Molecular analysis of receptor protein tyrosine phosphatase μ -mediated cell adhesion

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Type IIB receptor protein tyrosine phosphatases (RPTPs) are bi-functional cell surface molecules. Their ectodomains mediate stable, homophilic, cell-adhesive interactions, whereas the intracellular catalytic regions can modulate the phosphorylation state of cadherin/catenin complexes. We describe a systematic investigation of the cell-adhesive properties of the extracellular region of RPTP μ , a prototypical type IIB RPTP. The crystal structure of a construct comprising its N-terminal MAM (meprin/A5/ μ) and Ig domains was determined at 2.7 Å resolution; this assigns the MAM fold to the jelly-roll family and reveals extensive interactions between the two domains, which form a rigid structural unit. Structure-based site-directed mutagenesis, serial domain deletions and cell-adhesion assays allowed us to identify the four N-terminal domains (MAM, Ig, fibronectin type III (FNIII)-1 and FNIII-2) as a minimal functional unit. Biophysical characterization revealed at least two independent types of homophilic interaction which, taken together, suggest that there is the potential for formation of a complex and possibly ordered array of receptor molecules at cell contact sites.

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Introduction

Cell adhesion is fundamental to multicellular organisms, providing structural support and avenues for communication. Cell-adhesion molecules are specialized cell surface receptors capable of clustering at intercellular contacts via interactions,

mediated by their ectodomains, which can occur in *trans* (between opposing cells) as well as in *cis* (on the same cell surface). Reversible protein phosphorylation plays a prominent role in the signalling associated with these dynamic molecular assemblies (reviewed in Perez-Moreno *et al*, 2003). Most cell-adhesion molecules lack intrinsic kinase or phosphatase activity, but the type IIB receptor protein tyrosine phosphatases (RPTPs) can be classified both in terms of intercellular adhesive and intracellular signalling functions (reviewed in Beltran and Bixby, 2003; Johnson and Van Vactor, 2003). One of them, RPTP ρ (PTPRT), among all annotated human protein phosphatases, was recently found to have the highest occurrence of somatic mutations, affecting both the extracellular and intracellular regions, in colorectal tumours and to have the characteristics of a tumour suppressor (Wang *et al*, 2004). The type IIB RPTPs thus comprise a biologically very interesting family of proteins but the molecular mechanisms underlying their cell-adhesive function and the implications of these ectodomain interactions for phosphatase signalling are yet unclear.

RPTP μ is a prototypical type IIB RPTP (Gebbink *et al*, 1991), others being RPTP κ , RPTP ρ and PCP2 (RPTP ψ /π/λ). RPTP μ and RPTP κ have been shown to mediate homophilic cell adhesion (Brady-Kalnay *et al*, 1993; Gebbink *et al*, 1993; Sap *et al*, 1994; Cheng *et al*, 1997), a function probably also performed by RPTP ρ and PCP2 as the four homologues have highly similar ectodomain sequences (amino-acid identities between RPTP μ and RPTP κ , RPTP ρ and PCP2 are 54, 63 and 49%, respectively). These molecules display different spatial expression patterns throughout development (Fuchs *et al*, 1998; McAndrew *et al*, 1998), suggesting that they function in a strictly homophilic manner. This concurs with the absence of mixed cell aggregates in co-cultured cells expressing either RPTP μ or RPTP κ (Zondag *et al*, 1995). RPTP μ has been found in the nervous system where it appears to perform functions related to axon guidance within the visual system and retinal lamination (reviewed in Johnson and Van Vactor, 2003; Ensslen-Craig and Brady-Kalnay, 2004) and in tissues where tight, continuous cell–cell contacts are physiologically important, such as the vascular endothelia and the intercalated discs of cardiomyocytes (Bianchi *et al*, 1999; Koop *et al*, 2003), where it has been shown to be clustered at the intercellular boundaries (Sui *et al*, 2005). In this context, it has been shown that RPTP μ can interact with members of the cadherin family of cell-adhesion molecules (N, E, R and VE cadherins; Brady-Kalnay *et al*, 1998; Sui *et al*, 2005). Functionally, it is thought that such an interaction would bring the phosphatase activity of RPTP μ near the cadherin–catenin complexes, maintaining them in a dephosphorylated state and so stabilizing the intercellular contacts.

The type IIB RPTPs share the same domain structure: an ectodomain, an ~100-residue intracellular juxtamembrane region unique among RPTPs and two phosphatase domains. The ectodomain contains one MAM (meprin/A5/ μ) domain (Beckmann and Bork, 1993), one Ig domain, and four

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fibronectin type III (FNIII) repeats. The MAM fold is to date unknown, and is also present extracellularly in a functionally diverse range of proteins such as meprins, neuropilins and zonadhesins. This domain has been shown to participate in homo-oligomerization of neuropilin-1 and -2 (Chen *et al*, 1998; Nakamura *et al*, 1998) and meprin A (Ishmael *et al*, 2001). For RPTP μ , the MAM and the Ig domains have been separately implicated as having key roles in homophilic adhesive (*trans*) interactions (Brady-Kalnay and Tonks, 1994; Zondag *et al*, 1995). Recently, Cismasiu *et al* (2004) have proposed that the homophilic binding properties of the MAM domain make a particular contribution to the lateral (*cis*) receptor oligomerization.

