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Immunoglobulin superfamily cell adhesion molecules: zippers and signals

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The latest structural studies of immunoglobulin superfamily cell adhesion molecules are driving a shift in perspective; increasingly the view is not focused solely on the individual molecule but rather is on the molecular assembly. Two common themes are emerging, revealing mechanisms for ectodomain-dependent regulation of cell surface receptors' signalling abilities. The first is the propensity of many such molecules to arrange in zipper-type or array-type assemblies driven by a network of highly specific *cis* and *trans* interactions. The second is the use of the extracellular dimensions of a molecule or adhesion complex as properties which, in combination with characteristic intercellular spacings, can determine the co-localisation or exclusion of particular protein populations at cell interfaces and junctions.

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Introduction

Cell adhesion molecules (CAMs) perform a wide range of functions at cell contacts, ranging from mechanical support to target recognition, from differentiation and specialization of membrane microdomains to the initiation, organization and regulation of signalling platforms [1–3]. Two decades of structural biology research have resulted in the characterisation of domain topologies, and atomic level insights into binding mechanisms, for key members of all the main classes of CAMs: the immunoglobulin superfamily (IgSF), the cadherins, the integrins and the selectins [4,5,6••].

Analysis of the human genome reveals that Ig-like has the widest representation of any protein domain, being encoded in 765 genes [7]. Other species, such as *Drosophila melanogaster* and *Caenorhabditis elegans* also contain large numbers of IgSF proteins: 142 and 80, respectively [8]. Vogel and Chothia [9••] have recently identified 194 protein superfamilies (out of 1219 analysed in 38 eukaryotic species ranging from protozoa to man) for which comparisons between organisms indicate that increasing membership parallels increasing diversification of cell types. This correlation suggests that expansion in these families may have supported an increase in biological complexity. Interestingly, the IgSF ranks at the top, with the highest correlation coefficient ($R = 0.97$). Of course the functions of Ig-like domain containing proteins are not limited to cell adhesion. Nevertheless, analysis of *Drosophila* and *C. elegans* genomes reveals that the majority of IgSF members are cell surface proteins, and many of these (82/142 and 31/80, respectively) are likely to be CAMs [8].

This review is focused on significant results in the structural characterisation of IgSF CAMs from the past two years. Several important concepts have gained strength. One recurrent theme is the organization of ordered (zipper-like) arrays of CAMs via combined *cis* and *trans* interactions. A second theme is the role of size-exclusion mechanisms in dynamically shaping the molecular composition of intercellular contact regions.

