The MAM (Meprin/A5-protein/PTPmu) Domain Is a Homophilic Binding Site Promoting the Lateral Dimerization of Receptor-like Protein-tyrosine Phosphatase μ*

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The MAM (meprin/A5-protein/PTPmu) domain is present in numerous proteins with diverse functions. PTPμ belongs to the MAM-containing subclass of protein-tyrosine phosphatases (PTP) able to promote cell-to-cell adhesion. Here we provide experimental evidence that the MAM domain is a homophilic binding site of PTPμ. We demonstrate that the MAM domain forms oligomers in solution and binds to the PTPμ ectodomain at the cell surface. The presence of two disulfide bridges in the MAM molecule was evidenced and their integrity was found to be essential for MAM homophilic interaction. Our data also indicate that PTPμ ectodomain forms oligomers and mediates the cellular adhesion, even in the absence of MAM domain homophilic binding. Reciprocally, MAM is able to interact homophilically in the absence of ectodomain trans binding. The MAM domain therefore contains independent cis and trans interaction sites and we predict that its main role is to promote lateral dimerization of PTPμ at the cell surface. This finding contributes to the understanding of the signal transduction mechanism in MAM-containing PTPs.

The phosphorylation state of numerous signaling proteins is controlled by opposing activities of protein-tyrosine kinases and protein-tyrosine phosphatases (PTP)1 (1). The family of PTPs consists of soluble and receptor-like PTPs (RPTPs) (2). Whereas the intracellular region of RPTPs is relatively similar in all representatives containing either a single or two PTP domains, the extracellular region has a large diversity. PTPμ belongs to subclass IIb, called “MAM-containing PTP” (2). Besides the MAM domain (meprin/A5-protein/PTPmu domain; Ref. 3), their extracellular region contains a single immunoglobulin (Ig)-like domain and four fibronectin (FN) III repeats

1 The abbreviations used are: PTP, protein-tyrosine phosphatases; RPTP, receptor-like protein-tyrosine phosphatases; FN, fibronectin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; BS3, bis(sulfosuccinimidyl)suberate; IAA, iodoacetic acid; IAM, iodoacetamide; Ex, extracellular region.

(4). This structural architecture of ectodomain is similar to members of the cell-adhesion molecule superfamily.

PTPμ is strongly expressed in the endothelial cell layer of the arteries and continuous capillaries as well as in cardiac muscle, bronchial and lung epithelia, retina, and several brain areas (4–6). At the subcellular level, it is localized at sites of cell-cell contact (7). In this regard, it has been demonstrated that PTPμ restores E-cadherin-mediated cellular adhesion, when it is expressed in LNCaP human prostate carcinoma cells (8). Physiologically, PTPμ has been shown to be involved in promotion and regulation of neurite outgrowth (5, 9).

Numerous experiments have clearly demonstrated that the extracellular region of PTPμ promotes cell-cell aggregation in a Ca2+-independent manner (10, 11). The homophilic binding has been also evidenced in the ectodomains of PTPα (12) and PTPβ (13), strongly suggesting that these RPTPs may be involved in signal transduction through cell-to-cell contact in vivo. Evidence concerning the physiological role of PTPμ-mediated homophilic binding has been reported in a recent article (14) showing that homophilic interactions trigger rearrangements of the axonal growth cone. However, the molecular mechanism of this interaction remains largely unknown. In this respect, it is still unclear which regions of the ectodomains are responsible for homophilic binding. Brady-Kalnay and Tonks (15) suggested that the Ig-like region is sufficient for the homophilic binding and they did not find any role for the MAM region in this interaction. In contrast, Zondag et al. (16) have shown that the MAM domain is necessary for the PTPμ-mediated adhesion, especially in determining its specificity.

The MAM domain was also found in various, unrelated proteins like meprins, neuropilins, and zonadhesins. It was reported that the MAM domain in meprin is involved in oligomerization, as a result of covalent and non-covalent linkages (17). Also, the neuropilin MAM domain was demonstrated to be involved in lateral (cis) dimerization (18).

To investigate the role played by the MAM region in homophilic binding interactions of PTPμ, we analyzed by different methods the oligomerization capacity of the MAM domain and the whole extracellular region of PTPμ, both expressed in insect cells as secreted proteins. Also, the wild-type and mutant forms of the MAM domain were used to assess whether they are able to bind the extracellular region of PTPμ at the surface of insect cells expressing full-length PTPμ. Similar experiments were performed to establish the role played by the MAM domain in homophilic binding of the extracellular region of PTPμ. To compare our results to those reported on the controversial subject of the role of MAM domain in PTP-mediated adhesion, we included in our experiments a similar experimen-
EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The cDNA of human PTP\(\mu\) (pBS-hFl) was kindly provided by M. Gebbink (Netherlands Cancer Institute, Ref. 4). Using pBS-hFl as template, the cDNA fragments encoding either the complete extracellular region (Ex, bp 61–2227) or the MAM domain (bp 61–562) were amplified with oligonucleotides: 5′-GGGATCCGGAGACGGTCTCAGTGGTGGTGGTGAGGAGTCCTGGTACAT-3′ and 5′-CTTGGATATAGTCAATCTGAAGGTG-3′. The PCR products were cloned into a pBluescript vector (Stratagene), resulting in pBS-Ex and pBS-MAM, respectively. A Bsu36I-fragments from pBS-Ex was replaced with a similar one from pBS-MAM as template, and a pair of complementary primers containing the mutation. The sense primer used to change the TGT codon with the GCT codon was 5′-GATGAGCCGTATAGCACA-3′ and the reverse primers encode six histidine amino acids and a stop codon.