We describe here a systematic investigation of the cell-adhesive properties of the human RPTP μ extracellular region (Ex μ), aiming to map further the homophilic interaction sites and to understand the relative contributions of MAM and the other constituent domains. We determined the crystal structure of a construct containing the two N-terminal domains (MAM and Ig, hereafter called MIg) at 2.7 Å resolution. This allowed the identification of a distinctive surface on the MAM domain that, as subsequent mutagenesis studies suggest, contributes directly to cell adhesion. However, a serial domain deletion analysis revealed that further interactions are required for this function, involving domains beyond MIg. Our results are consistent with a multidomain interaction model for RPTP μ and, more generally, type IIB RPTPs-mediated cell adhesion. Any adhesion-induced oligomerization of the RPTP μ molecules will affect the relative spatial arrangement of the intracellular catalytic regions and may induce specific conformational changes, thus representing a potential regulatory mechanism for downstream signalling.

Results

Crystal structure of the MIg segment of RPTP μ

Crystals of selenomethionyl (SeMet) substituted and hexahistidine-tagged MIg were grown from protein expressed in human embryonic kidney (HEK293T) cells. There is one monomer per crystallographic asymmetric unit. The protein migrates as ~40 kDa on SDS gels, ~10 kDa larger than its theoretical molecular size, a difference accounted for entirely by *N*-glycosylation as confirmed by PNGase F treatment (data not shown). The current model (Figure 1) is complete for residues 21–279 (residues 1–20 constitute the secretion signal sequence) with only the C-terminal His₆ tag unobserved, and contains three glycosylation sites at Asn72, Asn92 and Asn131. It has been refined to $R_{\text{work}}/R_{\text{free}}$ of 22.4/27.4% at 2.7 Å (Table I, representative electron density is shown in Supplementary Figure S1).

The overall structure of MIg is roughly L-shaped, where the principal axes of the MAM (residues 21–184) and the Ig (residues 185–279) domains form an angle of ~104° (Figure 1A). The interdomain orientation is stabilized by a short linker (residues 180–184, XPCXX) whose proline and cysteine are conserved in type IIB RPTPs (Figure 2A). Pro181 is at the turning point of the linker as it exits the main body of the MAM domain. Cys182 serves to secure the linker via a disulphide bond (Cys96–Cys182, both conserved among the MAM domains). The Ig domain is oriented edge-on against the MAM domain, with the outer strand G of the MAM domain β -sheet GDIB hydrogen bonding to strand g of

the Ig domain β -sheet cfg to form a continuous sheet spanning both domains (Figure 1A). The interdomain interface contains 21 van der Waals contacts and 20 hydrogen bonds, 15 of which are main chain–main chain interactions.

The MAM domain fold was heretofore unknown. A DALI (Holm and Sander, 1995) search for structural homologues reveals that it belongs to the galactose-binding domain-like fold in the SCOP database (Andreeva *et al*, 2004). The 10-stranded β -sandwich is of jelly-roll topology (Supplementary Figure S2) and is structurally homologous to the ephrin-binding domain of murine EphB2 receptor tyrosine kinase (PDB ID 1KGY, chain A, 181 residues) (Himanen *et al*, 2001) and a host of carbohydrate-binding domains of bacterial proteins, for example that of laminarinase from *Thermotoga maritima* (PDB ID 1GUI, chain A, 155 residues) (Boraston *et al*, 2002) (Figure 1B). Sequence similarities among these proteins are minimal and are confined to the structural core. The Ig domain of MIg, similar to those in many CAMs, belongs to the I-set structural class of the immunoglobulin superfamily (reviewed in Chothia and Jones, 1997). The edge of the β -sandwich between strands c and d is the least conserved region in I-set domains and compared to other structures MIg lacks strand c' but has extended β -strands d and e (residues 229–244).

Structural comparisons of the MAM domain with the ephrin-binding domain of the EphB2 receptor and with the carbohydrate-binding domain of laminarinase provide insights into the location of putative functional sites for the MAM domains in general. The RPTP μ MAM domain, when structurally aligned with 1KGY:A, reveals 108 superposable residues with inter-C α atom distances <3 Å, root mean square deviation (r.m.s.d.) ~1.8 Å. With 1GUI:A, it shares 103 superposable residues, r.m.s.d. ~1.9 Å. Regions (loops >6 residues) that are not superposable with the MAM structure are all part of, or demarcate, the ligand-binding sites in EphB2 and laminarinase (Figure 1B). By analogy, in the MAM domain these regions are putative protein–protein interaction sites. They can be grouped into two subsites, located on opposing faces of the β -sandwich: L1 (residues 21–31) and L2 (53–67) on one side; L3 (108–113), L4 (122–127) and L5 (133–138) on the other. Notably, L1 (stabilized by a disulphide bond composed of two cysteines conserved in MAM domains, Cys27 and Cys36) and L2 are unique to the MAM-type jelly-roll fold, with L2 displaying a particularly high degree of sequence variability (Figure 2A and B). This suggests that they might play functional roles in all MAM-containing proteins, with L2 in particular conferring binding specificity. The structure of the L1 and the L2 loops is well ordered (Supplementary Figure S1); both are anchored to the β -sandwich main body via 64% (L1) and 42% (L2) of the side-chains (Figure 1C). The surfaces formed by these two loops are relatively flat, and are approximately coplanar with each other (Figure 1B). In addition, we have identified two metal ions (most likely sodium as judged from their coordination geometry) involved in multiple interactions with residues from L1 and L2 and therefore playing a stabilizing role (Supplementary Figure S3).

