Structures of the EphA2 Receptor at the Membrane: Role of Lipid Interactions

Highlights

- EphA2 receptor ectodomain mainly interacts with lipid bilayers via its FN2 domain

- Anionic lipids are recruited by positively charged residues in FN2

- Mutations K441E and R443E abolish the specific binding of FN2 to anionic lipids

- The FN2-bilayer interaction orients the EphA2 ectodomain relative to a membrane

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In Brief

Chavent et al. combine multiscale molecular dynamics simulations with a biochemical assay to describe the interactions of the EphA2 receptor FN2 domain with the anionic lipids of a model membrane. This provides insights into the dynamic behavior of the receptor at the cell surface.
Structures of the EphA2 Receptor at the Membrane: Role of Lipid Interactions

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SUMMARY

Ephs are transmembrane receptors that mediate cell-cell signaling. The N-terminal ectodomain binds ligands and enables receptor clustering, which activates the intracellular kinase. Relatively little is known about the function of the membrane-proximal fibronectin domain 2 (FN2) of the ectodomain. Multi-scale molecular dynamics simulations reveal that FN2 interacts with lipid bilayers via a site comprising K441, R443, R465, Q462, S464, S491, W467, F490, and P459–461. FN2 preferentially binds anionic lipids, a preference that is reduced in the mutant K441E + R443E. We confirm these results by measuring the binding of wild-type and mutant FN2 domains to lipid vesicles. In simulations of the complete EphA2 ectodomain plus the transmembrane region, we show that FN2 anchors the otherwise flexible ectodomain at the surface of the bilayer. Altogether, our data suggest that FN2 serves a dual function of interacting with anionic lipids and constraining the structure of the EphA2 ectodomain to adopt membrane-proximal configurations.

INTRODUCTION

The ephrin receptors (Ephs) are the largest group within the family of receptor tyrosine kinases (RTKs). Ephs play critical roles in many developmental processes (Herbert and Stainier, 2011; Lai and Ip, 2009) and are implicated in a number of cancers (Herbert and Ip, 2009) and experimental (Roberts et al., 2012) studies suggest that other RTKs may interact with lipid bilayers via their ectodomains. We have explored the interaction of the EphA2 FN2 domain with model lipid bilayers. Molecular dynamics (MD) simulations provide a powerful computational tool to explore the interactions between proteins and membranes (Biggin and Bond, 2015; Li et al., 2015; Stansfeld and Sansom, 2011b). This approach was previously used to define the interactions of peripheral membrane proteins and domains (e.g. PH [Lumb and Sansom, 2012], C2 [Jaud et al., 2007], PTEN [Kalli et al., 2013, 2014], and Talin [Kalli et al., 2010]) at the surface of model membranes, yielding results that are in good agreement with biophysical and mutational data. MD simulations have also been used to model adsorption of the FN34 domain of fibronectin on (non-biological) surfaces (Kubiak-Ossowska et al., 2014). Here, we used multi-scale MD simulations to define a potential membrane-binding motif on the surface of the EphA2 FN2 domain. We show that this membrane-binding motif includes positively charged residues that recruit negatively charged lipids to the site of membrane-protein interaction. Charge-swap mutations in the FN2...
membrane-binding site reduce the recruitment of negatively charged lipids in silico, and abolish preferential binding to negatively charged lipids in a biophysical binding assay. We further demonstrate that the interactions of FN2 with lipids stabilize the otherwise flexible EphA2 ectodomain in two main conformations relative to the membrane. This was extended to simulations of two different models of the EphA2 dimer: one corresponding to a liganded conformation of the ectodomain, and one to an unliganded conformation. Taken together, our results reveal a previously unknown selective lipid-binding motif on the EphA2 ectodomain that defines the receptor’s architecture at the membrane.

