Structural Basis for Semaphorin Signalling through the Plexin Receptor
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LETTER

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Structural basis for semaphorin signalling through the plexin receptor

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Semaphorins and their receptor plexins constitute a pleiotropic cell-signalling system that is used in a wide variety of biological processes, and both protein families have been implicated in numerous human diseases[1–4]. The binding of soluble or membrane-anchored semaphorins to the membrane-distal region of the plexin ectodomain activates plexin's intrinsic GTPase-activating protein (GAP) at the cytoplasmic region, ultimately modulating cellular adhesion behaviour[5]. However, the structural mechanism underlying the receptor activation remains largely unknown. Here we report the crystal structures of the semaphorin 6A (Sema6A) receptor-binding fragment and the plexin A2 (PlxnA2) ligand-binding fragment in both their pre-signalling (that is, before binding) and signalling (after complex formation) states. Before binding, the Sema6A ectodomain was in the expected 'face-to-face' homodimer arrangement, similar to that adopted by Sema3A and Sema4D, whereas PlxnA2 was in an unexpected 'head-on' homodimer arrangement. In contrast, the structure of the Sema6A–PlxnA2 signalling complex revealed a 2:2 heterotetramer in which the two PlxnA2 monomers dissociated from one another and docked onto the top face of the Sema6A homodimer using the same interface as the head-on homodimer, indicating that plexins undergo 'partner exchange'. Cell-based activity measurements using mutant ligands/receptors confirmed that the Sema6A face-to-face dimer arrangement is physiologically relevant and is maintained throughout signalling events. Thus, homodimer-to-heterodimer transitions of cell-surface plexin that result in a specific orientation of its molecular axis relative to the membrane may constitute the structural mechanism by which the ligand-binding 'signal' is transmitted to the cytoplasmic region, inducing GAP domain rearrangements and activation.

Both semaphorins and plexins contain, at the amino terminus of their ectodomain, a ~500-residue sema domain followed by a short (~50 residues) plexin–semaphorin–integrin (PSI) domain. Those regions corresponding to the sema plus PSI segment of Sema6A ($Sema6A_{SP}$, residues 19–570) and PlxnA2 ($PlxnA2_{SP}$, residues 38–561) that mediate ligand–receptor interaction were first expressed in mammalian cell lines, and then purified and crystallized (Supplementary Fig. 1). Structures of $Sema6A_{SP}$ and $PlxnA2_{SP}$ were determined at 2.5 Å and 2.1 Å resolution, respectively (Fig. 1a, b, Supplementary Tables 1 and 2, and Supplementary Results). In both proteins, the sema domain displays a seven-bladed β-propeller fold very similar to previously determined structures of Sema3A[6], Sema4D[7] and Met[8]. In addition to the long 'extrusion' within blade 5 described previously[7] (hereafter called extrusion 2), we noted a second insertion between blades 1 and 2 that proved characteristic to all sema domains (hereafter called extrusion 1) (Fig. 1e).

In the $Sema6A_{SP}$ crystal, monomers make contact with one another using the upper rim of the β-propeller, thereby assuming a 'face-to-face' dimer configuration (Fig. 1a). This dimeric configuration is essentially identical to that seen in the crystal structures of the Sema3A and Sema4D sema domains (Supplementary Fig. 2a). The location of the loops involved in the dimerization is precisely conserved among the three semaphorins, with the exception of the N-terminal region's participation in Sema6A (Supplementary Fig. 2b). Surprisingly, $PlxnA2_{SP}$ also assumes a dimeric configuration in the crystal, albeit with a markedly different mode compared to that observed in the semaphorin sema domains (Fig. 1b). The two $PlxnA2_{SP}$ fragments in the asymmetric unit are related by a non-crystallographic two-fold axis and interact with each other by using a flat surface located at the side of the β-propeller, exhibiting a 'head-on' configuration twisted orthogonally, in contrast to the face-to-face configuration observed in the known semaphorin structures. All the key residues involved in the dimerization are well conserved among the A-type plexin family (Supplementary Results and Supplementary Fig. 3), indicating the physiological relevance of the dimerization. Analytical ultracentrifugation sedimentation velocity experiments performed on the $Sema6A_{SP}$ protein confirmed that it does indeed form a dimer in solution with a dissociation constant (K_d) value of 3.5 μM (Supplementary Fig. 4). The dimerization affinity for $PlxnA2_{SP}$, however, was extremely low (K_d > 300 μM) and could not be definitively determined (Supplementary Fig. 5).