The formation of a crystal lattice requires protein–protein contacts and the major contact sites in the crystal can sometimes correspond to physiologically relevant interactions. The crystal packing of MIg, however, lacks extensive intermolecular interactions and no one site involves a

Figure 1 The Mlg crystal structure and analysis of the MAM domain. (A) Ribbon diagram of Mlg. The MAM-Ig linker in the MAM domain is highlighted in purple. Disulphide bonds (orange) and the N-glycosylation sites (CPK) are presented as stick models. The N- and C-termini are labelled. Inset shows the L-shape of the molecule. Arrowhead indicates the largest crystal contact site. (B) Comparisons of the MAM domain to two closely related β -sandwich structures. Ribbon diagrams are shown for comparisons of secondary structures and molecular surfaces are shown to display surface features of binding sites (marked by arrowheads). Structurally equivalent regions (inter-C α distances < 3.0 Å) are shown in green and structurally distinct regions are highlighted (blue in front of the β -sandwich and red at the back). In 1GUI:A, the blue loops demarcate the carbohydrate-binding groove. In 1KGY:A, the red loops surround the hydrophobic ephrinB2-binding pocket, which constitutes the primary dimerization site; the blue loop forms the second ephrinB2-binding site. Regions that are not superposable are coloured in grey. Disulphide bonds are shown as orange sticks. All three structures are shown from the same view upon superimposition on MAM. (C) Structural details of the MAM L1 and L2 loops. The L1 and L2 loops are depicted in stick representation whereas the remainder of the MAM domain is shown as a solid surface. Residues selected for mutagenesis studies are highlighted with yellow carbon atoms. Phe68 (shown in pink) corresponds to the F74S cancer-linked mutation in RPTP μ . As in panel A, linker residues are coloured in purple and cysteines in orange. Asparagine residues providing sites for N-linked glycosylation are distinguished by standard atom colouring (carbon: white, nitrogen: blue, oxygen: red). This figure was produced using Pymol (<http://pymol.sourceforge.net/>).

significant number of interatomic contacts suggestive of homophilic adhesion interactions. The largest contact site (~ 1000 Å² total buried surface area) involves MAM and Ig residues in 'elbow-to-elbow' interactions of the L-shaped Mlg

molecule (Figure 1A). Although this site involves the solvent-exposed side-chain of Arg211 and the main-chain of the buried Tyr273, which correspond to the cancer-linked K218T and Y280H mutations in RPTP μ (Wang *et al*, 2004),

Table 1 Data collection, structure determination and refinement statistics

Data collection statistics				
Data set	Peak	Inflection	Remote 1	Remote 2
Space group	I222			
Unit cell (Å)	$a = 58.7, b = 97.5, c = 134.1$			
Wavelength (Å)	0.9786	0.9789	0.8731	0.9763
Resolution (Å)	30–2.7 (2.8–2.7)	30–2.7 (2.8–2.7)	30–3.5 (3.6–3.5)	30–3.5 (3.6–3.5)
Completeness (%)	99.8 (99.3)	98.2 (99.3)	99.8 (98.6)	99.9 (99.7)
R_{sym} (%) ^a	13.7 (53.3)	13.1 (85.6)	6.2 (13.0)	7.9 (19.2)
Redundancy	10.5 (10.7)	4.0	3.3	4.3
I/σ overall (outer shell)	12.7 (4.1)	6.6 (0.9)	15.0 (4.6)	15.5 (4.9)
<i>Phasing statistics</i>				
Phasing power (iso)	0.2 (versus Inf), 0.3 (versus Rem1)	0.5 (versus Rem1), 0.2 (versus Rem2)	0.4 (versus Rem2)	—
Phasing power (anom)	0.5	0.1	0.3	0.4
Se sites	3			
FOM ^b after SOLVE	0.28			
FOM after RESOLVE	0.53			
<i>Refinement statistics</i>				
Resolution (Å)	30–2.7			
Reflections	10 473			
R_{work} (%) ^c	0.224			
R_{free} (%) ^c	0.274			
r.m.s.d. bonds (Å)	0.012			
r.m.s.d. angles (deg)	1.52			
No. of atoms per asymmetric unit (protein/sodium/carbohydrate/water)	2037/2/92/72			
Average B factors (Å ²) (protein/sodium/ carbohydrate/water)	46.9/38.5/72.6/41.2			

^a $R_{\text{sym}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

^bFOM = overall figure of merit determined using SOLVE/RESOLVE (Terwilliger, 2003).

^c R_{work} and $R_{\text{free}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$.

r.m.s.d. stands for root mean square deviation from ideal geometry. Numbers in parentheses refer to the appropriate outer shell. R_{free} equals the R-factor against 5% of the data removed before refinement.

it appears unlikely to be, by itself, sufficient for homophilic binding. Residues corresponding to the other three RPTP μ cancer-linked mutations in the Mlg structure have buried side-chains (Figures 1C and 2A); their effects are likely to be because of structural perturbation rather than direct involvement in homophilic interactions.

Mapping of an adhesion-competent ectodomain segment

To characterize further the structural requirements for RPTP μ ectodomain function, we used a cell-adhesion assay based on the induced aggregation of insect Sf9 cells expressing RPTP μ domain-deletion constructs on their surface (acronyms and schematics of domain composition are shown in Figures 2C and 3). These constructs have their extracellular regions serially truncated from the C-terminal end, and their second cytoplasmic phosphatase domain replaced by EGFP. We combined results from the structure prediction algorithm PSIPRED/THREADER (Jones *et al*, 1999), sequence alignment of the type IIB RPTPs and structural knowledge of FNIII domains in general, to delineate the domain boundaries (Figure 2C and Materials and methods). A previously un-noted tail region, which is ~50 residues long and follows the FNIII-4 domain, is apparent.