RESULTS

Interaction of the FN2 Domain with Lipids
We performed coarse-grained (CG) dynamics simulations (summarized in Table 1 and Figure S1) with a PC (phosphatidylcholine) zwitterionic lipid bilayer, using a protein/bilayer encounter protocol (Kalli and Sansom, 2014; Kalli et al., 2010) to determine the preferred orientation and interaction of FN2 with a lipid bilayer. The initial position of the FN2 domain was such that the protein was distant (C24120 Å) from the center of mass of the membrane (Figure 1B and Experimental Procedures). We performed multiple CG simulations, each lasting 6 μs, starting with different protein orientations with respect to the membrane. In three out of four simulations the protein interacted transiently with the membrane without adopting a stable orientation (Figure S2). In one simulation the FN2 domain bound to the membrane after C241.4 μs. We also used a membrane self-assembly approach (Bond and Sansom, 2006; Bond et al., 2008) (see Figure 1C).

Table 1. Summary of the Simulations

| System                          | Granularity | Particles | Duration (μs) |
|---------------------------------|-------------|-----------|---------------|
| FN2 WT + PC bilayer             | CG          | 17,063    | 4 x 6.0       |
| FN2 WT + PC bilayer (self-assembly) | CG         | 9,575     | 4 x 2.5       |
| FN2 WT + PC/PG bilayer          | CG          | 17,407    | 2 x 1.0, 2 x 2.0 |
| FN2 K441E + R443E + PC/PG bilayer | CG        | 17,430    | 1 x 1.0, 1 x 2.0, 2 x 3.0 |
| FN2 K441 + R443E + R465E + PC/PG bilayer | CG | 17,442    | 1 x 1.0, 1 x 2.0, 1 x 5.0, 1 x 6.0 |
| FN2 K441 + R443E + R465E + F490A + PC/PG bilayer | CG | 17,447    | 1 x 2.0, 1 x 4.0, 1 x 5.0, 2 x 6.0 |
| FN2 K441 + R443E + R465E + F490A + W467A + PC/PG bilayer | CG | 17,440    | 2 x 2.0, 2 x 7.0 |
| Ectodomain (“flat”) + TM + PC/PG bilayer | CG | 196,851   | 1 x 10.0      |
| Ectodomain (“upright”) + TM + PC/PG bilayer | CG | 295,445   | 1 x 10.0      |
| Ectodomain dimer (“flat”) + 2 TMs + PC/PG bilayer | CG | 189,251   | 1 x 10.0      |
| Ectodomain dimer (“upright”) + 2 TMs + PC/PG bilayer | CG | 292,430   | 1 x 10.0      |
| FN2 WT + PC/PG bilayer          | AT          | 89,156    | 1 x 300 ns    |
| FN2+TM WT + PC/PG bilayer       | AT          | 85,187    | 1 x 20 ns     |

Simulations were performed in mixed PC/PG or in PC bilayers. See also Table S1 for further details of FN2 domain interactions with the membrane.
Experimental Procedures) to evaluate whether the membrane-bound form observed was the most stable arrangement of the FN2 domain at a bilayer surface. In four simulations, each lasting ~2.5 μs, we observed the same stable interaction between the FN2 domain and the lipid bilayer (Figure S1C). The main interacting FN2 residues are positively charged (K441, R443, and R465), polar (Q462, S464, and S491), or aromatic (W467 and F490). Three prolines (P459–461) reach deep into the membrane where they interact predominantly with the aliphatic lipid tails (Figures 1A, S3A, and S3B).