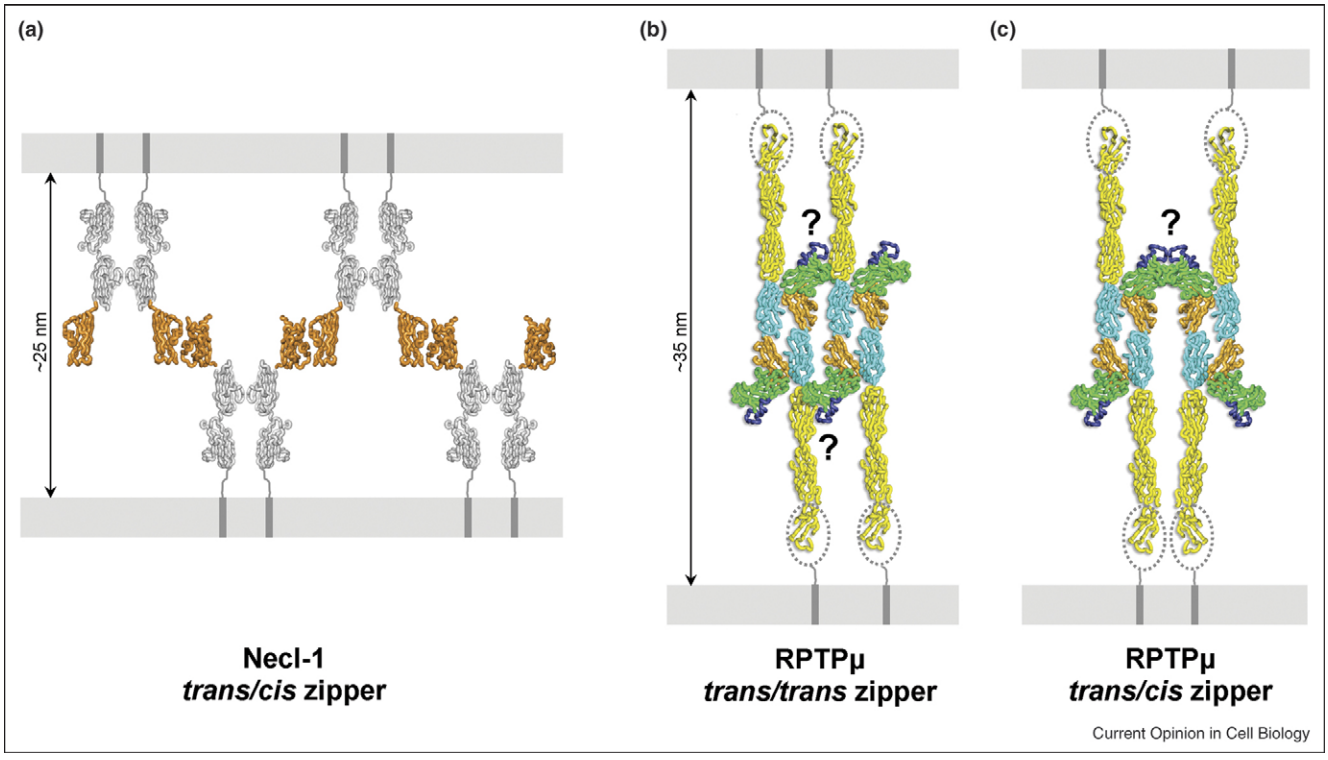
Homophilic protein interactions and CAM zippers

Crystal structures of functional (adhesive) extracellular fragments from several homophilic IgSF CAMs (for example the myelin protein P0 [10], axonin-1/TAG-1 [11], the neural cell adhesion molecule (NCAM) [12] and the junctional adhesion molecule (JAM) [13]) have previously led to the idea that ordered 1D (zippers) or 2D (arrays) of CAMs form at cell contacts. These assemblies require additional interactions beyond that of the primary homophilic binding site to combine contacts in *cis* (between molecules attached to the same cell surface) and *trans* (between molecules attached on opposite cell surfaces). A similar *cis–trans* interaction model has been proposed for the cadherins [14] and such zippers or arrays are certainly attractive candidates as mechanisms to stabilise and expand cell contacts (discussed in [11]). Moreover, since most CAMs span the plasma membrane and can associate with a large number of proteins via their intracellular regions, zipper/array-based assemblies could establish platforms for the organisation of signalling complexes or anchorage points for cytoskeletal components. Two recent studies reporting crystal structures of ectodomain constructs derived from nectin-like molecule-1 (Necl-1 or SynCAM3) [15••] and the receptor protein tyrosine phosphatase μ (RPTPμ) [16••] have underpinned fresh discussion of these ideas.

Necl molecules contain three Ig-like domains in their extracellular region and are expressed prominently at neural synaptic sites where they appear to play a driving role during synapse assembly [3] whilst the intracellular regions provide links to the actin cytoskeleton. The crystal structure of the N-terminal Ig-like domain (V-type) of Necl-1 revealed the *trans* homophilic interaction, supported by a large interface (1405 $Å^2$) mainly located to the C–C′–C″–D β-strands and built around a central Phe residue. Previous results on nectins have demonstrated their ability to form *cis* dimers via their second Ig-like domain. Given the high similarity between nectins and Necl-1, the combination of these two observations led to the persuasive proposal that preformed *cis* Necl-1 dimers interact in *trans* via their N-terminal Ig-like domains to create a zipper-like arrangement [15••] (Figure 1a).