Results of the cell-adhesion assays are presented in Figure 3 (expression levels were assessed by Western blotting, see Supplementary Figure S4). Only insect cells expressing constructs with extracellular regions corresponding to

Ex μ , MIF3 and MIF2 formed aggregates, demonstrating that MIF2 (MAM-Ig-FNIII-1-2) is the minimal ectodomain fragment capable of inducing cell aggregation. An MIF1-x-Fc (Fc = two tandem Ig domains, dimeric; x = 20-residue linker containing a 3C protease cleavage site and the IgGy1 hinge region) fusion construct was inactive in cell adhesion, arguing against the notion that the FNIII domains 2, 3 and 4 merely play a supportive role in cell adhesion by acting as a spacer between the adhesive part of the ectodomain and the plasma membrane. Brady-Kalnay *et al* (1993) and Gebbink *et al* (1993) have shown that constructs lacking the intracellular catalytic domains (but still containing 54 juxtamembrane residues) are functional in cell-adhesion assays; therefore, this activity is contributed predominantly by the ectodomain. We can now further refine this observation because an Ex μ transmembrane construct lacking all but the first 10 intracellular basic residues, which are responsible for membrane anchoring of the protein, is as active in inducing cell aggregation as its counterpart which contains the intracellular juxtamembrane region and D1 phosphatase domain (Figure 3).

Distinctive loops on the MAM domain are important for cell adhesion

Of the five loops in the MAM domain highlighted by structural and sequence comparisons two, L1 and L2, were the most distinctive features in this domain structure. To explore the role of this region, the Ex μ construct was mutated in the L1

Figure 2 Sequence alignments and secondary structure elements of the MAM and Ig domains. (A) Sequence alignment of the type IIB RPTPs MAM and Ig domains. Residue numbering begins with the signal sequence (not shown). Residues with more than three occurrences of sequence identity are highlighted and coloured by type. Conserved cysteines are marked by the symbol '▼' with disulphide pairs in the same colour and secondary structure elements are shown above the sequence. Special regions (denoted by thick lines) discussed in this report are also highlighted. Mutations in the MAM-specific loops (in red) and positions corresponding to RPTP ρ cancer-linked mutations discussed in the text (in black) are labeled. (B) Sequence alignment of the MAM domains from representative human proteins. Type IIB RPTPs, neuropilins (Nrp) and meprins are shown. Regions of the ligand-binding domain of Ephrin B2 receptor (EphB2, 1KG7:A) that are structurally equivalent to the RPTP μ MAM domain are also aligned (framed). The symbols '*' and '^' denote invariant and highly conserved residues among the human Ephrin receptors. A Phe residue (in blue frame) can be considered invariantly conserved between the two folds. The two disulphide pairs and the secondary structures of the RPTP μ MAM are also displayed. (C) Schematic representation of the domain structure of full-length RPTP μ . Keys: TM/JM = transmembrane/juxtamembrane region, PTP_D1/D2 = first/second phosphatase domain.

and the L2 loops (Figures 1C and 2A). In L1, two acidic residues (Asp30, Glu31), conserved in type IIB RPTPs, were mutated to lysines. In the more variable L2 sequence, there is a common occurrence of multiple prolines that may provide important conformational rigidity. Two such residues in RPTP μ , Pro57 and Pro61, were mutated to alanines. All four residues have solvent exposed side-chains in the Mlg crystal structure.

In parallel assays where aliquots of insect cells were infected with comparable numbers of baculoviral particles, cells expressing the L1 and the L2 mutants form smaller aggregates than wild-type Ex μ . A combined L1/L2 quadruple mutant further reduces this activity, resulting in very sparse and small cell aggregates (Figure 3). Analysis of the expression levels indicated that the cells produced similar amounts

Figure 3 Cell-adhesion assays. Insect Sf9 cells expressing transmembrane RPTP μ EGFP-fusion constructs were observed by fluorescence microscopy. All RPTP μ fusion constructs (except for Ex μ -TM-EGFP) have JM-D1-EGFP in the intracellular region, and are expressed at the plasma membrane (indicated by white arrows); whereas EGFP alone (control) is expressed uniformly in the cytosol. The bright intracellular signal indicates overexpression of the constructs in the endoplasmic reticulum and the Golgi apparatus. Note the localization of MIF2, MIF3, Ex μ and its mutants (L1m, L2m) at the cell-cell contact regions (also see Figure 5B and C). IF14t and F14t fusion constructs (not shown) did not induce cell aggregation. The Ex μ -TM-EGFP construct contains only the first 10 intracellular residues of RPTP μ . Scale bar: 250 μ m for the epifluorescence images; 20 μ m (for constructs that form cell aggregates) and 10 μ m (others) for the confocal images.

of the wild-type and mutant proteins (Supplementary Figure S4). The ectodomain of the L1/L2 mutant is expressed at normal (wild type) levels by 293T cells and also is a dimer in solution at pH 8.0 (see below), confirming its overall structural integrity. These results suggest that both the L1 and L2 loops contribute to a MAM domain-mediated interaction that is necessary for cell adhesion. However, because residual binding activity was observed, even for the L1/L2 mutant, this region of the ectodomain does not appear to be the sole contributor to the adhesive interaction.