The point mutation Cys\(^{54}\) → Ala in pBS-MAM was made using the QuickChange™ site-directed mutagenesis kit (Stratagene). DNA amplification was performed using Pfu polymerase, pBS-MAM as template, and a pair of complementary primers containing the mutation. The sense primer used to change the TGT codon with the GCT codon was 5′-GATGAGCCGTATAGCACA-3′ and the reverse primers encode six histidine amino acids and a stop codon.

To obtain the pBS-ExmutC36A plasmid, the pBS-MAMmutC36A was subjected to DNA sequencing. A Bsu36I-HindIII fragment was extracted from pBS-ExmutC36A and cloned into pBluescript vector (Stratagene), resulting in pBS-MAMmutC36A. The presence of the mutation in plasmid pBS-MAMmutC36A was confirmed by sequencing.

To obtain the pBS-ExmutC36A plasmid, pBS-MAMmutC36A and inserted into pBS-Ex to produce the pBS-ExmutC36A plasmid.

To obtain the pBS-Exmut5Cys plasmid, four amino acids (Cys-Gly-Pro-Ala) were inserted into the MAM region, between Pro\(^{55}\) and Trp\(^{62}\), using the DNA oligonucleotide 5′-CATGCGGCGGCCG-3′. This short sequence is complementary to itself, generating a double stranded DNA fragment with two cohesive ends. Because the ends are complementary to those generated by the restriction enzyme NcoI, the pBS-MAM plasmid was digested with this enzyme and religated in the presence of the oligonucleotides. The new plasmid pBS-MAMmut5Cys was subjected to DNA sequencing. A Bsu36I-HindIII fragment was extracted from pBS-MAMmut5Cys and inserted into pBS-Ex to produce the pBS-Exmut5Cys plasmid.

To obtain the pAc-GSTMAM baculovirus transfer vector, the cDNA coding for the MAM domain was inserted into BamHI and KpnI sites of a modified form of pAcSecG2T (BD Pharmingen). All recombinant constructs were expressed in insect cells under the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus. Genes inserted in pVL93MelFlag and pVL93MelMyc transfer vectors were preceded by an in-frame prepromelittin signal sequence to allow secretion of the corresponding proteins into the supernatant. Similarly, the pAc-GSTMAM baculovirus transfer vector contained upstream of the GST gene an in-frame g6p7 signal sequence.

Cell Cultures and Baculovirus Generation—The Sf9 insect cells were routinely maintained at 28 °C in Grace's insect medium (Invitrogen), supplemented with 3.3 g/liter lactalbumin hydrolysate (Sigma), 3.3 g/liter yeastolate (Sigma), 30 μg/ml gentamicin (Sigma), and 10% fetal calf serum. For the suspension cultures, the medium was supplemented with 6 mM (final concentration) of sodium phosphate buffer at a flow rate of 0.5 ml/min. The cell cultures were grown in 250 ml Erlenmeyer flasks at 220 rpm at 25 °C with aeration at 1.5 l/min.

Protein Expression and Purification—For protein expression, an Sf9 suspension culture (2 × 10\(^6\) cells/ml) was infected with the appropriate recombinant baculoviruses at multiplicity of infection of 10 and harvested at 48 h post-infection. The Sf9 cells were resuspended in ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH 7.4) supplemented with a protease inhibitor mixture (Roche Diagnostics) and lysed by sonication 3 times for 10 s.

For purification of soluble, secreted GST-MAM protein, the culture medium was 10-fold concentrated on a centrifugal filter device, Centricon YM-10 (Millipore). The supernatant was incubated with glutathione-Sepharose™ 4B (Amersham Biosciences; 20 μl of gel per 10 ml of culture) for 4 h. After extensive washing of the resin with PBS, the immobilized protein was subjected to thrombin digestion (Sigma) in PBS for 3 h at 25 °C. Each mg of fusion protein was cleaved with 20 NIH units of thrombin in 1 ml of the clarified supernatant. After 4 h, the beads were washed 6 times with 10 volumes of ice-cold Tris-buffered saline (TBSA: 50 mM Tris-HCl, 500 mM NaCl, pH 7.4) containing 15 mM imidazole. The adsorbed proteins were eluted with TBSA supplemented with 300 mM imidazole. After addition of CaCl\(_2\) (2 mM final concentration) and centrifugation (10,000 × g, 10 min), the supernatant was incubated for 2 h with anti-FLAG (M1) affinity agarose (Eastman Kodak Co.). The beads were washed with ice-cold Tris-buffered saline B (TBSB: 50 mM Tris-HCl, 150 mM NaCl, pH 7.2) plus 2 mM CaCl\(_2\), and the bound proteins were eluted 3 times with 4 volumes of PBS plus 2 mM EDTA. The eluted proteins were concentrated on Centricon YM-30 devices. All steps of protein purification were carried out at 4 °C if not otherwise specified. Protein concentration was measured at a wavelength of 280 nm.