We next crystallized the Sema6A–PlxnA2 complex by mixing $Sema6A_{SP}$ and $PlxnA2_{SP}$ at an equimolar concentration, obtaining a structure at 3.6 Å resolution (Supplementary Results, Supplementary Table 3 and Supplementary Fig. 6). The $Sema6A_{SP}$ and $PlxnA2_{SP}$ molecules constitute a 2:2 complex in the crystal, which contained a crystallographic two-fold symmetry (Fig. 1c). The two $Sema6A_{SP}$ molecules in the complex formed the same face-to-face dimer as was observed in the plexin-free state (Supplementary Fig. 7a). On the other hand, the $PlxnA2_{SP}$ head-on homodimer was no longer present in the complex, and the two plexin molecules independently docked onto the two Sema6A monomers with their carboxy-terminal PSI domains emanating away diagonally. Despite their participation in different molecular interactions, there were no major changes in the structure of individual $Sema6A_{SP}$ and $PlxnA2_{SP}$ monomers, including the conformation of the loops at the interface, upon the complex formation (root mean squared deviation of 0.7Å for $Sema6A_{SP}$ and 0.8Å for $PlxnA2_{SP}$, respectively; Supplementary Figs 7 and 8). At the interface, the Sema6A side showed positively charged surface potentials whereas the PlxnA2 side was negatively charged, indicating that complex formation is driven mainly by electrostatic interactions (Fig. 1d).

We subsequently mutated a select number of interface residues on Sema6A to see whether these mutations disrupt plexin binding. The H212N mutation is expected to create a novel N212-D213-S214 glycosylation sequon and place a large carbohydrate obstacle at the heart of the interface (Fig. 1d and Supplementary Fig. 9). Another mutation, K393E, is expected to convert the electrostatic interaction between

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The following is a comment on the published paper shown on the preceding page.

Structural Basis for Semaphorin Signalling through the Plexin Receptor

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Introduction

Cells receive various 'signals' from outer environment, and change their location, behavior, and fate by these 'signals'. Many of these 'signals' come as a form of extracellular protein, while the 'sensors' are proteins located on cell surface generally called receptor. Although numerous "ligand-receptor" systems that play fundamental roles in the intercellular signal transduction are known, the mechanisms for the information transmission from extracellular to intracellular space remain poorly understood. Semaphorins ('signals') are a large family of cell surface or secreted proteins that are implicated in development and homeostasis of various tissues by regulating cellular migration behavior[1-4]. Semaphorins are divided into 8 distinct classes according to their domain structure, of which five (classes 3 to 7) are present in mammals[5]. Among mammalian semaphorins, classes 4-6 are type I transmembrane proteins, class 7 is GPI-anchored, and class 3 is secreted. Each semaphorin binds to and signals through specific receptors belonging to the plexin family transmembrane proteins ('sensors')[1-4]. At the intracellular side, plexins have a split GTPase-activating protein (GAP)-homology domain intervened by a Rho-binding domain. It is believed that ligand-activated plexin GAP domain deactivates R-Ras, leading to the down-modulation of integrin-mediated cell adhesion. The mechanistic linkage between the extracellular semaphorin binding and the cytoplasmic GAP activation, however, remains unclear. Class 6 semaphorins (Sema6s) comprise of 4 members (A-D), and are regarded as "prototype" semaphorins, because they show the greatest structural similarity to invertebrate semaphorins and directly bind to type A plexins as is the case for invertebrate semaphorins. Signal transduction by Sema6s-plexin A system has been implicated in the control of neuronal migrations, cardiogenesis, navigation of neural crest cells, bone homeostasis, and immune responses. Furthermore, Sema6s and their receptors have been implicated in the pathology of various kinds of human diseases including autoimmunity, osteopetorosis, inflammation, and neurodegenerative diseases.