Type IIB RPTPs (see ref. [17••] for a recent review) occupy a special niche among IgSF CAMs due to their dual role: the ectodomains are involved in homophilic (adhesive) interactions while the intracellular domains carry a catalytic (phosphatase) function. RPTPμ is thought to play a stabilising role at cellular adherens junctions since all its substrates known to date are components of the cadherin–catenin complexes [18•]. Type IIB RPTPs share a common architecture: their extracellular region contains one meprin/A5/μ (MAM), one Ig-like and four FN-III domains. Previous studies [19,20] suggested that either MAM or Ig-like domains are necessary or sufficient for homophilic binding and specificity. Therefore, it was hoped that a construct containing these two domains (termed MIg) would reveal the structural determinants for adhesion. The crystal structure of MIg [16] duly provided the architecture of a MAM fold (a 10-stranded β-sandwich of jelly-roll topology, similar to the ligand-binding domain of Eph receptors) and showed that MIg forms a tightly packed structural unit where a continuous β-sheet spans both domains, but failed to deliver

Figure 1



Novel IgSF CAMs proposed to form homophilic adhesive zippers. **(a)** Crystal structure of the Necl-1 N-terminal Ig domain (coloured in orange) revealed the mechanism of *trans* adhesive interactions. The second and third Ig domains of the Necl-1 extracellular region are shown in grey (modelled on the NCAM N-terminal two Ig domains, PBD code 1EPF [55]). The second Ig domain promotes *cis* dimerisation in nectins, and it is likely to perform a similar function in Necl-1, leading to the *trans–cis* model proposed [15••]. The intermembrane distance predicted, ~25 nm, is compatible with observed synaptic spacings. **(b)** and **(c)** Zipper models for the adhesive phosphatase RPTPμ were built based on mutagenesis, cell-adhesion assays and surface plasmon resonance binding data [16••]. The RPTPμ ectodomain structure (2V5Y), however, reveals only the *trans* homophilic interaction [22••]. The MAM domain is shown in green, the Ig domain in light orange, the FN1 domain in cyan and the remaining three FN domains in yellow (the membrane proximal FN domain, disordered in the 2V5Y structure, is circled in grey; domain colouring scheme was chosen to match the one used in ref. [16••]). The L1 and L2 loops, predicted to be involved in low-affinity adhesive contacts are shown in dark blue. Since L1 and L2 may participate in either *trans* or *cis* interactions (marked by question marks), two zipper models have been proposed [16••]. Plasma membranes (shown as thick grey lines and drawn approximately to scale), linker and transmembrane regions (dark grey) are only schematic representations. Intracellular regions are not shown in order to simplify the figure.

a view of the primary homophilic dimerisation interface. However, mutagenesis data based on the MIg structure as well as analogies with previous findings on the meprin MAM domain, have suggested the involvement of two MAM loops (termed L1 and L2) in higher order interactions, in either *cis* or *trans* orientations [16••,21] prompting two possible models for RPTPμ zippers to be suggested [16••] (Figure 1b and c). Meanwhile, biophysical and cell-adhesion assays have demonstrated that contributions from two additional FN-III domains are required for the primary (high affinity) *trans* adhesive interaction [16••]. This finding recently received further support from the elucidation of a full-length RPTPμ ectodomain crystal structure [22••]. This structure revealed all the determinants required for the *trans* homophilic (adhesive) interaction and the mechanism for ectodomain-controlled localization of RPTPμ at cell contacts ([22••] and see ''Size matters'' section). However, the weaker interactions involving the MAM L1 and L2 loops [16••] have not been observed in this crystallographic arrangement. Further work will therefore be required to validate the proposed higher order assemblies.

Zippers and glycans

NCAM is one of the most extensively studied IgSF CAMs and plays important roles in brain development and function [23•]. It is expressed in several isoforms, but all membrane-attached versions (NCAM-120, 140 and 180) contain five Ig-like domains and two FN-III domains in the extracellular region. A complex pattern of interactions involving the Ig-like domains is responsible for the homophilic binding properties and successive zipper-type models have been proposed with each advance in the structural characterisation of this system (reviewed in ref. [24]). The largest construct solved to date contains just the three N-terminal Ig-like domains, therefore, further refinements of the NCAM zipper models may well prove necessary.