Using the purification procedure described above, 1.2 mg of Flag-Ex protein and 0.5 mg of MAM domain fragment were obtained per 1 liter of suspension culture. To remove N-linked glycosyls, the cell lysate or the purified proteins were incubated with peptide-N-glycosidase F (PNGase F, Roche Diagnostics) according to the manufacturer’s protocol.

Electrophoresis and Immunoblotting—Samples were solubilized in SDS loading buffer, separated by SDS-PAGE, and either stained by Coomasie Blue R-250 or transferred to polyvinylidene difluoride membranes Immobilon-P (Millipore). After blocking with 5% nonfat dry milk in TBSB buffer, the immunoblots were probed sequentially with primary and anti-mouse alkaline phosphatase-conjugated secondary antibodies (Promega). The following monoclonal antibodies were used in these studies: the BK9 antibody (kindly provided by S. Brady-Kalnay, Case Western Reserve University), directed against the MAM domain of PTP\(\mu\); the anti-myc (clone 9E10), anti-GST (clone GST-2), and anti-poly-His (clone HIS-1) antibodies, purchased from Sigma, and the anti-FLAG (M2) antibody, from Eastman Kodak Co.

Analytical Gel Filtration Chromatography—All gel chromatography experiments were performed using the Biologic System (Bio-Rad). The columns were equilibrated in buffers used for protein elution.

Multimers of the purified PTP\(\mu\) ectodomain were fractionated on a Superdex 200 HR 10/30 column (Amersham Biosciences; separation range 10–700 kDa) and eluted with PBS (as such or supplemented with 1 M NaCl, 2 M urea, or 25 mM DTT) at a flow rate of 0.5 ml/min. The elution calibration curve was established using standard globular proteins delivered by Amersham Biosciences: ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (689 kDa).

Chemical Cross-linking—The entire procedure was performed at 25 °C with the MAM domain fragment at 0.7 mg/ml concentration using a freshly prepared stock cross-linker solution: 10 mM bisulfosuccinimidylsuberate (BS\(_2\); Sigma) in PBS (pH 7.4).

The cross-linking reaction was carried out for various periods with a 10-fold molar excess of BS\(_2\) (0.3 μM). The reactions were quenched by addition of 10-fold molar excess of DTT at a flow rate of 0.5 ml/min. The samples were subjected to SDS-PAGE.

Protein Alkylation Procedure—The one- and two-step alkylation procedures and subsequent protein electrophoresis were performed as described by Takahashi and Hirose (20). Protein denaturation was done with 8 M urea.
In the first step, 10 µg of denatured protein was alkylated with 30 mM iodoacetamide (IAA) at 37 °C for 20 min. The protein was precipitated with cold acetone, washed, and dissolved in PBS supplemented with 8 mM urea and 5 mM DTT. In the second step, the fully reduced protein was alkylated with 10 mM iodoacetamide (IAM) at 37 °C for 10 min. In the control experiment, the procedure was identical except IAA was omitted.

In the one-step procedure, equal amounts of denatured and fully reduced protein were alkylated simultaneously with different molar ratios of IAA to IAM (30/0, 22.5/2.5, 15/5, 7.5/7.5, and 0/10 mM/mM). Alkylation reaction was allowed to proceed 20 min at 37 °C and finally all samples were mixed. The alkylated proteins were analyzed by electrophoresis on a discontinuous acrylamide slab gel (9% polyacrylamide) in the presence of 8 mM urea and stained with Coomassie Blue B-250.

Cell Aggregation and Homophilic Binding Assays—A suspension culture of Sf9 insect cells was infected with the Ac-MycPTPµ, Ac-MycPTPµMutC36A, Ac-MycExTJ, Ac-MycExTJmut5Cys, or Ac-MycExTJmutC36A/5Cys recombinant baculoviruses at a multiplicity of infection of 10. At 24 h post-infection, the medium was replaced with a fresh one. At 48 h post-infection, the culture medium containing the soluble, secreted proteins was analyzed by immunoblotting. All expressed proteins were glycosylated as indicated by the fact that treatment with PNGase F yielded shifts to lower molecular weights in SDS-PAGE (Fig. 1).