Therefore, the structural information about the Sema6-plexin A interaction as well as the elucidation of the signalling mechanism would not only set the basis for the mechanistic understanding of semaphorin-plexin systems utilized in various biological contexts, but also contribute to define the potential therapeutic targets for human diseases.

In the present study, we report the crystal structures of Sema6A receptor-binding fragment and plexin A2 ligand-binding fragment, in their pre-signalling state as well as in the signalling state (i.e., after complex formation). The structures provide a detailed picture of the interaction between the two proteins, as well as a possible signalling mechanism involving a "partner switch" at cell surface.

Crystal structures of Sema6A and PlxnA2 and their oligomeric state in solution

Structures of Sema6A and PlxnA2 were determined at 2.5Å and 2.1Å resolution, respectively (Figs. 1a and b). In both proteins, the sema domain displays a seven-bladed β-propeller fold very similar to previously determined structures of Sema3A, Sema4D and Met. In the Sema6A crystal, monomers make contact with one another using the upper rim of the β-propeller, thereby assuming a 'face-to-face' dimer configuration (Fig. 1a). This dimeric configuration is essentially identical to that seen in the crystal structures of the Sema3A and Sema4D sema domains. Surprisingly, PlxnA2 also assumes a dimeric configuration in the crystal, albeit with a markedly different mode compared to that observed in the semaphorin sema domains (Fig. 1b). The two PlxnA2 fragments in the asymmetric unit are related by a non-crystallographic two-fold axis and interact with each other by using a flat surface located at the side of the β-propeller, exhibiting a 'head-on' configuration twisted orthogonally, in contrast to the face-to-face configuration observed in the known semaphorin structures.

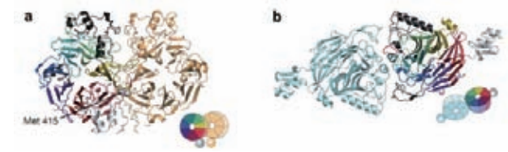

Fig.1 Crystal structure of Sema6A and PlxnA2 ectodomain fragments in pre-signalling states. (a) Structures of the Sema6A face-to-face homodimer and (b) PlxnA2 head-on dimer. Individual propeller blades are coloured differently in one monomer. Arrangement of the toroidal propeller domains within the structure is schematically depicted in the cartoon next to each ribbon presentation.

Analytical ultracentrifugation sedimentation velocity experiments performed on the Sema6A protein confirmed that it does indeed form a dimer in solution with a dissociation constant (Kd) value of 3.5μM (Fig. 2). The dimerization affinity for PlxnA2, however, was extremely low (Kd>300 μM) and could not be definitively determined.

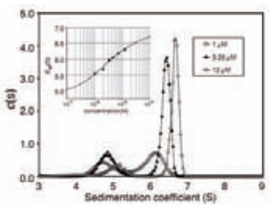

Fig.2 C(s) distribution from sedimentation velocity analytical ultracentrifugation experiments performed at three different concentrations of Sema6A. The concentration-dependent change observed in the sedimentation coefficient distribution confirms the presence of a monomer-dimer equilibrium. The weight-average sedimentation coefficients were plotted against protein concentration, yielding a Kd value of 3.5 μM (inset),