The presence of positively charged residues in the FN2-lipid interaction surface prompted us to also perform simulations using an anionic model membrane, thus mimicking anionic surfaces found in mammalian cell membranes. Negatively charged lipids such as the glycosphingolipid GM3 (Posse de Chaves and Sipione, 2010) are known to modulate RTK activity, for example that of the epidermal growth factor receptor (EGFR) (Coskun et al., 2011). However, such glycolipids exhibit rather complex behavior, including nanoscale clustering, in mixed lipid bilayers (Ingólfsson et al., 2014; Koldsø et al., 2014). We therefore decided to use, as a first approximation to the anionic membrane surface, a simpler lipid, namely phosphatidylglycerol (PG). We therefore constructed a model bilayer of 60% zwitterionic PC and 40% anionic PG (Figure S1B). With this anionic lipid bilayer, we observed stable interactions for all four of the simulations performed (Figures 1B and 1C). The FN2 surface interacting with the anionic bilayer was essentially the same as previously observed using neutral PC bilayers (Figure 2A). The N-terminal region of the FN2 membrane-binding motif contains a positively charged motif, including K441 and R443 (Figure 3A), which interacts preferentially with anionic lipids (Figure 2A). At the end of the simulation, the PG lipids formed a negatively charged “halo” around the bound FN2 domain (Figure 2B). This preference for anionic lipids was quantified by calculating radial distribution functions (RDFs) for the lipids around the protein (Figure 3B). This analysis yielded a ratio of the first RDF peak (corresponding to direct protein/lipid interactions) for PG/PC of 2.5:1. Given the composition of the bilayer, this result correspond to a ~4-fold binding preference of FN2 for anionic over neutral (i.e. zwitterionic) phospholipids. To refine our model, we converted the FN2 domain, as found in the last frame of the CG simulation, into an atomistic (AT) model (see Experimental Procedures). We simulated the resultant atomistic system for 0.3 μs. In this AT model, FN2 remained stably bound to the membrane without significant changes in orientation (Figure S5). Compared with the CG models, only minor differences were found, namely small increases in N-terminal interactions with charged residues alongside small decreases in interactions of apolar and aromatic residues (Figure S3C). We did not
observe differences in the density of PG lipids around the FN2 domain after 0.3 μs of simulation.

To validate our results experimentally, we performed lipid vesicle pull-down assays with isolated FN2 domain, expressed in and purified from HEK293T cells (Figure 3C). We used lipid vesicles composed of either PC or PG or of a 1:1 mixture of PC and PG. Consistent with the in silico results, we found that FN2-binding to these vesicles correlates with the ratio of PG/PC, with a preference for the negatively charged lipid PG. We note that there is no significant difference between PC + PG and PG liposomes. The anionic lipid-binding sites may be already fully saturated in the mixed PC + PG liposomes, such that no further increase is seen with the pure PG vesicles.

Effects of Mutations on the FN2 Preference for Anionic Lipids

Our simulation highlighted a strong spot of anionic lipid attraction around the residues K441 and R443 (see curve in Figure 2A). We mutated both residues to glutamic acids (K441E + R443E) to assess the contribution of charge effects on FN2-lipid binding specificity (Figure 3A). We performed four simulations with the mixed PC-PG bilayer. The simulations showed that mutant FN2 still interacts with the membrane via the same motif as with the wild-type domain, although the time taken to reach the membrane-bound orientation was longer (Table S1). Importantly, the mutations had an impact on the FN2 domain’s preference for anionic lipids. RDFs revealed less anionic lipid around the mutant FN2 domain compared with the wild-type: g(r) for the first peak changes from 7.5 for the wild-type (Figure 3B) to 5.5 for the mutant. The density of PG lipids at the membrane-protein interface was lower for the mutant than for the wild-type (Figure 2C). In contrast, the K441E + R443E mutations had no significant effect on FN2 interaction with PC lipids. We extended the charge-swap mutations to include R465E (Figure S4A). The protein still interacted with the membrane, although for one repeat it did not form a stable interaction after 6 μs of simulations. The anionic lipid preference profile for this triple mutant (i.e. K441E + R443E + R465E) was very similar to that of the K441E + R443E mutant. We continued to add further mutants, adding F490A and W467A to the triple mutant (Figures S4B and S4C). Again, these mutants did not fully abolish the interaction with the membrane, but the time elapsed before an interaction was formed did increase slightly in comparison with the other mutants (Table S1). Thus, the clearest effect was for the double K441E + R443E mutant, which seems to almost fully abolish the preference for anionic lipids.

To validate our simulation results, we produced the double mutant protein FN2 K441E + R443E in vitro and performed vesicle pull-down assays. Unlike the wild-type, mutant FN2 protein did not bind better to vesicles of PG or of PC + PG compared with PC (Figure 3C). We conclude that the preferential interaction of FN2 with anionic lipids is dependent on the membrane-binding motif described above, containing K441 and R443.