Previously, it was reported that PTPµ, expressed at the surface of insect cells, promotes cell-cell aggregation by homophilic trans interactions (10, 11). To test the expression and the adhesive function of the full-length PTPµ, the extracellular region, and the MAM domain, respectively, were expressed in baculovirus-infected insect cells as secreted proteins or, in the case of constructs containing the transmembrane region, on the cell surface. Constructs were either N-terminal or both N- and C-terminal labeled using different tags as shown schematically in Fig. 1. The affinity purification of the proteins produced single bands corresponding to the expected molecular weight as assessed by Western blot analysis with tag-specific antibodies. All expressed proteins were glycosylated as indicated by the fact that treatment of purified proteins with PNGase F yielded shifts to lower molecular weights in SDS-PAGE (Fig. 1).

RESULTS

Expression and Purification of PTPµ and Its Fragments in Insect Cells—The different constructs, encompassing the full-length PTPµ, the extracellular region, and the MAM domain, were expressed in baculovirus-infected insect cells as secreted proteins or, in the case of constructs containing the transmembrane region, on the cell surface. Constructs were either N-terminal or both N- and C-terminal labeled using different tags as shown schematically in Fig. 1. The affinity purification of the proteins produced single bands corresponding to the expected molecular weight as assessed by Western blot analysis with tag-specific antibodies. All expressed proteins were glycosylated as indicated by the fact that treatment of purified proteins with PNGase F yielded shifts to lower molecular weights in SDS-PAGE (Fig. 1).

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MAM Domain Interacts Homophilically in Solution—To investigate under in vitro conditions the capacity of the soluble MAM domain fragment to self-associate, it was cross-linked using the homobifunctional reagent BS3 (a water-soluble cross-linking agent that reacts covalently with primary amino groups). The cross-linking experiments were performed under different reaction times but with a constant, 10-fold molar excess of BS3. Fig. 2 shows that under relatively mild cross-linking conditions the dimer can be detected after 1 min of incubation. Also, the MAM dimers were detected after 3 min of incubation with only 2-fold molar excess of reagent (data not shown). These results suggest that the MAM region of PTPµ has the capacity to interact with itself even in the absence of other regions of the PTPµ ectodomain.
FIG. 2. The MAM domain interacts homophilically in solution. The purified protein at 0.7 mg/ml was incubated with 0.3 mM BS3 at 25 °C. After different periods of reaction, 10 μg of the cross-linked protein were recovered and subjected to 10% SDS-PAGE. This panel is an immunoblot using anti-poly-His antibody. The arrows indicate the oligomeric and monomeric forms of the cross-linked protein. The control experiment (C) was performed under similar conditions except BS3 was not added.

MAM Domain Contains Two Intramolecular Disulfide Bridges—Within the amino acid sequence of the MAM domain there are four conserved cysteine residues (3), which can, in principle, be involved in inter- or intramolecular disulfide bridges. To determine whether one or more of the cysteine residues forms intermolecular disulfide linkages, the purified MAM domain was analyzed by SDS-PAGE in the presence or absence of the reducing agent (DTT). In both situations the proteins run according to the molecular weight of a monomer (data not shown), suggesting that the cysteine residues of MAM are not involved in intermolecular disulfide bridges.

We also examined whether the MAM domain contains intramolecular disulfide linkages using the two-step alkylation procedure (20). This procedure is based on the following principle: both IAM and IAA react only with free sulphydryl groups but iodosacetic acid introduces into the protein molecule an additional charge, thus increasing the electrophoretic mobility of the molecule, as analyzed by urea gel electrophoresis. The IAA cannot react with the non-reduced protein as illustrated in Fig. 3A (lanes 1 and 2, step I). Consequently, there are no free cysteine residues within the MAM domain or, in other words, all four cysteine residues are involved in intramolecular linkages. The protein treated with a mixture of IAM and IAA can be separated into five electrophoretic bands, proving that the protein contains all four predicted cysteines (Fig. 3A, lane 3). Cross-linking experiments in the presence or absence of DTT, evidenced the role played by the disulfide bridges in preserving the self-binding capacity of the MAM domain: when the purified MAM domain fragment was first treated with DTT and then cross-linked with BS3, the dimer form was not still observed on SDS-PAGE gel (Fig. 3B, lane 2). Altogether, the above results indicate that the MAM domain has two intramolecular disulfide bridges, which are essential for MAM domain self-interaction.

MAM Domain Interacts with the PTPμ Extracellular Region, at the Cell Surface—To further investigate the homophilic binding characteristics of the MAM domain, we performed a protein-protein interaction assay, where one of the interacting partners is expressed at the cell surface as a transmembrane protein and the other one is in the culture medium (secreted protein).

As a positive control for this binding assay, we checked first if the interaction between the full-length PTPμ and the secreted PTPμ ectodomain can be detected under our experimental conditions. To this purpose, the insect cells were infected with recombinant baculoviruses carrying full-length PTPμ. Separately, Flag-Ex protein was expressed as a secreted protein and then, the medium containing it was mixed with cells expressing Myc-PTPμ. Fig. 4 shows that the homophilic binding between Flag-Ex and PTPμ does take place, as expected, whereas there was no interaction between insect cells infected with non-recombinant baculoviruses and soluble ectodomain (lanes 1 and 5, respectively).

