along on a second edge flanked by FVIIa's K60a and K192, makes another turn at Leu38, and exits at the third edge ended at FVIIa's N37. K192, although not participating in either electrostatic interaction or hydrogen bond, significantly shapes the binding pocket with the hydrophobic extent of its side-chain. Inside the binding pocket, there are two deep mini pockets, one hosting PAR2's Arg36 and the other hosting Ile39. The former is anticipated and involves residues H57, D189, S190, C191, K192, G193, D194, S195, V213, S214, W215, G216, G219, C220, and G226. The latter is novel and includes residues Q40, W141, G142, Q143, T151, K192, and G193.

Interestingly, there are no stable PAR2-FVIIa interactions before PAR2's Arg31 or after Glu56. These two ends contribute most to the RMSD fluctuations of PAR2 (Figure 3). Thus, Arg31 and Glu56 define the extent of the PAR2 substrate region that interacts with FVIIa. They also contribute to the charge complementarities between PAR2 and FVIIa. PAR2's Arg31, Lys34, and Arg36 are close to the only two negatively charged FVIIa residues on the binding pocket surface: D170g and D60, while PAR2's Asp43 and Glu56 are close to the positively charged FVIIa residues K60a, K60c, and R62. This charge complementarity, which involves long-range electrostatic interactions, presumably plays an important role in aligning the PAR2 n-terminal polypeptide correctly on the FVIIa protease domain during binding.

6. Antibody Study

In order to address potential exosite interactions of PAR2 with the basic FVIIa residues Arg62, Arg84, Arg247, we evaluated the inhibitory effects on TF-FVIIa signaling of two monoclonal antibodies that were previously mapped to bind to this region of the FVIIa

protease domain. The epitopes of 12C7 and 12D10 are overlapping, but located outside the catalytic cleft³¹. The epitope of 12C7 contains FVIIa residues Arg247, Arg62, Arg84, and Gln87, while the epitope of 12D10 contains FVIIa residues Arg62, Arg84, and Asn63. Both antibodies have minimal effects on chromogenic substrate hydrolysis, but 12D10 produces more steric hindrance with macro molecular substrate factor X (FX) binding to FVIIa. Accordingly, 12D10 was a more potent inhibitor of Xa generation in comparison to 12C7 (Figure 6). Nevertheless, both antibodies completely inhibited TF-FVIIa signaling through PAR2 (Figure 6). These data provide evidence that the PAR2 binding to this FVIIa protease domain basic region makes significant contributions to the efficient orientation of the substrate to the enzyme.

To understand the inhibition mechanism, we highlight the epitope residues in our FVIIa-PAR2 model in Figure 7. The FVIIa residue Arg62 in the epitopes of both 12C7 and 12D10 is seen to be interacting with PAR2, while the rest of the epitope residues do not (12D10 epitope residue Asn63's side-chain is only ~4 Å away from PAR2's Val53 but does not form any significant interaction with PAR2). Arg62 has an electrostatic interaction with PAR2's Glu56 (Figure 4) one of the key interactions that we see from the molecular dynamics simulations. The other major FVIIa-PAR2 interaction nearby is the hydrogen bond between FVIIa's Asn60d and PAR2's Val53 (Table 1 & Figure 5). Thus, 12C7 and 12D10 could inhibit TF-FVIIa signaling through PAR2 by blocking the electrostatic interaction between FVIIa's Arg62 and PAR2's Glu56 and probably the hydrogen bond between Asn60d and Val53 as well. However, charge reversal mutations (R62E/R84E) made to the FVIIa basic region do not inhibit PAR2 activation, as measured by the TR3 gene induction event (Figure 6). While this result does not eliminate the possibility that electrostatic interactions in this region help to guide substrate orientation under physiological conditions, it indicates that the specific interactions seen in the simulation are not essential for PAR2 cleavage to take place.

DISCUSSION

The derived PAR2-FVIIa interactions presented here may help in the design of small molecule inhibitors that block the PAR2 cleavage by FVIIa. Comparisons of these interactions with those present in FVIIa's binding to other substrates can serve to guide the design of inhibitors that would selectively block a particular FVIIa substrate. The key PAR2-FVIIa interactions identified here include electrostatic interactions between stably folded regions of the PAR2 ectodomain and residues within the catalytic cleft. Most of the contacts are consistent with typical docking of substrates to serine protease domains. We find an insertion of PAR2's P3' Ile39 into a mini binding pocket formed by FVIIa residues Gln40, Trp141, Gly142, Gln143, Thr151, Lys192, and Gly193. This is somewhat unusual and transition state structures of serpin-serine protease domains typically show binding of P2' Leu38 into this approximate area. The predicted interactions will require further mutagenesis studies to precisely confirm the energetic contributions of P and P' interaction, as well as accessory contributions by the somewhat more remote charged residues.