Modeling EphA2 Signaling Units in a Lipid Bilayer Environment

The FN2 domain orientation at the membrane surface was then used to construct more complex assemblies, including monomers and dimers of EphA2 receptors (i.e. the ectodomain + TM helix) with the membrane, thus integrating structures from both crystallography (Seiradake et al., 2010) and nuclear magnetic resonance (NMR) (Bocharov et al., 2010) experiments.

We first constructed an atomistic model of the FN2 domain attached to the TM helix corresponding to the NMR structure of the TM dimer (Bocharov et al., 2010) (see Experimental Procedures). We performed a short (20 ns) AT simulation to relax the
The TM helix remained tilted in the membrane, as previously described for the dimeric TM domain structure (Bocharov et al., 2010). The linker remained close to the FN2 domain during the simulation. Residues L535, A536, and V537, which adopt a helical conformation in the NMR structure (Bocharov et al., 2010), had a tendency to unfold at the bilayer surface during our MD simulation, but the rest of the helix remained stable.

Based on this model, we then constructed two atomistic configurations of the monomer comprising the EphA2 ectodomain + TM helix embedded in the lipid bilayer. This corresponds to a construct that, in vitro, is functionally capable of clustering at the cell surface in response to ligand stimulation (E.S., unpublished data). We generated models based on the liganded conformation (PDB: 2X11; but without the ephrin ligand) and on the unliganded (PDB: 2X10) conformation (see also Experimental Procedures). It was previously reported that the FN1-FN2 linker can undergo substantial conformational changes, leading to different structures for the FN1-FN2 domains with the FN2 domain rotated by \( \sim 70^\circ \) between the two models (Seiradake et al., 2010). The rest of the ectodomain (i.e. the LBD to EGF domain) appears to be less flexible. Here, the positioning of the FN2 domain onto the membrane surface as directed by our simulations provided a novel insight into the potential consequences of this structural difference for receptor orientation at the cell surface. The anchoring of the FN2 domain to the membrane surface yields two very different orientations of the receptor as a whole driven by the FN1-FN2 linker difference. The unliganded configuration lies almost flat on the membrane surface, while the ephrin-bound conformation adopts a more upright orientation (Figure 4B). Neither orientation leads to major steric clashes of the ectodomain with the membrane, although loop residues 367–375 of the FN1 domain, which were not ordered in the original structure, may point toward and form close contacts with the membrane (Figure 4B, inset).

We used the monomeric EphA2 ecto + TM models described above to initiate two CG simulations (each of 10 \( \mu \)s duration; see Figure 4C and Experimental Procedures). The model in which the ectodomain is lying “flat” on the surface of the membrane (derived from the unliganded EphA2 ectodomain structure, PDB: 2X10) resumed a stable position in which both the FN2 domain and the LBD domain interact with the membrane. The LBD residues involved in this interaction were largely aromatic. Both the FN2 and LBD domain interactions attract anionic lipids (Figure 6A). The ephrin ligand-binding site remains accessible in this conformation, i.e. it faces upward, away from the membrane (Figure 6B). During the 10-\( \mu \)s simulation of the “upright” model (derived from the ephrin-bound EphA2 ectodomain structure, PDB: 2X11, albeit without the ligand present during the simulation), the flexible FN1-FN2 linker allowed for movement of the EphA2 ectodomain, although the receptor remained in a generally upright orientation (Figures 4C and 5A). The distance between the LBD domain and the membrane ranged from 75 to 150 Å (Figure 5B). As previously shown for the FN2 domain alone, the RDF calculation for the two ectodomain configurations highlighted a preference of anionic lipid around the FN2 domain (data not shown). We then simulated a K441E + R443E mutant, as previously done for the FN2 domain only, also in the context of the entire ectodomain in the upright conformation. Interestingly, this mutant is destabilized in comparison with the wild-type, and after 6 \( \mu \)s the ectodomain lies flat on the membrane, resembling the configuration of the unliganded structure (Figure 5C). Following this change in orientation, the interactions of this mutant with the membrane are very similar to those of the unliganded structure (Figure 5D). Taken together, our data suggest that, while the FN2 domain is constrained by tight interactions with the lipid bilayer surface, flexible regions upstream of FN2 allow for a range of EphA2 ectodomain orientations relative to bilayer/membrane.