In addition to the protein–protein interactions, glycans are known to play essential roles in regulating NCAM-based adhesion. These glycans include polysialic acid (PSA), heparin and chondroitin sulphate [24,25•]. Recent structural work has shed some light on the mechanisms by which these molecules exert their regulatory effects on NCAM. PSA is typically attached to the *N*-glycosylation sites present in the fifth Ig-like domain (Ig5) and has an anti-adhesive function [25•]. The adjacent FN-III domain (FN1) is essential for polysialylation, presumably because it contributes an interaction site for the two enzymes responsible for PSA addition. The crystal structure of FN1 [26••] revealed two distinctive features, which could be involved in this regulatory process: an acidic surface patch and an α-helix linking the fourth and fifth β strands of the domain. Subsequent mutagenesis experiments confirmed that the acidic patch is important for polysialyltransferase attachment, while the α-helical region plays an intriguing role: a mutation in this structural element shifted PSA addition from the *N*-glycans in Ig5 to the O-glycans in the FN1 domain. This finding suggests that the helix mediates an Ig5-FN1 interaction vital to the correct positioning of Ig5 *N*-glycans for polysialylation [26••]. Heparan and chondroitin sulphate proteoglycans can also modulate NCAM homophilic interactions, and a mechanistic understanding of this process has been provided by the recent characterisation of their binding site using NMR [27•]. Both glycans can bind to the same site in the Ig2 domain, which also overlaps with a site for NCAM homophilic interaction in *cis* between Ig1 and Ig2 and hence provides a satisfying explanation for the modulatory role of these glycans in NCAM-dependent neuronal growth and differentiation [27•].

Homophilic and pseudohomophilic interactions without zipper formation

Adhesion mediated by members of the CD2 family of IgSF CAMs does not appear to trigger zipper/array formation. These molecules, which include CD2, CD48, CD58 and members of the SLAM (signalling lymphocytic activation molecule) subfamily, are widely expressed on hematopoietic cells and are important regulators of immunity [28,29•]. Within the SLAM subfamily binding is mainly homophilic, while interactions between CD2-CD48 and CD2-CD58, due to the high homology of the partners, can be considered pseudohomophilic [4].