RPTP μ ectodomain forms a high-affinity, pH-dependent homodimer

We expressed our series of domain-deletion ectodomain constructs as secreted, soluble proteins in HEK293T cells, and subjected them to gel filtration analysis (chromatograms shown in Supplementary Figure S5) to evaluate their oligomeric state and structural integrity. The results are summarized in Figure 4A. At pH 8.0, both MAM and Mlg are monomeric whereas MIF1 (MAM-Ig-FNIII-1) and MIF2 are exclusively dimers, and MIF3 and Ex μ have apparent sizes of a tetramer. The apparent size of Ex μ has been reported to be halved at pH < 6 as compared to pH 8 (Cismasiu *et al*, 2004), therefore all C-terminal domain-deletion constructs were also

chromatographed at pH 6.0. All constructs that had previously been dimeric or apparently tetrameric converted to monomers or apparent dimers, respectively, at this pH. Elongated molecules have larger sizes as estimated by gel filtration. However, analytical ultracentrifugation (data not shown) confirmed that Ex μ is dimeric at pH 8.0 and monomeric at pH 6.0. These results indicate that MIF1 constitutes the minimal dimerization unit. The pH-dependent switch accords with previously reported observations based on aggregation assays (Gebbink *et al*, 1993). As such, it is likely that the pH-sensitive dimers are in a *trans* ('head-to-head') orientation. The equilibrium pH for Ex μ dimer-monomer conversion was found to be 6.2–6.3 as analysed by dynamic light scattering (Figure 4B). This pH range is close to the pK_a of histidine, suggesting a role for one or more such residues in homodimer stabilization.

Most N-terminal domain-deletion constructs, IF14t, F14t and F24t, though soluble, form nondiscrete oligomers (Figure 4A), suggesting that these constructs bear hydrophobic surfaces that cause nonspecific molecular aggregation. An exception is F34t which is monomeric, implying that this fragment does not contribute to the pH-dependent *trans* homodimerization. The L1/L2 mutant construct was also found to be dimeric at pH 8 and monomeric at pH 6, implying

Figure 4 Oligomeric states of RPTP μ ectodomain constructs. (A) Schematic representation of the deletion constructs and Coomassie-stained gel showing the purified soluble constructs, and estimated M_r . The indicated number of N-glycosylation sites is based on computer analysis. (B) Evaluation of pH-dependent homodimerization of non-His₆-tagged Ex μ by dynamic light scattering. The averaged hydrodynamic radius (R_h) is plotted against solution pH. Error bars represent standard errors of triplicate measurements.

that the mutations did not disrupt the overall structure of the MAM domain sufficiently to cause nonspecific cell aggregation (Supplementary Figure S5). Thus, these results imply that Ex μ contains at least two independent binding sites that

contribute to the intercellular homophilic interactions. One of them (at least in part) involves the L1/L2 region, whereas the second is pH-dependent and appears to have a very high affinity (dimers on gel filtration were observed at concentrations as low as 10 nM).

Solution binding studies of the domain deletion constructs

To further investigate the contribution of individual domains to homophilic interactions, the equilibrium binding affinities of each domain-deletion mutant were measured by surface plasmon resonance (SPR). The ligands were immobilized on streptavidin-coated sensor chips via a C-terminal biotin tag to mimic their functional orientation. Ectodomain fragments (IF14t, F14t and F24t) that displayed polydisperse behaviour in solution exhibited tight, nonspecific binding to streptavidin-coated sensor chips, reflecting the nonspecific nature of the aggregates formed. These constructs were therefore not included for further analysis. All SPR measurements were performed at pH 7.4 where MAM and MIG are monomeric, whereas all the larger constructs are dimers. Therefore, the results presented here refer to pH-independent interactions. The results are summarized in Table II (binding curves and Scatchard plots are provided in Supplementary Figure S6).

Our primary set of binding experiments involved Ex μ as the immobilized ligand. All soluble fragments (analytes) dissociated readily from the ligand-coated sensor chip at the end of the injection. We did not detect any binding of MIG to Ex μ up to a concentration of 50 μ M. The reverse experiment with Ex μ binding to MIG gave similar results (data not shown). All homodimeric ectodomain constructs (MIF1, MIF2, MIF3 and Ex μ) showed measurable (in the low μ M range) binding to Ex μ . To evaluate the effect of the L1/L2 mutations, a second set of binding experiments were carried out with wild-type and mutant ectodomains taken both as analytes and ligands (Supplementary Figure S7). The mutations affected binding to wild-type Ex μ in both orientations (i.e. as analyte or ligand); while measurable, the weakness of the interactions precluded the determination of K_d values. However, even between L1/L2 mutant molecules some residual binding was apparent, consistent with the reduction, but not total loss of function, observed in the cell-adhesion assays.

In taking the analysis of the solution binding data further, there are clearly limitations regarding the interpretation of SPR experiments in the case of homophilic molecules, in particular in situations where the protein is already a dimer (possible interpretations are discussed in Supplementary data). Thus, we cannot draw definitive conclusions regarding the precise number and location of sites involved in the homophilic interactions of Ex μ based on SPR alone. However, we can be confident about the order of magnitude and domains required for the pH-independent interactions. All the results converge towards the idea that the RPTP μ homophilic interactions are complex, with at least two components—a high affinity pH-dependent interaction and the lower affinity interaction(s) (with low μ M K_d values) measurable by SPR—and involve minimally three domains (MIF1) in *in vitro* assays while, for cell adhesion, a further domain (FNIII-2) is required.