We extended our study of the monomeric receptor by simulation of crystal-structure-based dimers of the liganded and unliganded forms (see Experimental Procedures). We note that, as for the monomeric models, the creation of the dimers did not lead to any major steric clashes with the membrane. After 10 \( \mu \)s of simulations we noted dynamic differences when compared with the monomer simulations. For the upright dimer the configuration stayed very stable during the whole simulation, unable to adopt a conformation as flexible as the monomer
For the unliganded dimer, as was seen for the monomer, the ectodomains quickly (within a few tens of nanoseconds) moved down onto the bilayer surface where they remained for the whole 10 ms. One monomer is positioned on top of the other, resulting in asymmetrical interactions with the membrane (Figure 6C). The first monomer presents interactions equivalent to those seen in the monomeric simulations, interacting mainly with the membrane through the FN2 domain and LBD (Figure 6D). The asymmetry is also visible in terms of lipid preference between the two monomers. Interestingly, asymmetric receptor dimers are suggested to occur in other signaling systems, such as the EGFRs (Arkhipov et al., 2014).

DISCUSSION

We have presented MD simulations and biophysical data showing that the EphA2 ectodomain harbors a membrane-binding motif in the FN2 domain which preferentially interacts with anionic lipids. The cell membrane is composed of different lipids, often arranged into specific domains, whose biological functions are only beginning to emerge. For example, lipid (nano) domains (sometimes denoted as “lipid rafts”) are densely packed regions that are enriched in signaling proteins and play important roles in membrane signaling events (Simons and Toomre, 2000). The lipid composition of these nanodomains differs from the average, overall neutral, composition of the mammalian cell membrane (van Meer et al., 2008). Nanodomains are often enriched in anionic lipids (Pike et al., 2002), such as glycolipid GM3 (Koldso et al., 2014). Activated EphA2 is known to localize to lipid nanodomains (Chakraborty et al., 2012) and requires the presence of negatively charged lipids for at least some of its functions (Tawadros et al., 2012). As demonstrated previously for the EGFR (Hofman et al., 2008), interaction of EphA2 with specific anionic lipids could act as a mechanism to trigger the coalescence of lipid nanodomains to form more extended signaling platforms. Conversely, the clustering of anionic lipids around EphA2 could guide the receptor toward specific lipid domains, in agreement with the lipid shells theory (Anderson and Jacobson, 2002).

The simulations of the EphA2 ecto + TM domains suggest that the receptor adopts at least two different conformations at the cell surface. In one arrangement the receptor ectodomain sits “upright” on the membrane while in the second it lies “flat” on...
the membrane surface. Both conformations are consistent with known biological receptor functions. In the upright conformation the receptor LBD is in a position presumably compatible with binding ephrin ligand presented by a neighboring cell (Figure 7). This conformation also allows packing of the receptor into dense signaling clusters as previously proposed based on crystal structures (Himanen et al., 2010; Seiradake et al., 2010). Our simulations suggest that the FN2-membrane interaction we describe here has a stabilizing effect on this EphA2 conformation, which can be removed by mutation of the relevant membrane-binding site on FN2. The flat orientation of the EphA2 ectodomain on the membrane surface could represent a functionally distinct receptor conformation, as postulated for EphA4 based on a recent crystal structure data, and seems to also exist for the EphA2 receptor, perhaps in a less stable conformation due to a smaller interface between the FN2 and LBD domains (Xu et al., 2013). As recently suggested by Nikolov et al. (2014), the two EphA2 conformations we describe may reflect clustering at different receptor densities, with the “flat” configuration corresponding to the formation of less tightly packed clusters compared with the “upright” conformation. Cluster density may in turn have an effect on the signaling outcome (Lohmüller et al., 2013). Xu et al. (2013) have postulated that the “flat” configuration may also help to create a larger platform to propagate ephrin-Eph signaling. Nevertheless, as seen for other RTKs, such as EGFR, it is still not clear how membrane lipid composition may favor active or inactive forms (Arkhipov et al., 2013, 2014; Kaszuba et al., 2015; Kästner et al., 2009; Webb et al., 2008). Furthermore, the membrane environment (Arish et al., 2015) and/or the glycosylation of the protein (Kaszuba et al., 2015) may impact on the behavior of RTKs. Interestingly, the “flat” EphA2 conformation appears to be compatible with ephrin ligand binding, as the main ephrin-binding site is not obscured. This conformation could, for example, promote in cis interaction with ephrins, also found in lipid nanodomains (Gauthier and Robbins, 2003; Levental et al., 2010). This would be in addition to a second in cis ephrin-binding site thought to exist at the Eph FN domains (Carvalho et al., 2006; Falivelli