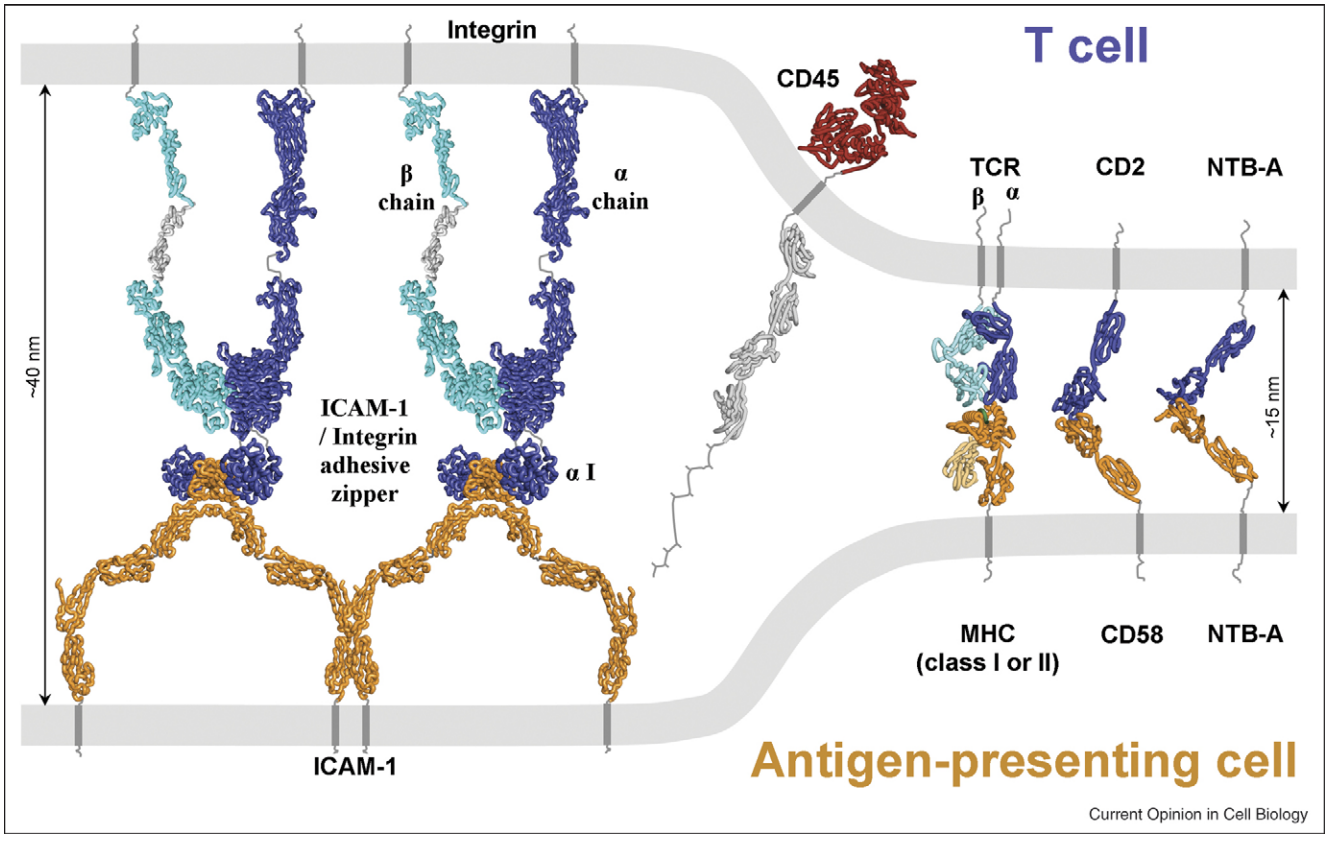
Structural analysis of CD2 (reviewed in ref. [4]) and the CD2-CD58 complex [30] suggested a general paradigm for the low affinity adhesion interactions mediated by this family, where electrostatic rather than surface-shape complementarity governs specificity. This view was recently bolstered by the results of a crystallographic characterisation of CD48 and analysis of the putative CD2–CD48 interface [31••]. Interestingly, the crystal structure of the homophilic NK-T-B-antigen (NTB-A, a SLAM subfamily member), despite overall structural similarities, reveals significant differences in the detailed organisation of the adhesive interface: a larger buried surface area than the CD2–CD58 dimer, an improved surface complementarity and a significant number of hydrophobic contacts [32••]. Examination of more SLAM subfamily members is likely to reveal additional determinants of specificity, since K_D values of the homophilic interactions vary over several orders of magnitude: from 200 μM in the case of SLAM (CD150), to 2 μM in NTB-A, further dropping to the nM level in CD84 [32••].

Structural analyses of CD2 family members triggered much of the early discussion concerning physical constraints on the overall size of adhesion molecules and their relationship with subcellular localisation. Recent developments providing further fuel for these ideas are reviewed below.

Heterophilic interactions

Several IgSF CAMs can establish *trans* adhesive interactions with members of the integrin superfamily, these include ICAMs (intercellular CAMs), VCAMs (vascular CAMs) and MAdCAMs (mucosal addressin CAMs) [6••,33]. All these molecules are expressed on vascular endothelial cells and function in leukocyte attachment and transendothelial migration at inflammatory sites. ICAMs, in particular, bind to integrins that contain an 'inserted' (I) domain in their α chain, such as $\alpha_L\beta_2$ and $\alpha_M\beta_2$ [6••,33]; this interaction is thought to favour the formation of a heterophilic adhesive zipper [6••] (Figure 2). The crystal structure of a complex between the N-terminal Ig-like domain of ICAM-1 (D1) and the α_L I domain (α_LI) revealed the essential determinants for this interaction: a Glu residue, located in the centre of a relatively flat surface of D1 that coordinates a Mg^{2+} ion from the MIDAS (metal ion-dependent adhesion site) of α_cI and a Lys_{D1}-$Glu_{\alpha LI}$ salt bridge [34]. The recently published crystal structure of the ICAM-3 D1 in complex with the α_LI [35••] yielded important new details for this type of adhesive interface (not least due to an improved resolution, 1.65 Å versus 3.3 Å). Firstly, despite the common docking mode for the two ICAMs, the binding affinity varies significantly (ICAM-1 > ICAM-3): this is due to the landscape surrounding the key MIDAS-coor-