Table II Equilibrium binding studies of RPTP μ constructs using surface plasmon resonance

Analyte	Dissociation constant ^a (μM)		Goodness-of-fit for nonlinear regression (χ ²)	
			One-site model	Two-sites model
<i>Immobilized ligand: Exμ</i>				
	<i>K_{d1}</i>	<i>K_{d2}</i>		
MIF1	7.9 ± 0.9 (9.3 ± 0.8)	NA	0.4	0.2
MIF2	8.1 ± 0.5 (4.1 ± 0.4)	0.33 ± 0.04 (0.61 ± 0.06)	1.1	0.02
MIF3	9.5 ± 1.0 (4.9 ± 0.5)	0.27 ± 0.06 (0.52 ± 0.03)	2.8	0.1
Exμ	5.5 ± 0.4 (3.4 ± 0.2)	0.18 ± 0.05 (0.60 ± 0.06)	4.8	0.3
Mlg, F34t	No binding up to 50 μM			
IF14t, F14t	ND ^b			
<i>Immobilized ligand: MIF1</i>				
	<i>K_{d1}</i>	<i>K_{d1'}</i>		
MIF1	1.1 ± 0.08 (3.0 ± 0.3)	33 ± 2 (17 ± 1)	7.3	0.05

^aExcept for MIF1 binding to immobilized Ex μ , which was fitted with a 1:1 binding model, nonlinear fitting for the Req binding curves was performed with the simultaneous two-sites binding model: $\text{Req} = \text{Conc} \cdot \text{Rmax1} / (K_{d1} + \text{Conc}) + \text{Conc} \cdot \text{Rmax2} / (K_{d2} + \text{Conc})$. Values in parentheses were obtained when nonlinear fitting of the Req binding curves was performed separately for the two binding sites, as guided by the two linear segments in the Scatchard plots.

^bNot determined as the proteins exhibited polydisperse behaviour and nonspecific binding to sensor chip.
NA, not available.

Discussion

RPTP μ is a bi-functional molecule directly linking cell adhesion and signal transduction. However, in the absence of structural information, it is difficult to understand how this molecule, or indeed other members of the type IIB RPTPs, works. The MAM and Ig domains have long been recognized as essential for the adhesive function of RPTP μ (Brady-Kalnay and Tonks, 1994; Zondag *et al*, 1995; Cismasiu *et al*, 2004). However, these studies (based at least in part on treating the two domains as separate entities) came to rather divergent conclusions, placing differing emphasis on the MAM or Ig domain. Our crystallographic results, as well as providing the architecture of a MAM fold, reveal that Mlg is a tight structural unit, with MAM and Ig domains intimately juxtaposed such that they should be viewed as interdependent for stability and function. Despite our expectations, based on the previous work in the field cited above, this two domain unit is not—in isolation—capable of mediating homophilic interactions. For our series of domain deletion constructs, biophysical characterization reveals that the addition of at least one FNIII domain to Mlg (i.e. the MIF1 construct) is required for formation of stable homodimers, whereas a construct containing Mlg plus two FNIII domains is the minimal functional unit in live-cell-adhesion assays. Why might the second FNIII domain be important for biological function? Three factors could contribute, either individually or in combination: (i) the threshold affinity for homodimer formation may be insufficient for cell-adhesive function (i.e. the second FNIII contributes extra affinity), (ii) adhesive function is highly sensitive to the orientation of the molecule on the cell surface resulting from addition of the second FNIII domain and (iii) additional interactions (possibly in *cis*) are required and these are at least in part mediated by the second FNIII domain.

We can minimally describe two types of homophilic interactions involving the RPTP μ extracellular region. The first one is very tight ($K_d < 10$ nM), could be observed by gel filtration, dynamic light scattering and analytical ultracentrifugation and is pH-dependent, that is, can be readily and

reversibly dissociated by a switch of pH from 7 to 6. This agrees with the results obtained by Cismasiu *et al* (2004) and effects observed in cell-aggregation assays (Gebbinck *et al*, 1993), leading us to believe that it is a *trans* interaction. We could not measure its affinity by SPR because of experimental design constraints and we could not see it in the crystal structure as, as we found, it minimally requires the MAM, Ig and FNIII-1 domains. The second interaction involves the L1 and L2 loops of the MAM domain. Mutations in these loops reduce cell adhesion, although the overall domain integrity does not appear to have been disturbed. Thus, the L1/L2 region, plus the second FNIII domain (see above), both appear to contribute effects that add to a simple *trans* interaction mediated by the pH-dependent homodimer. Because homodimer-dependent (high affinity) and additional (low affinity) interactions have effects on cell adhesion, one could imagine two possible arrangements: both in *trans* or one in *trans* and one in *cis* (Figure 5A). MAM domains of neuropilin-1, -2 and meprin A have been associated with *cis* interactions (Chen *et al*, 1998; Nakamura *et al*, 1998; Ishmael *et al*, 2001) and previous results (Cismasiu *et al*, 2004) suggest that L2 is important for *cis* interactions in RPTP μ . The second (*trans/cis*) model therefore appears more probable.

What are the implications of a *trans/cis* model for the adhesive mechanisms? A primary, strong, *trans* interaction could play an anchoring role and initiate clustering of RPTP μ at the cell contacts, as observed by Gebbinck *et al* (1995) and Bianchi *et al* (1999); the second, lower affinity interaction, in *cis*, could order the relative orientation of the molecules within the cluster and possibly initiate a zipper-like receptor array, in a manner previously suggested for other cell-adhesion molecules such as NCAM (Soroka *et al*, 2003), axonin (Kunz *et al*, 2002) and cadherins (Gumbiner, 2005). Adhesive events involving highly mobile cells such as leucocytes are based on multiple weak interactions. However, less dynamic cell-cell interactions may require higher affinity binding, for example as reported for NCAM (Kiselyov *et al*, 2005) and our data point to RPTP μ showing similar characteristics. Indeed for RPTP μ , it is possible that linear zippers could achieve