Figure 6. EphA2 Ectodomain Interacting with the Membrane Starting from the Unliganded Conformation
(A) Histogram showing the normalized average number of contacts (see Experimental Procedures for details) to the bilayer as a function of residue number for the LBD and FN2 domain, which are interacting with the membrane (Figure 4C).
(B) Main residues of the LBD domain interacting with the membrane. The ephrin-binding site is not involved.
(C) A snapshot of the unliganded dimer at the end of the simulation showing the asymmetric interaction of the two monomers with the membrane.
(D) Histogram of the normalized average number of contacts for the two monomers starting from the unliganded dimer conformation.
See also Figure S6.
Thus, the data presented here form the basis for future work exploring in cis interactions of Eph receptors with ephrin and other cell-surface molecules.

Eph receptors are known to dimerize and further cluster upon ligand-induced activation. Our simulations show that the upright orientation seemed to be less flexible than for the monomeric receptor, a characteristic that may help to retain a favorable conformation to propagate formation of a signaling array by addition of monomers (Seiradake et al., 2010). Singh et al. (2015) proposed recently that the formation of unliganded dimers might reduce EphA2 pro-tumorigenic signaling by reducing the supply of free monomers. In our simulations, we can see that the unliganded dimer configuration might sequester monomeric forms at the membrane and potentially hide (by interactions with the membrane surface) important binding sites for the seeding effect (see Seiradake et al., 2010) including residues 246–248, 254–255–257, or 378 and 380 (Figure 6D, monomer 1). In our model, the distance between the two TM helices is ~52 Å, which is in agreement with a separation of ~48 Å from fluorescence resonance energy transfer measurements (Singh et al., 2015). We postulate that this configuration may impose some steric constraints: on the extracellular LBD side the extension of the signaling platform suggested by Xu et al. (2013) might be prevented; and on the cytosolic side of the membrane the kinase domains might be held apart, preventing them from signaling (Figure 6D).

The CG simulation protocol uses an elastic network model to maintain the tertiary structure of the proteins (Bond et al., 2007; Periole et al., 2009). This may dampen the larger-scale dynamic of the systems, so that in the future extended atomistic simulations could be used to further validate our model. Furthermore, the use of a CG force field might be anticipated to restrict the spatial and chemical resolution of protein/lipid contacts (Marrink and Tieleman, 2013). It is encouraging that we obtain a good correlation between simulation and experimental studies of the anionic lipid-binding preference. Our results using this PC/PG system will act as a stepping stone toward constructing more biologically relevant models using multicomponent lipid mixtures that better mimic the compositional complexity of living cell membranes (Ingólfsson et al., 2014) as well as the study of glycosylation effects (as in recent studies of EGFR [Kaszuba et al., 2015]).

Taken together, the biophysical and computational data presented here increase our molecular understanding of how EphA2 receptors are oriented and function within a model lipid membrane. Significantly, the model presented provides an essential stepping stone toward the analysis of full-length Eph receptor structure at the cell surface, its clustering (Figure 7), and the mechanism of how Eph receptors transfer signals across the cell membrane.