Figure 2



A size-dependent sorting mechanism operates at local zones of contact between T cells and antigen-presenting cells. The NTB-A crystal structure [32••] revealed a *trans* adhesive dimer of a length comparable with other synaptic molecules such as the TCR-pMHC complex and the CD2-CD58 complex. Molecules with larger ectodomains, such as the CD45 phosphatase (crystal structure of the intracellular catalytic region is shown in red), are excluded from the narrow synaptic area to allow tyrosine phosphorylation of the TCR-CD3 complex and downstream signalling [41••]. Larger molecules such as the $\alpha_L\beta_2$ integrin and its ICAM-1 ligand occupy a peripheral zone with a larger intermembrane distance [57]. The coloured domains (in orange, red, dark blue and cyan) are all crystal structures, while the grey regions are either models (EFG repeats 1 and 2 in the integrin β chain or the four FN type III domains in the CD45 extracellular region) or purely schematic (the mucin-like N-terminal domain of CD45, as well as the linker, transmembrane and intracellular regions). The stoichiometry of the integrin:ICAM-1 interaction is 1:1, however for clarity only αI domain of the integrin is shown for the molecules in the background. The following Protein Data Bank (PDB) entries were used to create this figure: 2IF7 for NTB-A [32••]; 1HNF for CD2 [58]; 1QA9 for the CD2–CD58 complex [30]; 1OGA for the TCR–MHC class I complex [59]; 1YGR for the CD45 intracellular region [60]; 1FNF for the model CD45 ectodomain [56]; 1P53 for the ICAM-1 Ig domains 3, 4 and 5 [57]; 1MQ8 for the ICAM-1 Ig domains 1 and 2 in complex with the I domain of integrin α_L chain [34]; 1M1X for the integrin α chain and membrane-proximal region of the β chain [61]; 1TYE for the N-terminal region of the integrin β chain [62]. The integrin molecules are drawn in an extended ('high affinity') conformation, as discussed in [6••,62]. The CD45 ectodomain (four FN type III and a mucin-like domain) appears to have an overall length of ~28 nm, as revealed by rotary shadowing and electron microscopy [63], and is drawn to scale. Plasma membranes (shown as thick grey lines and drawn approximately to scale), linker and transmembrane regions (dark grey) are only schematic representations.

dinating Glu being dominated by hydrophilic residues in ICAM-3 versus hydrophobic ones in ICAM-1. Secondly, the ICAM-1 D1 carries no glycans, while three N-linked sugars encircle the binding surface of ICAM-3; it appears that these sugars impair the binding affinity to α_LI primarily by lowering the k_{on} [35••]. Detailed knowledge of the ICAM- α_LI interface also opened the way for a rational design of ICAM-1 mutants with significantly increased affinities for α_LI [36•]. Such constructs have the potential to develop into therapeutic agents or serve as leads for small-molecule design of competitive inhibitors of the ICAM-integrin interactions, a strategy currently tested for the treatment of autoimmune diseases and prevention of immune rejection in organ transplantation [37].

Many IgSF CAMs have been hijacked by viruses as cell attachment receptors and the ICAMs are no exception. Several viruses of the *Picornaviridae* family, including coxackieviruses (CVA) and human rhinoviruses (HRV) bind to ICAM-1 [38••]. The recently published crystal structure of CVA21 and a cryo-electron microscopy reconstruction, at 8 Å resolution, of CVA21 bound to ICAM-1 variant, ICAM-1^{Kilifi} [38••] provides an important advance in our understanding of this interaction. The previously determined ICAM-1 crystal structure could be accurately fitted into the cryo-EM density and four regions of ionic interaction networks in ICAM-CVA21 interface were defined. These networks involve residues well conserved within CVA and HRV family members that bind ICAM-1 and are very likely to provide the specificity determinants. Functional studies to validate these predictions may soon become available.