Second, we tested whether the MAM domain, expressed as a soluble protein, is able to interact with the PTPμ extracellular region at the insect cell surface. Thus, in a similar experiment, the GST-MAM protein was used as a protein secreted into medium and mixed afterward with a suspension of insect cells expressing Myc-PTPμ. Soluble GST-MAM binds to PTPμ ectodomain expressed at the surface of insect cells (Fig. 4, lane 4). To check that the GST or insect cell surface proteins are not involved in this interaction, the same experiment was repeated but using either soluble GST or cells infected with non-recombinant baculoviruses. Binding was not detected in any of the two control experiments (Fig. 4, lane 5, and data not shown). This result suggests that the MAM domain contains at least one specific binding site, which promotes its adhesion to the PTPμ ectodomain.

PTPμ Ectodomain Interacts Homophilically in Trans Even in the Absence of MAM Domain Self-binding—To confirm that the PTPμ ectodomain-MAM domain interaction is a direct consequence of MAM-to-MAM binding, we analyzed if the interac-
results were obtained in four independent experiments. The insect cells infected with baculoviruses carrying this mutant and is also glycosylated, like the wild-type protein (Fig. 5B). The mutant protein is expressed at the expected molecular weight and is also glycosylated, like the wild-type protein (Fig. 5A). The insect cells infected with baculoviruses carrying this mutant still displayed the capacity to form cellular aggregates (Fig. 5A). Insect cells infected with baculoviruses carrying this mutant still displayed the capacity to form cellular aggregates (Fig. 5A). This result indicates that the mutant full-length PTPμ is expressed at the cell surface and that it retains the trans binding capacity of the wild-type protein.

We examined then the capacity of the secreted, non-mutated MAM domain (GST-MAM) to interact with the mutant receptor Myc-PTPμMutC36A at the cell surface. Fig. 5C evidences the lack of interaction between the soluble protein and the PTPμ ectodomain when the last one has an altered disulfide bridge within the MAM region. Because the self-binding capacity of the MAM domain can be abolished by reduction of disulfide bridges (Fig. 3C), this result indicates that the PTPμ ectodomain-MAM domain interaction is based on MAM-MAM binding. Interestingly, these results indicate that the cellular adhesion driven by the trans interactions of the PTPμ takes place even in the absence of MAM-MAM binding (Fig. 5, B and C).

**MAM Domain Can Interact with PTPμ in the Absence of Ectodomain Trans Binding**—It was previously reported that the cellular aggregation mediated by PTPμ can be reversibly blocked by decreasing the pH of culture media below 6 (11). The question is whether the self-binding capacity of the MAM domain has the same pH sensitivity as in case of trans interactions of the whole PTPμ ectodomain.

To answer this question we performed the protein-protein binding assay, in which the interaction between GST-MAM and the PTPμ at the cell surface was tested at two different pH values of the medium. Fig. 6 summarizes our results, demonstrating that binding of MAM to the ectodomain exposed on the surface of insect cells is not pH-dependent. Thus, even at pH 5.9, where the trans binding of PTPμ is abolished (Fig. 6A), the MAM domain can still interact with the ectodomain (Fig. 6B). Therefore, MAM-to-MAM binding can take place under conditions in which the trans interaction of the PTPμ is abolished. Based on the last two experimental observations: (i) the homophilic trans interaction of the ectodomain can take place while the MAM self-binding is blocked; and (ii) the MAM-to-MAM interaction is still occurring when the ectodomain trans interaction is blocked, it can be suggested that the self-binding capacity of MAM domain is not required for the homophilic trans interactions of PTPμ.

**PTPμ Ectodomain Forms Oligomers in Solution, in a pH-dependent Manner**—To confirm the results described above, we investigated the homophilic binding properties of the PTPμ ectodomain by a different approach: the whole extracellular region of PTPμ was expressed as a secreted protein, purified, and analyzed by analytical gel filtration chromatography.

Under physiological conditions (PBS buffer, pH 7.2), the protein elutes as a single peak, which can be predicted to
contain an oligomeric form having an apparent molecular mass of 375 kDa (probably the dimer; Fig. 7A). Repeating this experiment under similar conditions but adding in the running buffer (1 M NaCl, 25 mM DTT, or 2 M urea, respectively), practically identical chromatograms were obtained (data not shown). Thus, the oligomer seems to be relatively resistant to ionic strength, DTT, or relatively low concentrations of urea. However, performing this gel filtration experiment in a running buffer at pH 6, the unique peak of the chromatogram was shifted to a lower elution time, corresponding to an apparent molecular mass of 180 kDa (Fig. 7B). Thus, the dissociation of the oligomeric form was induced by decreasing the pH from 7.2 to 6.