Figure 5 Hypothetical schemes of Ex μ adhesive interactions. (A) Possible arrangements of Ex μ homophilic interactions that are consistent with the presence of two adhesion sites in the MIF1 region (one pH-dependent and one involving the L1/L2 loops). The FNIII-2 is required for efficient cell adhesion—possibly by facilitating the extension of an adhesive zipper. (B) 3D view of insect cells expressing the Ex μ -TM-D1-EGFP construct. Note the extended planarity of the plasma membrane at the intercellular contact sites (arrowheads) that may be indicative of a 2D-adhesive array. The slab of cell layer (3.6 μ m in depth) amidst a cell aggregate was imaged 0.2 μ m apart by fluorescence confocal microscopy. This projection view is tilted 15° with respect to the 2D, single image shown in Figure 3 (* denotes the same cell in the two images). (C) Cartoon outlining the cellular structures observed in the (B) image: plasma membrane (blue), endoplasmic reticulum and Golgi apparatus (green).

a further level of organization, for example by parallel alignment, leading to a 2D ('Velcro'-like) array, which would be entirely consistent with the large and apparently planar contact surfaces that we have observed between adherent cells (Figure 5B and C). Such an arrangement could strengthen the adhesion (through avidity effects) but would require at least one additional type of interaction between the RPTP μ molecules. Potentially, there are yet further levels of complexity to the system as RPTP μ is also thought to interact directly with cadherins (including N, E, R and VE) via the intracellular region (Brady-Kalnay *et al*, 1998; Hellberg *et al*, 2002; Sui *et al*, 2005). Considering the oligomerization models postulated here for RPTP μ and previously for various cadherins (Chothia and Jones, 1997; Gumbiner, 2005), more studies will be required to understand their interplay in various circumstances and the structural arrangements of the molecules in such complexes.

Taken together, all observations to date point towards a complex, multidomain, interaction model for the RPTP μ -mediated cell adhesion. The direct consequence of such a model is an ordered clustering of receptors at cell-cell contact regions, a significantly increased half-life of RPTP μ in confluent cultures (described by Gebbink *et al*, 1995) and, more importantly, a direct effect on signalling properties. Such an effect could be via steric modulation of the catalytic (intracellular) region, the control of subcellular localization, bringing RPTP μ into proximity with its substrates (members of the cadherin-catenin complex such as p120ctn (Zondag

et al, 2000) or VE-cadherin (Sui *et al*, 2005)) or clustering of downstream scaffolding proteins—such as RACK1 (Mourton *et al*, 2001)—at intercellular contact areas.

The distribution of RPTP ρ mutations from tumour isolates (targets of selection during tumorigenesis; Wang *et al*, 2004) provides possible insights into how in the RPTP family misfunction of cell adhesion may have direct consequences for downstream signalling. Mutations are found in both the RPTP ρ extracellular and intracellular regions. The intracellular mutations are predicted to result in the loss of catalytic domains or a reduction of the catalytic activity (Wang *et al*, 2004). Of the extracellular mutations, five can be mapped to equivalent residues in the RPTP μ Mlg structure. We predict that most of these mutations cause significant perturbations of the RPTP ρ structure as they dramatically alter the character of buried side-chains (F74S, A209T, F248S and Y280H; Figures 1C and 2A). As such they may exert their effects either by preventing surface expression of a misfolded RPTP ρ or by disrupting a functional portion of the exposed ectodomain. However, one mutation, K218T, is at the Ig domain surface; this mutation is therefore compatible with a correctly folded structure implying that it may impact directly on a protein-protein interaction. The principle of extracellular interactions regulating intracellular signalling in RPTPs was first demonstrated by the enforced dimerization of the EGFR-CD45 hybrid receptor (Desai *et al*, 1993) and further confirmed by experiments with RPTP α (Jiang *et al*, 1999). Intra- and intermolecular interactions between the

catalytic domains of many RPTPs have been identified and been shown to modulate their enzymatic activity (Blanchetot and den Hertog, 2000; Feiken *et al*, 2000; Aricescu *et al*, 2001). The crystal structure of the first catalytic domain of RPTP α revealed a self-inhibitory dimer (Bilwes *et al*, 1996); conversely the structural determination of the equivalent domain in RPTP μ (Hoffmann *et al*, 1997) revealed a different dimerization mode (one which the authors point out may be due to the crystal lattice contacts). Thus, many questions remain concerning the effect of receptor oligomerization on catalytic function in the RPTP family. *Trans/cis* models, which are suggestive of higher order oligomerization of the RPTP ectodomains, point to extracellular-based mechanisms that can modulate the intracellular interactions, thus potentially influencing their signalling ability.

The true importance of the type IIB RPTP family of receptor enzymes is just beginning to emerge, with several recent reports linking them to tumour suppressor functions (Wang *et al*, 2004; McArdle *et al*, 2005). It is therefore important to understand the organization and regulatory mechanisms of these signalling assemblies. Further structural and functional studies must aim to characterize the molecular architecture of RPTP μ adhesive complexes and their consequences for downstream signalling.

Materials and methods

Ectodomain constructs

The cDNA sequences encoding deletion constructs of the RPTP μ ectodomain were amplified by PCR using the pFL vector (Gebbinck *et al*, 1993) as a template (a gift of M Gebbinck, Utrecht University). They are MAM: residues 1–185; MIG: 1–279; MIF1: 1–375; MIF2: 1–481; MIF3: 1–585; Ex μ : 1–742; IF14t: 189–742; F14t: 281–742; F24t: 379–742 and F34t: 482–742. The last four constructs, lacking the native secretion signal, were fused to the secretion signal of chicken RPTP σ (residues 1–28, GenBank accession number L32870). Site-directed mutagenesis was performed according to Stratagene's QuickChange protocol. All mutants have been verified by DNA sequencing.

Protein expression in HEK293T cells

A vector, pHL, a derivative of the pCA β -EGFP plasmid containing the chicken- β -actin promoter (gift of J Githorpe, Kings College London), was constructed for high-level expression of secreted, C-terminally His $_6$ -tagged ectodomain constructs. Transient transfection was carried out using polyethyleneimine (Durocher *et al*, 2002). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma), L-glutamine and nonessential amino acids (Gibco). The concentration of FCS was lowered to 2% immediately after transfection in 90% confluent cells.