The PAR2-FVIIa interaction and the relatively well defined structure of the carboxyl aspect of the PAR2 ectodomain raises an important question, i.e. how the FVIIa protease domain, which is well above cell membrane, approaches the PAR2 ectodomain that is directly anchored in the cell membrane. The distance between the active site of TF-bound FVIIa and the membrane is experimentally measured to be 75 ± 2 Å³³. However, from this simulation study the distance between PAR2's Arg36 (in the FVIIa catalytic site) and Gly71 (four residues above membrane) is only 33 ± 5 (Figure 8). The large discrepancy between these two distances suggests that the TF-FVIIa complex must be tilted toward the cell membrane, potentially by additional protein interactions in order for the FVIIa protease domain to bind

and cleave the PAR2 extracellular tail. Although our antibody inhibition data are consistent with potential interactions of VIIa exosite regions during the initial docking of PAR2, another possibility is that the antibody inhibition of PAR2 activation results from steric interference with the required tilting of the TF-FVIIa complex.

CONCLUSIONS

In this work, we have simulated the binding of PAR2 extracellular ectodomain by the FVIIa protease domain through molecular modeling and a 4-ns implicit-solvent molecular dynamics followed by a 54-ns explicit-solvent molecular dynamics. These simulations maintain the transition-state interactions of the serine protease catalytic site with the substrate and reveal the molecular interactions that can facilitate PAR2-FVIIa binding. They also identify folded regions of the PAR 2 ectodomain that constrain the distance between the cell membrane and the activation cleavage site. The derived model thus implies that the TF-FVIIa complex must be tilted towards cell membrane in order to access and cleave the PAR2 extracellular tail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Interactions of PAR2's Arg36-Ser37 with FVIIa catalytic site after implicit-solvent energy minimization (Left) and explicit-solvent molecular dynamics (Right)
The hydrogen bonds are shown in purple dashed lines. The FVIIa residues forming hydrogen bonds with PAR2 Arg36-Ser37 are shown and labeled. The figures are generated in InsightII (Accelrys) and edited in Inkscape (www.inkscape.org).

Figure 2. The structure of FVIIa protease domain complexed with PAR2 ectodomain (residues 1 to 71) after energy minimization (A), 4-ns implicit solvent molecular dynamics simulation (B), and 54-ns explicit solvent molecular dynamics simulation (C)

The FVIIa protease domain is shown in secondary structure or blue surface, and the PAR2 extracellular tail as an orange ribbon. Five PAR2 residues are shown in either CPK or ball-and-stick: Met1, Asn30-NAG (glycosylated Asn30), Arg36, Ser37, and Gly71. The calcium in the FVIIa protease domain is shown as a ball but it is never approached by the PAR2 tail. In (A) the FVIIa residues forming hydrogen bonds with PAR2's Arg36 and Ser37 are shown in thin ball-and-stick together with disulfides. In both (A) and (B) the complex structure is taken from the last snapshot of the simulation, while in (C) the complex structure is averaged over the last 18 ns of the simulation. The figures are generated in InsightII (Accelrys) and edited in Inkscape (www.inkscape.org).

Figure 3. RMSD of FVIIa protease domain (Top) and PAR2 ectodomain (Bottom) as a function of simulation time in explicit solvent molecular dynamics

The four vertical lines mark four events in the simulation: (1) the constraint on FVIIa (except the rigid residues) and PAR2's Arg36-Ser37 is removed at 11 ns, (2) the atomic charges of the Gly71 carboxyl group are changed to zeros at 15 ns to mimic, (3) the atomic charge of the Cl⁻ ion is changed to zero at 19 ns, (4) the system is raised from 300 K to 310 K at 33 ns.

Figure 4. Electrostatic interactions between PAR2 and FVIIa protease domain

Top: Distances between selected charged residues of PAR2 and FVIIa as a function of simulation time in explicit-solvent molecular dynamics. **Bottom:** Complex structure averaged over the last 18 ns of simulation. The FVIIa protease domain is shown as secondary structure while the PAR2 extracellular tail as an orange ribbon. All charged PAR2 residues (in ball-and-stick) are shown and labeled in orange; the charged FVIIa residues within 5 Angstrom of the PAR2 charged residues are shown and labeled in green. There are three residue pairs of electrostatic interactions between PAR2 and FVIIa: Arg31—Asp170g (inside blue dashed ellipse), Arg36—Asp189 (not visible but in Figure 1), and Glu56—Arg62 (inside red dashed ellipse). The figure on top is plotted in Python using the matplotlib module (matplotlib.sourceforge.net); the figure on bottom is generated in InsightII (Accelrys) and edited in Inkscape (www.inkscape.org).