Crystal structure of Sema6A-PlxnA2 complex

We next crystallized the Sema6A–PlxnA2 complex by mixing Sema6A and PlxnA2 at an equimolar concentration, obtaining a structure at 3.6Å resolution. The Sema6A and PlxnA2 molecules constitute a 2:2 complex in the crystal, which contained a crystallographic two-fold symmetry (Fig. 3a). The two Sema6A molecules in the complex formed the same face-to-face dimer as was observed in the plexin-free state. On the other hand, the PlxnA2 head-on homodimer was no longer present in the complex, and the two plexin molecules independently docked onto the two Sema6A monomers with their carboxy-terminal PSI (plexin–semaphorin–integrin) domains emanating away diagonally. Despite their participation in different molecular interactions, there were no major changes in the structure of individual Sema6A and PlxnA2 monomers, including the conformation of the loops at the interface, upon the complex formation. At the interface, the Sema6A side showed positively charged surface potentials whereas the PlxnA2 side was negatively charged, indicating that complex formation is driven mainly by electrostatic interactions (Fig. 3b).

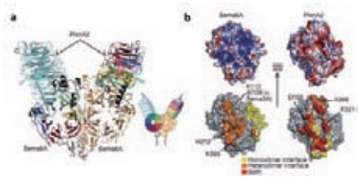

Fig.3 Crystal structure of Sema6A-PlxnA2 2:2 complex. (a) The complex representing post-signalling states. (b) Open-book view of the Sema6A–PlxnA2 interface surface coloured by electrostatic potential (top panel) and by the residue-wise contribution to the interface (bottom panel).

Mutational experiments based on the crystal structure

Cell-based activity measurement using mutant ligands/receptors confirmed that the current complex structure captures genuine receptor-ligand interactions. In addition, functional importance of Sema6A dimer was also indicated (Fig. 4). These mutational experiments based on the crystal structure showed that the hetero-tetrameric configuration of the Sema6A-PlxnA2 complex seen in the crystal structure represents a signalling–competent conformation maintained throughout the signal transduction process.

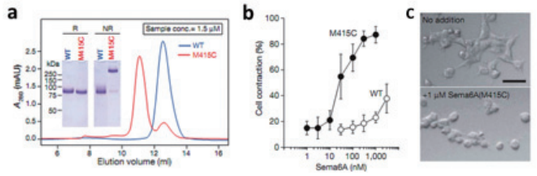

Fig.4 The Sema6A face-to-face homodimer represents a signalling-competent active conformation. (a) Sema6ASP ectodomain fragments (WT or M415C mutant) were subjected to gel filtration chromatography. The peak elution positions for the wild type and M415C mutant corresponded to 106 and 219 kDa, respectively. SDS–PAGE analysis under reducing (R) or non-reducing (NR) conditions (inset) confirmed the 90% formation of a disulphide-linked homodimer in theM415C mutant. (b) Signalling activities of soluble Sema6A proteins. Purified Sema6ASP proteins (WT or M415C) were tested for their ability to induce contraction of HEK293T cells stably expressing PlxnA4. (c) Representative images of cell morphologies both before and after the stimulation are also shown (right). Scale bar, 50 mm.

Conclusion

Plexins on the resting cell surface assume an 'auto-inhibited' state, with their cytoplasmic GAP domain activity suppressed. It is also accepted that ligand engagement at the extracellular side somehow activates GAP. Although it is still unclear how the activity of the GAP domain is structurally regulated (for example, by a monomer/dimer exchange or conformational changes within a single domain), our current structure clearly identifies the structural change that takes place at the extracellular side. In the resting state, plexin assumes an auto-inhibited conformation, possibly by structural constraints stemming from head-on dimerization (Fig. 5, left). Upon semaphorin engagement, the orientation of the two plexin heads becomes more perpendicularly aligned to the membrane (Fig. 5, right).

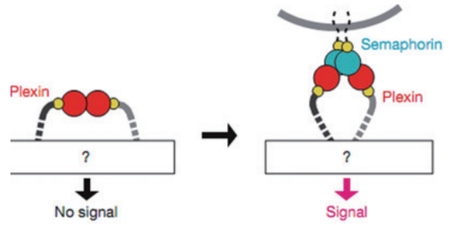

Fig.5 Possible structural mechanism of semaphorin-induced plexin signalling. Transition from the head-on cis homodimer of plexin (left) to the semaphorin-engaged complex (right) changes the relative orientation of the plexin molecular axis. This conformational change is transmitted through the stalk region (thick dotted line) and alters the conformation (for example, dimerization state) of the cytoplasmic GAP domain, resulting in signal initiation. The closer positioning of the two plexin tails in the active conformation is drawn arbitrarily and should be taken as an example, because the association states of the transmembrane and cytoplasmic regions before and after receptor activation remain unknown.