Because the cellular adhesion mediated by PTP\(\mu\) can be blocked in a pH-dependent manner (Ref. 11 and Fig. 6A), it can be assumed that the Flag-Ex oligomerization at pH 7.2 and its dissociation at pH 6 reflect in vitro the homophilic trans interaction of PTP\(\mu\). When the second conserved cysteine of the MAM domain was mutated within the Flag-Ex, the gel filtration experiment demonstrated that this mutant protein is still able to form similar oligomers as the wild-type protein (Fig. 6C). In addition, DTT treatment of Flag-Ex did not result in dissociation of the oligomeric form (data not shown). Thus, under conditions when the self-binding capacity of MAM domain is blocked, formation of the Flag-Ex oligomer with the apparent molecular mass of 375 kDa is not substantially altered. Assuming that Flag-Ex oligomerization in solution takes place by trans binding, these results are consistent with the previous finding that the self-binding capacity of the MAM domain is not involved in the homophilic trans interaction of the PTP\(\mu\) ectodomain.

**MAM Domain Can Promote Lateral (cis) Dimerization of PTP\(\mu\)**—The previous results demonstrated that the MAM domain has the capacity of self-binding, but this feature seems not to be involved in the homophilic trans interaction of the PTP\(\mu\) ectodomain. Consequently, a question can be raised whether the MAM domain of PTP\(\mu\) is involved in formation of the other type of homophilic interaction, i.e. the lateral (cis) dimerization.

The MAM domain of meprin contains, besides the four highly conserved cysteines, an additional cysteine that was proved to participate in the homophilic interaction between meprin subunits, through formation of an intermolecular disulfide bridge (21). To test formation of cis dimers in the case of PTP\(\mu\) through MAM-to-MAM binding, we attempted to generate covalently linked dimers by employing the approach of disulfide cross-linking. To this purpose, a mutant construct (Mut5Cys) was obtained, containing an additional cysteine between Pro61 and Trp62 of the MAM domain of PTP\(\mu\) (Fig. 8A). The insertion was placed at this position based on the sequence alignment between the MAM regions of meprin and PTP\(\mu\) (Fig. 8A). Structure prediction for the MAM domain of PTP\(\mu\) displays the lack of secondary structural elements in this region, suggesting
the presence of a loop having ~15 amino acids (data not shown). As the predicted loop is shorter than in the case of meprin, the additional cysteine residue was introduced together with three other amino acids (Fig. 8A). One of them was a proline, to avoid formation of an α-helix or a β-sheet within the mutated region.

One reason for introducing the “5Cys” mutation in the full-length PTPμ was to test whether the corresponding protein (Myc-PTPμMut5Cys) is still able to interact on the cell surface with soluble, wild-type MAM domain. Fig. 8B shows that the mutation did not alter the self-binding capacity of the MAM domain.

To test whether this mutation affects the trans interaction of the PTPμ ectodomain, we examined the cellular aggregation of insect cells expressing on their surface either the wild-type or the mutated extracellular region of PTPμ (Myc-ExTJ or Myc-ExTJmut5Cys). Fig. 9A shows that the 5Cys mutation did not abolish the capacity of the PTPμ ectodomain to promote formation of cellular clusters. (Uninfected Sf9 cells do not form cellular clusters, as already reported (10).) The cells used in the electrophoretic analysis were plated at a non-confluent density, to avoid cellular aggregation (homophilic interactions). In addition, iodoacetamide was included in all buffers to prevent the Myc-ExTJmut5Cys dimerization after cell lysis. Thus, it is reasonable to assume that the 280-kDa band corresponds to dimers formed as a result of homophilic cis interactions among PTPμ ectodomains.

The alignment between MAM sequences of PTPμ and meprin was done using the program Clustal 1.81. The first studies on the adhesive role of MAM-containing RPTPs were initiated because of the existing similarities between their extracellular regions and cell-adhesion molecules, both types of proteins having Ig-like and FN III-like domains (10, 11). Although the capacity of RPTP type II to mediate cellular adhesion by homophilic trans interactions has been demonstrated, the role of the MAM domain in this process is still unclear. The presence of a MAM domain in molecules such as meprin (17) and neuropilin (18) appears to be correlated with their ability to interact in a homophilic manner. However, an adhesive role of MAM has not been reported so far in the case of zonadhesin (22), MAEG (23), nephronectin (24), and DAIk (25).

Data reported here provide the first evidence that the MAM domain of PTPμ has homophilic binding properties. Thus, in vitro experiments demonstrate that MAM forms oligomers in solution and the homophilic binding experiments at the cell surface confirm the self-binding capacity of this region. It is still not clear what types of forces govern the MAM oligomerization, but the MAM adhesion capacity does not depend on the pH value of the medium. Hence, it can be speculated that the contribution of electrostatic forces to MAM-to-MAM interactions is less important.

**FIG. 8.** The insertion of an additional cysteine into the MAM domain did not block its self-binding capacity. A, schematic representation of the 5Cys mutation within the MAM domain. The Cys residue involved in formation of the intermolecular disulfide bridge of meprin is marked by an asterisk. The alignment between MAM sequences of PTPμ and meprin was done using the program Clustal 1.81.

B, both wild-type (WT) and mutant (Mut5Cys) forms of PTPμ bind the soluble GST-MAM protein, at the cell surface. This panel is an immunoblot with the BK9 antibody.