Size matters

The idea that CAMs may play a role in determining the inter-membrane spacings at cellular contacts emerged over a decade ago. The P0 crystal structure revealed a pattern of homophilic interactions that could help maintain a constant intermembrane distance, as observed in compact myelin [10]. Size predictions of adhesion receptors in the immune system [39], and early structural work [40], suggested the existence of two classes of molecules that can cluster in distinct areas of cell contacts: the TCR–pMHC (T cell receptor–peptide-MHC complex) and CD2–CD58 complexes at narrow contacts (~15 nm), versus integrins–ICAM complexes and proteins with large, glycosylated, ectodomains at contacts ~40 nm wide. Subsequently, insights into the link between tyrosine phosphorylation of the TCR–CD3 and T-cell activation led to the proposal of a 'kinetic-segregation' model for TCR triggering (recently reviewed in ref. [41••]). This underlines the importance of size-sorting mechanisms for the actual signalling process, where protein tyrosine phosphatases with large ectodomains (such as CD45) are excluded from the narrow contact zones (Figure 2). A significant piece of evidence supporting this model was recently provided by a demonstration that artificial elongation of the pMHC ectodomain, resulting in an increase in the overall length of the TCR–pMHC complex, greatly reduces TCR triggering without affecting the TCR–pMHC ligation [42••]. The NTB-A crystal structure (discussed above) further supports this idea, revealing an overall length for the *trans* dimer length of ~100 Å [32••], in line with the previously reported dimensions of CD2 family adhesion complexes (first exemplified by CD2 itself [40]). With the exception of Ly-9, it now appears that all members of the CD2 family exhibit a similar overall organisation, with two Ig-like domains and a maximal linear dimension of *trans* adhesion dimer that is consistent with that of other pairs of signalling molecules, such as the TCR-pMHC and CTLA-4-B7 [32••,41••], present at local zones of contact between T cells and antigen-presenting cells (Figure 2). The NTB-A structure also revealed some limited variation (~14°) in the relative orientation of the two Ig domains within a molecule was possible. This is reminiscent of observations originally reported for the prototypic rat CD2 structure, where the domains were seen to be able to flex by some 7° relative to each other [40]. Together with some intrinsic flexibility in juxtamembrane linker regions, this inter-domain hinge point may serve an important function under physiological conditions, allowing the receptors some leeway to adapt to a dynamic environment (where forces caused by cell movements tend to oppose adhesion) without rupturing the *trans* interface.

A similar mechanism for size-sorting, with direct signalling consequences, may operate at other cell contacts, such as the adherens junctions. We have recently solved the crystal structure of the full-length RPTPμ ectodomain [22••] and observed an extended architecture which mediates *trans* dimerisation through a large interface. The only flexible region appears to be in the linker between the juxtamembrane FN type III domain and the rest of the ectodomain [22••] (Figure 1b, c). Cell surface expression of a series of domain-deletion constructs (still preserving the adhesive interface) revealed a direct correlation between the RPTPμ ectodomain length and intercellular spacings [22••]. In vivo, RPTPμ is excluded from narrow contacts such as tight junctions. Moreover, full length RPTPμ trans dimers match the reported dimensions of cadherin adhesive interactions [14] and RPTPμ phosphatase activity has been reported to act on cadherin-catenin assemblies [18]. This led us to propose a model whereby the rigid, ruler-like ectodomain of RPTPμ acts as a sensor of intercellular distances matching the cadherin-initiated cell contacts. The *trans* adhesive interaction acts as a spacer clamp, locking the phosphatase activity in proximity with its target substrates. This stabilises the junctions both physically and via dephosphorylation of the cadherin-catenin complex (known to be required for junctional integrity [18]).