**EXPERIMENTAL PROCEDURES**

**System Construction**

**FN2 Domain Interacting with the Membrane**

We used two different protocols. We coarse grained the FN2 domain (Figure 5A) using an in-house protocol. An elastic network was applied to further constrain the tertiary structure of the protein, using a cut-off of 7 Å. We then positioned the domain ~120 Å away from the center of mass of the membrane (Figure 1B) rotating it about the x, y, or z axes to create four different starting orientations (Table S1). We used this protocol to assess the interaction of the FN2 domain with a palmitoyloleoylphosphatidylcholine (POPC) membrane, and with a mixed POPC/palmitoyloleoylphosphatidylglycerol (POPG) (3:2) membrane. We also used the four different starting positions of the FN2 domain in a self-assembly approach whereby POPC lipids were initially in random positions around the protein. We identified the most representative structure of the FN2 domain interacting with the mixed PC/PG bilayer and converted the system to an atomistic representation using a CG2AT protocol, which has been used for a number of systems (Stansfeld and Sansom, 2011a). We then extracted one TM helix from the NMR structure of the EphA2 TM dimer (PDB: 2K9Y) (Bocharov et al., 2010) to generate an AT model of the FN2 + TM in the membrane. We then extended the lipid bilayer to create a large 4 × 4 patch of membrane. The resultant model was then converted to coarse grain using an in-house script. We used the VMD plugin mutator (http://www.ks.uiuc.edu/Research/vmd/plugins/mutator/) on
the atomic model of the liganded monomer to construct the K441E + R443 mutant, then coarse grained it as described above.

**Ectodomain Dimers Interacting with the Membrane**

Based on the atomic monomer models, we constructed each dimer by superimposing each of two ectodomain monomers onto the PDZ structures of the ectodomain + TM dimers, then placing the full dimer in the membrane. We coarse grained the membrane and the proteins (making sure that we did not link the monomers together by the elastic network) as described above. We then applied the Alchembed methodology (Jefferys et al., 2015) to remove clashes due to the insertion of the new monomer in the membrane.

**CG MD Simulations**

CG MD simulations were performed using GROMACS versions 4.5 and 4.6 (www.gromacs.org) (Hess et al., 2008) and the MARTINI 2.1 force field (Monticelli et al., 2008). For the PC membrane, a pre-assembled membrane composed of 249 POPC molecules was used for the encounter protocol while the self-assembly method involved 242 lipids. Water and counterions were added to equilibrate the system. For the mixed PC/PG bilayer, we used a pre-assembled bilayer from previous work (Kalli et al., 2010) composed of 151 POPC and 98 POPG lipids. After 100 steps of steepest descent, we performed 5 ns of equilibration before the production runs (see Tables 1 and S1 for details). In the case of the self-assembly method, after the 500 ns of self-assembly we performed 2 μs of simulation to assess the stability of the system. The electrostatic/coloumbic interactions were shifted to zero between 0 and 12 Å and the Lennard-Jones interactions between 9 and 12 Å. A Berendsen thermostat with a reference temperature of 310 K in combination with a Berendsen barostat with a coupling constant of 1.0 ps, a compressibility of 5.0 x 10^{-6} bar^{-1}, and a reference pressure of 1 bar was used in the equivalent protocols published recently (Kalli et al., 2011, 2013). The integration step was 40 fs. For the CG simulations of monomers and dimers we used the same parameters as for the single FN2 domain simulations with a coupling constant of 1.0 ps, a compressibility of 3.0 x 10^{-6} bar^{-1}, and a reference pressure of 1 bar to be consistent with recent CG simulations of large membranes (Ingolfsson et al., 2014; van Eerden et al., 2015). The integration step was 20 fs.