The cells used in the electrophoretic analysis were plated at a non-confluent density, to avoid cellular aggregation (homophilic trans interactions). In addition, iodoacetamide was included in all buffers to prevent the Myc-ExTJmut5Cys dimerization after cell lysis. Thus, it is reasonable to assume that the 280-kDa band corresponds to dimers formed as a result of homophilic cis interactions among PTPμ ectodomains.

The first studies on the adhesive role of MAM-containing RPTPs were initiated because of the existing similarities between their extracellular regions and cell-adhesion molecules, both types of proteins having Ig-like and FN III-like domains (10, 11). Although the capacity of RPTP type II to mediate cellular adhesion by homophilic trans interactions has been demonstrated, the role of the MAM domain in this process is still unclear. The presence of a MAM domain in molecules such as meprin (17) and neuropilin (18) appears to be correlated with their ability to interact in a homophilic manner. However, an adhesive role of MAM has not been reported so far in the case of zonadhesin (22), MAEG (23), nephronectin (24), and DAIk (25).
All four conserved cysteine residues within the MAM domain are involved in disulfide bridges, as suggested by the two-step alkylation experiment. According to our results, they should play a role in preserving the tridimensional conformation of MAM, which confers its self-adhesive capacity. Thus, either reduction of disulfide bridges with DTT or canceling one of these bridges by site-directed mutagenesis led to the abolishment of MAM self-binding.

Our results suggest that the MAM-to-MAM interaction is not involved in trans binding of the PTP\(\mu\) ectodomain. This conclusion came from the experiments performed with a mutant ectodomain in which the MAM domain self-binding does not take place (MutC36A). First, this mutant ectodomain expressed at the cell surface is able to induce cellular clustering by homophilic trans interactions. Second, gel filtration experiments show that the soluble, secreted mutant ectodomain is still an oligomer in solution and is eluted at a similar molecular weight like the wild-type form. The oligomer should be formed by trans interactions, because its stability is pH-sensitive, like in case of cellular aggregation. Together, these results suggest that the abolishment of MAM binding capacity did not lead to blocking of the ectodomain trans interaction. Consequently, the trans interaction of PTP\(\mu\) involves the participation of other domains of the extracellular region, i.e., the Ig-like and the FN III-like domains. This fact is in agreement with the conclusions of Brady-Kalnay and Tonks (15) and Zondag et al. (16) that the Ig-like and/or FN III-like domains should participate in the PTP\(\mu\) homophilic binding.

Moreover, our results suggest that the homophilic binding capacity of the MAM domain does not require prior formation of the trans PTP\(\mu\) interactions. This observation is supported by the binding experiments performed in conditions of culture medium for which the trans interactions are abolished (pH below 6). Under these conditions, the secreted MAM domain fragment is still able to bind the PTP\(\mu\) ectodomain expressed at the cell surface.

To our knowledge, no reports have been published so far in regard to the possibility of cis interaction of the MAM-containing PTPs. Given that MAM-to-MAM binding is not required for ectodomain trans interactions, we have analyzed if the MAM domain of PTP\(\mu\) could be involved in lateral dimerization of this protein at the cell surface. Here we provide experimental evidence indicating that the MAM domain can promote PTP\(\mu\) cis interactions. Thus, insertion of a supplementary Cys residue into the MAM sequence yielded PTP\(\mu\) dimers stabilized by intermolecular disulfide bridges. Because the experiment was conducted in such a way as to avoid trans interactions, the disulfide-linked PTP\(\mu\) dimers could be produced only by cis interactions. Under similar experimental conditions, when the self-binding capacity of the MAM domain was blocked, the lateral, covalent dimerization of PTP\(\mu\) could not be detected. Consequently, the MAM-to-MAM interaction is essential for the PTP\(\mu\) cis dimerization. In addition, the results reported here support the idea that the cis and trans interactions of PTP\(\mu\) are independent of each other within the experimental system described herein.

Previously, Brady-Kalnay and Tonks (15) found that the MAM domain did not bind homophilically to MvLu cells expressing PTP\(\mu\) at their surface. A possible explanation of the discrepancy between their results and those reported in this article could be the different expression systems used to generate the soluble MAM domain fragment: whereas Brady-Kalnay and Tonks (15) obtained this protein in the cytoplasm of the Sf9 insect cells, we produced the GST-MAM construct as a

![Figure 9: The MAM domain promotes lateral binding of PTP\(\mu\) ectodomain.](image-url)
secreted protein. Thus, the MAM domain used in our experiments should possess post-translational modifications and conformation much closer to the native protein. The importance of the conformation for the adhesive properties of MAM has been also addressed above. Possibly, the improper folding of the MAM domain expressed as non-secreted protein prohibited its homophilic binding in the experiments reported by Brady-Kalnay and Tonks (15).