Further challenges: large signalling complexes and adhesive puzzles

Structural studies have so far focused on the *trans* and/or *cis* adhesive interactions however there is an ever increasing body of evidence suggesting that CAM interactions are far more complex. Not only do adhesion assemblies involve a large number of intracellular adapter/signalling molecules but also other plasma membrane proteins. NCAM, L1 and *N*-cadherin, for example, can all interact in *cis* with FGF (fibroblast growth factor) receptors, via ectodomain-mediated interactions, and even induce signalling in the absence of FGF [43]. Interestingly, although such interactions can take place over most of the cell surface, they are not seen at the points of intercellular contact where the adhesion molecules typically accumulate to form stable junctions and zippers [44•]. In pancreatic tumour cells NCAM was found in a large signalling complex, that also includes FGF receptor 4, *N*-cadherin, phospholipase Cγ, the adaptor protein FRS2, $pp60^{c\text{-}src}$, contactin and GAP-43 [45]. L1, another IgSF CAM, binds homophilically in *trans* but also has multiple heterophilic-binding partners such as axonin-1/TAG-1, contactin, neurocan and integrins [1]. Moreover, L1 also appears to interact with neuropilin-1 and serve as co-receptor for semaphorin 3A [1,46]. Structural analysis of such complexes (these are just a few examples selected for illustrative purposes) will be highly challenging but also essential for a complete understanding of the IgSF CAMs' signalling mechanisms. Another challenge is raised by the high sequence variability, caused by alternative splicing, of certain CAMs. An extreme example is the Down syndrome CAM (Dscam), an axon guidance receptor with a very large diversity: over 38 000 isoforms can potentially be generated through alternative splicing [47]. Three of the four splicing hotspots affect Ig-like domains in the extracellular region. Importantly, various isoforms bind in a strict homophilic fashion, even those sharing very similar amino acid sequences, and this specificity is crucial for the correct patterning of dendrites in the peripheral nervous system [48••,49••,50••,51••]. It would be of great interest to understand this extremely complex and precise mechanism of specificity in structural terms.

Conclusions

Significant recent progress has been made in the structural characterisation of IgSF CAMs and insights into important concepts such as the supramolecular organisation and size-dependent sorting of surface receptors at intercellular contacts have been reinforced. The complexity of the network of interactions that CAMs participate in still poses significant challenges for structural biology. Technological advances in large-scale mammalian cell expression [52], control of N-linked glycosylation [53] and high-throughput robotic crystallization [54] should facilitate crystallographic analysis of full-length ectodomains, *cis*/*trans* ectodomain complexes and, ultimately, transmembrane constructs and complexes.

Note added in proof

A very important step towards solving the Dscam adhesive puzzle mentioned above has very recently been reported. Meijers *et al.* determined the crystal structures of four amino-terminal Ig-like domain constructs (D1-D4) derived from two Dscam isoforms [64••]. These constructs contain distinct variants of exons 4 and 6, two of the splicing hotspots affecting the second (D2) and third (D3) Ig-like domains, respectively. In both cases, the four domains are arranged in a horseshoe configuration reminiscent of the axonin-1/TAG-1 structure [11] and the structures reveal a surface epitope that is important for determining the specificity of Dscam homophilic interactions. Importantly, this involves residues contributed by both D2 (a long A′ strand) and D3 (a distinct A-A′ protrusion), encoded by the alternatively spliced exons. Swapping the peptides encoding this epitope resulted in a full switch in the homophilic binding specificity between the two isoforms analysed. Phylogenetic comparison of the alternatively spliced exons among *Drosophila* species revealed a high conservation of residues contributing to the homophilic interface, presumably an effect of selection pressure. In contrast, a second epitope also contributed by the 2 exons but not involved in homophilic binding shows marked divergence. Exon 4/6 combinations result in 576 possible isoforms, but the system is far more complex, considering the additional 33 alternatives of exon 9 (encoding the 7-th Ig-like domain) that may also contribute to homophilic binding. Further structural work will hopefully lead to a complete understanding of this puzzle.

Acknowledgements

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