This conformational change is then transmitted, through the long stalk and the transmembrane domain, to the cytoplasmic region, leading to activation of the GAP domain and/or recruitment of Rho family GTPases. Although it remains possible that most of the cell-surface plexins in the resting state do not form a head-on dimer and the inactive phenotype is maintained by another type of mechanism, the structural conservation observed between the two semaphoring-plexin 'terminal' complexes is strongly indicative of the fundamental importance of this conformation in plexin signal transduction. More structural data are needed regarding the rest of the molecule, particularly the stalk region and the GAP domain, under different activation states, in order to understand fully the mechanism underlying semaphorin-induced plexin signal transduction. Such information may lead to the discovery of novel points of semaphorin signal intervention not limited to the receptor–ligand interface.

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SHARPIN is a Component of the NF-κB-activating Linear Ubiquitin Chain Assembly Complex
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SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex

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***Cpdm* (chronic proliferative dermatitis) mice develop chronic dermatitis and an immunodeficiency with increased serum IgM[1–3], symptoms that resemble those of patients with X-linked hyper-IgM syndrome and hypohydrotic ectodermal dysplasia (XHM-ED), which is caused by mutations in NEMO (NF-κB essential modulator; also known as IKBKG)[4–6]. Spontaneous null mutations in the *Sharpin* (SHANK-associated RH domain interacting protein in postsynaptic density)[7] gene are responsible for the *cpdm* phenotype in mice[8]. SHARPIN shows significant similarity to HOIL-1L (also known as RBCK1)[9,10], a component of linear ubiquitin chain assembly complex (LUBAC), which induces NF-κB activation through conjugation of linear polyubiquitin chains to NEMO[10–13]. Here, we identify SHARPIN as an additional component of LUBAC. SHARPIN-containing complexes can linearly ubiquitinate NEMO and activated NF-κB. Thus, we re-define LUBAC as a complex containing SHARPIN, HOIL-1L and HOIP (also known as RNF31). Deletion of SHARPIN drastically reduced the amount of LUBAC, which resulted in attenuated TNF-α- and CD40-mediated activation of NF-κB in mouse embryonic fibroblasts (MEFs) or B cells from *cpdm* mice. Considering the pleomorphic phenotype of *cpdm* mice, these results confirm the predicted role of LUBAC-mediated linear polyubiquitination in NF-κB activation induced by various stimuli, and strongly suggest the involvement of LUBAC-induced NF-κB activation in various disorders.**

NF-κB is a transcription factor involved in various functions, including inflammation and cell survival. NF-κB is shown to be activated by various stimuli[14–16]. CD40-mediated degradation of inhibitor of κBα (IκBα, also known as NFKBIA) leads to the activation of NF-κB. This pathway is severely impaired, but not completely abolished, in patients with XHM-ED, a syndrome caused by mutations in NEMO[5,6], a crucial regulator of the canonical NF-κB pathway[17]. CD40-mediated degradation of IκBα was impaired, but not completely abolished in splenic B cells from *cpdm* mice carrying the *Sharpin* null mutation[1–3,8] (Fig. 1a), which indicated that loss of SHARPIN impairs, but does not completely abolish NF-κB activation. The ubiquitin-like (UBL) and NPL4 zinc-finger (NZF) domains of SHARPIN showed significant similarity to those of HOIL-1L, a component of LUBAC that has a crucial role in TNF-α-mediated NF-κB activation[9–12] (Fig. 1b and Supplementary Fig. 1). TNF-α-induced activation of IκB kinase (IKK), phosphorylation and degradation of IκBα, and nuclear localization of the p65 subunit of NF-κB, which are hallmarks of NF-κB activation, were impaired, but not completely abolished, in *cpdm* MEFs (Fig. 1c–e). In addition, expression of NF-κB targets was markedly reduced in *cpdm* MEFs (Supplementary Fig. 2). These results clearly indicated that deletion of *Sharpin* also attenuates TNF-α-induced NF-κB activation.