**AT MD Simulations**

Atomic simulations were carried out using the GROMOS96 43a1 force field (Van der Spoel et al., 1998). Water and counterion molecules were added to equilibrate the system. Then a 5,000-step steepest descent minimization was performed followed by an equilibration phase. The production run was then performed for 300 ns. Long-range electrostatics (beyond 10 Å) were modeled using the particle mesh Ewald procedure. The same cut-off distance was used to model van der Waals interactions. The reference temperature was 310 K. The simulation was performed at constant temperature, pressure, and particle number using Lennard-Jones interactions between 9 and 12 Å. A Berendsen thermostat with a reference temperature of 310 K in combination with a Berendsen barostat with a coupling constant of 1.0 ps, a compressibility of 5.0 x 10^{-6} bar^{-1}, and a reference pressure of 1 bar was used in the equivalent protocols published recently (Kalli et al., 2011, 2013). The integration step was 40 fs. For the AT simulations of monomers and dimers we used the same parameters as for the single FN2 domain simulations with a coupling constant of 1.0 ps, a compressibility of 3.0 x 10^{-6} bar^{-1}, and a reference pressure of 1 bar to be consistent with recent CG simulations of large membranes (Ingolfsson et al., 2014; van Eerden et al., 2015). The integration time step was 2 fs.

**Simulation Analysis**

VMD (Humphrey et al., 1996) was used to render system structures and was combined with Tcl scripts to analyze the simulations. Each simulation was centered on the protein using the trjconv command provided by the GROMACS 4.5.5 package. We used Tcl scripts in VMD and take a frame every 2 ns to calculate the number of contacts for each residue. We then concatenated the results of each simulation and normalized them to obtain the graphs in Figures 3 and S3. We used the VMD plugin to perform radial distribution calculation using NC3 beads for PC and GLH beads for PG lipids. We used the Volmap VMD plugin on the last 200 ns of simulation to create the density plot shown in Figure 2B.

**FN2 Expression and Mutagenesis**

A construct coding for human EphA2 FN2 (residues 436–534, Uniprot: P293317) was cloned into the AgeI-KpnI cloning site of a pHLsec vector (Aricescu et al., 2008), also coding for an N-terminal secretion signal sequence and a C-terminal polyhistidine tag. Point mutants (K441E + R443E) were generated using PCR techniques.

**Protein Purification**

We expressed EphA2 FN2 (wild-type or mutant) transiently in HEK293T cells, in the presence of kifunensine (Aricescu et al., 2006). Proteins were purified from cell culture medium using Ni-affinity chromatography. EphA2 FN2 domain does not contain native glycosylation sites, but the sequence of the vector tag, together with the last residue in our construct (N534), introduced an artificial glycosylation site at N534. This site was glycosylated in the wild-type and mutant proteins (not shown). To remove the artificial glycan, we treated our samples with EndoH (Gruninger-Leitch et al., 1996), and re-purified the proteins using size-exclusion chromatography.

**Liposome Pull-Down Assay**

We prepared lipid vesicles by drying dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), or a 1:1 (w/w) mixture of DOPG and DOPC under constant argon flow to avoid oxidation. We re-suspended the resultant lipid film in buffer (100 mM NaCl, 20 mM Tris [pH 7.5]) and sonicated the mixture for 15 min to generate vesicles. Final lipid concentrations were 2 mg/ml. We mixed 100 μl of vesicle suspension with equal amounts of wild-type or mutant EphA2 FN domain 2 protein, incubated these at room temperature for 10 min, and centrifuged them at ~20,000 g for 10 min. We repeated the experiments seven times. For each set the bound protein fractions were visualized by western blot using mouse anti-pentaHis antibody (Qiagen). Data analysis was done with ImageJ (Schneider et al., 2012). In each set, we normalized the data using the corresponding value measured for wild-type protein pelleted with DOPC vesicles. After normalization, we calculated averages and SEM from all experiments.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Information, 3D Molecular Model, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.11.008.

**AUTHOR CONTRIBUTIONS**

M.C. performed and analyzed simulations. E.S. performed experiments. M.C., E.S., E.Y.J., and M.S.P.S. designed the research and together wrote the manuscript.

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