Zondag et al. (16) suggested that the MAM domain is necessary for the cellular adhesion mediated by PTPβ. Under our experimental conditions, the homophilic binding property of this domain is not required for the cellular aggregation induced by PTPβ. However, we cannot rule out the hypothesis that the MAM domain could indirectly contribute to the PTPβ capacity of promoting cellular clustering. In this respect, cell surface expression of a PTPβ-truncated construct, lacking the MAM domain, was found to be unable to induce cell-cell aggregation (16). A possible explanation might be that, because of the absence of the MAM domain, the spatial conformation of the remaining extracellular part is altered, thus impairing the homophilic binding capacity of Ig-like and/or FN III-like domains. The importance of the MAM domain in the folding of the native proteins was in fact evidenced for a related RPTP (13) and for meprin (26).

The MAM domain appears to have similar self-binding properties in different proteins. Thus, the MAM domain of meprin is involved in oligomerization both by non-covalent interaction and by disulfide bridge formation (17). In addition, the MAM domain of neuropilin mediates the lateral (cis) dimerization of this receptor (18). Similarly, according to data reported here, the MAM domain of PTPβ has the capacity to self-interact. Meprin, neuropilin, and PTPβ are structurally and functionally different proteins, the presence of the MAM domain being their only common feature. Taking also into account that the topological position of MAM in these proteins is different, it is reasonable to suppose that the MAM domain can be considered an independent module for which the self-binding capacity does not require additional structural elements.

There are a couple of elements suggesting that cis-dimerization of PTPβ might be involved in the signal transduction mechanism. The current opinion about PTPβ is that this transmembrane protein plays a role in signaling, in response to cell-cell adhesion. Although the signaling pathway downstream of PTPβ is still unclear, the interaction of its intracellular region with specific ligands like cadherins (27), p120ctn (28), and the scaffold protein RACK1 (29) is well established. In addition, PTPβ seems to be up-regulated as a function of cell density. Thus, the protein is rapidly cleared from the cell surface in subconfluent cultures, but in high density cultures PTPβ is accumulated at the cell-cell contact sites (30). At high cell density, the PTPβ-RACK1 interaction is increased and RACK1 is recruited at the intercellular contacts (29). Therefore, it could be speculated that the high PTPβ density at contact sites may promote ectodomain cis-dimerization. Consequently, dimerization of the corresponding intracellular regions could be induced, which in turn may promote a conformation favorable to binding of signaling molecules like RACK1. The catalytic activity of PTPβ might also be regulated by the induced dimerization. Thus, Feiken et al. (31) demonstrated that the juxtamembrane region of PTPβ can interact either with membrane-proximal domain D1 or with membrane-distal domain D2. Also, it was proved that the kinetic phosphatase activity of D1 is negatively modulated and its ligand binding capacity is sensibly modified by domain D2 (32). Based on these findings, it was suggested that the activity of PTPβ might be regulated by the intramolecular interaction between the juxtamembrane region and the catalytically active domain D1 or the regulatory domain D2. It can be supposed that the induced dimerization of the intracellular region (as a consequence of the lateral dimerization of the ectodomain) may favor the interaction of the juxtamembrane region with either D1 or D2 domains, thus modifying the catalytic activity of PTPβ. This model, in combination with the hypothesis of cell-density controlled cis-dimerization, suggests a potential link to the cadherin-dependent adhesion. Indeed, p120ctn has been proved to be dephosphorylated both in vitro and in intact cells by PTPβ (28). On the other hand, p120ctn plays a key role in maintaining normal levels of cadherins in mammalian cells (33). Thus, modification of the PTPβ catalytic activity against p120ctn, as driven by increased cell-density (via cis interaction of extracellular regions of PTPβ), may lead to modification of cadherin-mediated adhesion.

Receptor dimerization has been established as a common mechanism for the regulation of many families of cell surface proteins. One major unsolved issue is whether such a mechanism is also involved in regulation of the RPTP activity. Several studies demonstrate that RPTPs can form homo- and heterodimers by intracellular interactions (34–40). In addition, experimental evidence indicates that the catalytic activity of PTPα and CD45 can be down-regulated by receptor dimerization (41–43). These findings provide support for the model in which RPTPs are regulated by the intracellular region-mediated dimerization. However, this model is subject to debate, because the crystal structures of PTPβ and LAR intracellular domains did not show dimers like in the case of PTPα (44, 45). Data reported here support the hypothesis that PTPβ activity may be regulated by the receptor dimerization but, if this is the case, the lateral (cis) interaction is mediated by the ectodomains rather than by the intracellular regions.

In summary, we demonstrate that the MAM domain of PTPβ is a homophilic binding module of the extracellular region. It contains two intramolecular disulfide bridges, which are essential for the adhesive capacity of the MAM domain. We have also shown that the PTPβ ectodomain can homophilically interact not only in trans, but also in cis. Our data indicate that the self-binding capacity of the MAM domain is not involved in trans interaction, whereas it participates in the lateral dimerization of PTPβ. Further studies are necessary to identify the physiological consequences of PTPβ cis interaction as well as its specific role in signal transduction mechanisms.

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The MAM (Meprin/A5-protein/PTPmu) Domain Is a Homophilic Binding Site Promoting the Lateral Dimerization of Receptor-like Protein-tyrosine Phosphatase µ
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