The UBL domain of HOIL-1L is critical for complex formation with HOIP, the catalytic component of the linear polyubiquitination[10,11]. We proposed that SHARPIN forms a ubiquitin ligase complex with HOIP (SHARPIN–HOIP) and activates NF-κB[10]. Indeed, SHARPIN co-immunoprecipitated with HOIP, but not HOIL-1L (Supplementary Fig. 3). Introduction of SHARPIN–HOIP, but not SHARPIN alone or SHARPIN and HOIL-1L, induced NF-κB transcriptional activity (Supplementary Fig. 4a). Nuclear translocation of p65 was observed in HEK293T cells expressing SHARPIN–HOIP (Supplementary Fig. 4b). Furthermore, SHARPIN–HOIP showed linear polyubiquitination activity in an *in vitro* polyubiquitination assay, and the SHARPIN UBL domain was indispensable for HOIP binding and ubiquitination activity (Supplementary Figs 5 and 6). The NZF domain of SHARPIN seemed to be indispensable for NF-κB activation, but not for linear ubiquitination, and the UBA and RING-IBR-RING regions of HOIP were crucial for NF-κB activation mediated by SHARPIN–HOIP (Supplementary Figs 5d and 6). Given that UBA and RING-IBR-RING domains have crucial roles in HOIP binding to SHARPIN and in linear polyubiquitination, these results indicated that the SHARPIN–HOIP complex can activate NF-κB via linear polyubiquitination. In fact, SHARPIN–HOIP catalysed linear polyubiquitination of NEMO almost as efficiently as HOIL-1L–HOIP when overexpressed in HEK293T cells, as assessed by mass spectrometry and an anti-linear chain specific antibody (Supplementary Fig. 7).

We have noticed that NF-κB was more strongly activated in 293T cells expressing SHARPIN–HOIL-1L–HOIP as compared to cells co-expressing HOIL-1L–HOIP or SHARPIN–HOIP (Supplementary Fig. 4a). Co-immunoprecipitation assays to determine whether SHARPIN formed a ternary complex with HOIL-1L and HOIP showed that SHARPIN co-immunoprecipitated with both HOIP and HOIL-1L when co-expressed with either protein (Supplementary Fig. 3). Moreover, immunoprecipitation of endogenous LUBAC from HEK293T cells revealed that endogenous SHARPIN forms complexes with both HOIP and HOIL-1L (Fig. 2a). In HEK293T and Jurkat cells, SHARPIN co-fractionated primarily with HOIL-1L and HOIP in the relative molecular mass ~600,000 fraction (M_r 600K; Fig. 2b). Sequential precipitation of the ~600K fraction from HEK293 cells that stably expressed Flag–HOIP, HOIL-1L–His_6–haemagglutinin (HA), and Myc–SHARPIN confirmed that HOIL-1L, HOIP and SHARPIN form a ternary complex (Supplementary Fig. 8). These results conclusively indicated that SHARPIN forms a ternary complex with HOIL-1L and HOIP, and we re-defined LUBAC as a 600K complex containing HOIL-1L and/or SHARPIN together with HOIP. Sequential precipitation of HEK293T cells expressing Flag–HOIP, His_6–HOIP and Myc–HOIP showed that there were at least three HOIP molecules per LUBAC complex (Supplementary Fig. 9). SHARPIN–HOIP formed complexes with HOIL-1L–HOIP more efficiently than SHARPIN alone (Supplementary Fig. 10). Thus, LUBAC seems to be composed of a mixture of HOIL-1L–HOIP and SHARPIN–HOIP complexes, although we cannot exclude the possibility that LUBAC may break down into HOIL-1L–HOIP and

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