Figure 5. Partial residue map of FVIIa protease domain complexed with PAR2 ectodomain

The structure is averaged over the final 18 ns of explicit solvent molecular dynamics, and hydrogens are not shown for simplicity. FVIIa is shown as a molecular surface built by MSMS³⁵ and colored by DG colors (carbon atoms in white, charged nitrogen and oxygen in saturated blue and red, uncharged nitrogen and oxygen in pale blue and pink, and hydrogens colored the same as the atoms to which they are bonded). PAR2 is shown as secondary structure representation in orange. The PAR2 residues Arg31-Asp43, Val53, and Glu56 are shown in ball-and-stick with Arg36 and Ile39 in a thicker model. Labeled residues include (1) all residues involving inter-molecular electrostatic interactions or hydrogen bonds except those FVIIa residues that interact with PAR2 Arg36 and Ser37 (see their positions in Figure 1); (2) the key residues that form the binding pocket for PAR2; (3) the FVIIa residues that form a mini binding pocket for PAR2's Ile39. The labels on FVIIa residues are in either black or white and use the one-letter residue code. The labels on the PAR2 residues are orange and have use the 3 letter code. The figure is generated in PMV (mgltools.scripps.edu) and edited in Inkscape (www.inkscape.org).

Figure 6. Effects of antibodies 12C7 and 12D10 on TF-FVIIa signaling through PAR2 (Left) and FX activation (Middle). Signaling of the VIIa mutant Arg62Glu/Arg84Glu was determined with the same methodology (Right).

Figure 7. Interactions of PAR2 with FVIIa residues in the epitopes of antibodies 12C7 and 12D10. FVIIa and PAR2 shown as in figure 4. The epitope residues are rendered in stick, and the PAR2 residues Glu56 and Arg36 are rendered in ball-and-stick.

Figure 8.

Time plot (Left) and structures (Right) showing **distance between PAR2's Arg36 and Gly71 in the explicit-solvent MD simulation**. The distance is measured between the alpha-carbons of Arg36 and Gly71. The average distance in the last 18 ns is 33 ± 5 Å. The four vertical lines in the plot mark the four events in the simulation (see Figure 3 for details). FVIIa is shown in blue secondary structure and PAR2 extracellular tail in red ribbon. The solid red ribbon represents the PAR2 tail structure averaged over the last 18 ns of the simulation, while the lined red ribbons represent 3 consecutive average structures along the 18 ns. The model shows that the two ends of the PAR2 tail are relatively flexible, but the conformation of the carboxyl aspect of the PAR2 ectodomain maintains a compact, well defined structure. The structure figure is generated in InsightII (accelrys.com).

Table 1
Hydrogen bonds between PAR2 extracellular and FVIIa protease domain

Each hydrogen bond is computed with an occupancy (percent of time the hydrogen bond is intact) over the last 18 ns of the explicit solvent molecular dynamics simulation. The criteria of a hydrogen bond are that the donor-acceptor distance is not greater than 3.35 Å and the donor-hydrogen-acceptor bond angle is greater than or equal to 135°. Hydrogen bonds with occupancy less than 25% are not reported, and occupancies less than 50% are right aligned in the last column. Each acceptor or donor is listed with its residue type, residue number, and atom name (in AMBER nomenclature¹⁷).

PAR2	FVIIa	Occupancy (%)
ARG31:NH2	ASP170g:OD1	99.0
ARG31:NH2	ASP170g:OD2	45.8
ARG31:NH1	ASP170g:OD2	99.3
ARG31:NH2	GLN217:OE1	38.1
SER32:O	GLY219:N	44.3
ARG36:N	HID57:NE2	61.7
ARG36:NH2	ASP189:OD1	26.2
ARG36:NH2	ASP189:OD2	91.3
ARG36:NH1	ASP189:OD1	92.8
ARG36:NH1	ASP189:OD2	29.1
ARG36:NH1	SER190:OG	83.0
ARG36:O	GLY193:N	99.7
ARG36:O	SER195:N	67.1
ARG36:N	SER214:O	28.8
ARG36:NH2	GLY219:O	92.2
LEU38:N	LEU41:O	60.0
ILE39:O	GLN143:NE2	29.9
ASP43:OD1	ASN37:ND2	30.3
ASP43:OD2	ASN37:ND2	29.4
VAL53:O	ASN60d:ND2	97.9
GLU56:OE1	ARG62:NH2	40.4