To prepare SeMet-substituted MIG protein, the cells were washed once with phosphate-buffered saline just before transfection, which was carried out in Met-free DMEM (ICN) supplemented with 30 mg/l L-SeMet (Acros Organics), 2% dialyzed FCS, L-glutamine and nonessential amino acids (Gibco).

Protein purification

His $_6$ -tagged proteins were purified 3–4 days post-transfection from the media using Ni $^{2+}$ -affinity and gel filtration chromatography. Fc-tagged proteins were purified by protein A affinity chromatography. The proteins were cleaved on the resin with 3C protease, eluted and further purified by gel filtration. All buffers were at pH 8.0.

Cell-adhesion assays

The method was as previously described (Brady-Kalnay *et al*, 1993; Gebbinck *et al*, 1993). Insect Sf9 cells were grown in suspension in Sf900II medium (Invitrogen). A shuttle vector, pFBR, was modified from pFastBac-1 (Invitrogen) for expression of transmembrane (TM), EGFP-tagged proteins, resulting in pFBR-x-TM-D1-EGFP

(x = insertion site of ectodomain constructs, D1 = first phosphatase domain). A pFBR-x-(3C)-Fc-TM-D1-EGFP vector was created by subcloning a cDNA fragment encoding the 3C protease cleavage site followed by the human IgG γ 1 Fc region from vector pRMHA-mIL1 α -(3C)-Fc (gift of JK Heath, Birmingham University). The Bac-to-Bac system (Invitrogen) was used with standard protocols to produce recombinant baculoviruses. These were titred for each construct and equal amounts were inoculated to 20 ml suspension cultures (1.4×10^6 cells/ml, 100 r.p.m.). Cells were visualized live 40–48 h post-infection using an inverted microscope equipped with epifluorescence (Nikon DIAPHOT 300) and a confocal laser scanning microscope (Bio-Rad MRC 1024).

Crystallization and crystal structure determination

Crystals of SeMet-substituted MIG-His $_6$ were grown by sitting-drop vapour diffusion at 22°C. The drops contained 1 μ l each of protein (10 mg/ml, in 100 mM Tris-HCl, pH 8.0, 250 mM NaCl) and mother liquor (100 mM tri-sodium citrate, pH 6.4, 100 mM ammonium chloride, 30% PEG 4000). The crystal was cryo-cooled (by nitrogen gas stream) in the original mother liquor. MAD data, collected on beamline BM-14 at the ESRF (Grenoble, France), were integrated and scaled using the HKL package (Otwinowski and Minor, 1997), analysed and merged using XPREP (Bruker AXS, Madison). Heavy atom location, refinement, phasing and density modification were performed using SOLVE/RESOLVE (Terwilliger, 2003). The experimental map showed clear regions of secondary structure but poor connectivities in the loop regions and ambiguous side-chain densities. Results of automatic tracing and sequence assignment done by either the programs RESOLVE, or ARP/wARP (Morris *et al*, 2003), alone, were not satisfactory. However, when main-chain fragments produced by cycles of ARP/wARP-REFMAC routine were fed into RESOLVE for sequence assignment in an iterative manner, ~90% of the protein sequence could be assembled in a semiautomatic fashion. The rest of the model was manually built using the program O (Jones *et al*, 1991) coupled with cycles of refinement by CNS (Brunger *et al*, 1998) and REFMAC (Murshudov *et al*, 1997). In the final stages of the refinement, a limited number of water molecules (picked with ARP/wARP and verified by manual inspection) were included based on decrease of R_{free} . The final model (residues 21–279, 72 water molecules, three N-linked glycosylation sites and two monovalent cations that, on the basis of coordination geometry, have been modelled as Na; Harding, 2002) has an R -factor of 0.224 (R_{free} = 0.274) using all data between 30.0 and 2.7 Å. The stereochemical properties of the structure were assessed by PROCHECK (Laskowski *et al*, 1993) and showed no residues in disallowed regions of the Ramachandran plot. Final refinement statistics are given in Table I. The coordinates have been deposited in the Protein Data Bank (entry 2C9A).

Analytical gel filtration and dynamic light scattering

A Superdex 200 10/30 column standardized with molecular weight markers (Amersham Biosciences) was used. Buffers contained either 25 mM Tris-HCl, 500 mM NaCl, pH 8.0 or 25 mM MES, 500 mM NaCl, pH 6.0. Samples were equilibrated to the same pH before loading. A lower NaCl concentration (150 mM) produced the same results. Non-His $_6$ -tagged Ex μ (obtained by proteolytic cleavage of the Fc fusion construct) was used for dynamic light scattering analysis.

Surface plasmon resonance

Steady-state binding experiments were carried out in duplicate at 25°C on a BIAcore 2000 instrument (BIAcore AB) using the HBS-EP buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) (BIAcore AB). All ligands and analytes were produced from HEK293T cells as secreted, soluble proteins. C-terminally biotinylated ligands were immobilized on streptavidin-coated CM5 sensor chips (BIAcore AB) via amine coupling, immobilization level ~3000–4000 response units. The intrinsic complication of self-association of the molecules, both as analytes and as ligands, has to be considered. The latter issue was addressed by immobilizing three different Ex μ ligand levels on consecutive flow cells. The medium ligand level was found to be optimal (Supplementary Figure S6) and was used for the MIF1 ligand. All curve-fitting procedures were carried out with the BIAevaluation software (version 3.0, BIAcore AB).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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