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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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Valeriu B. Cismasiu‡§, Stefan A. Denes‡, Helmut Reiländer¶, Hartmut Michel¶, and Stefan E. Szedlacsek‡‖

From the ‡Department of Enzymology, Institute of Biochemistry, Spl. Independentei 296, Bucharest 060031, Romania and the ¶Department of Membrane Biology, Max-Planck Institute for Biophysics, Heinrich-Hoffmann Str. 7, Frankfurt am Main, 60528, Germany

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 (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 Ca^{2+}-independent manner (10, 11). The homophilic binding has been also evidenced in the ectodomains of PTPκ (12) and PTPλ (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 ectodomain 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-

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§ Present address: Albany Medical College, 47 New Scotland Ave., Albany, NY 12208.

‖ To whom correspondence should be addressed: Dept. of Enzymology, Institute of Biochemistry, Spl. Independentei 296, Bucharest 060031, Romania. Tel.: 40-21-2239069; Fax: 40-21-2239068; E-mail: stefan.szedlacsek@biochim.ro.

[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; BS^3, bis(sulfosuccinimidyl)suberate; IAA, iodoacetic acid; IAM, iodoacetamide; Ex, extracellular region.

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tal model: expression of receptor PTPμ at the surface of insect cells and then, testing their capacity to form cellular aggregates. Our results indicate that the MAM domain of PTPμ has the capacity to self-interact and has two intramolecular disulfide bridges, which are necessary to preserve the binding properties of this domain. In addition, we found that the MAM domain is not involved in the *trans* interaction but can instead promote the lateral (*cis*) dimerization of PTPμ.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The cDNA of human PTPμ (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′-GGGGATCCCGAGACGTTCTCAGGTGGCTGC-3′ and 5′-CCCAAGCTTCAGTGGTGGTGGTGGTGGTGTTTAACTGTATGGTCTGTCTGTTTC-3′ or 5′-TTGGTACCTCAGTGGTGGTGGTGGTGGTGAGGAGTCCTGGTACAT-3′. The last two primers encode six histidine amino acids and a stop codon. The amplified fragments were cloned into a pBluescript vector (Stratagene), resulting in pBS-Ex-pcr and pBS-MAM, respectively. A Bsu36I-Tth111I fragment from pBS-Ex-pcr was replaced with a similar one from pBS-hFl yielding pBS-Ex. The PCR products were amplified with Pfu polymerase (Stratagene) and sequenced.

The point mutation $Cys^{36} \rightarrow$ Ala in pBS-MAM was made using the QuikChange™ 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**GC**TGGATATAGTCAATCTGAAGGTG-3′. The presence of the mutation in plasmid pBS-MAMmutC36A was confirmed by sequencing. A Bsu36I-HindIII fragment was extracted from 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^{61} and Trp^{62}, using the DNA oligonucleotide 5′-CATGCGGGCCCG-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. Similarly, the double mutant pBS-ExmutC36A/5Cys was obtained starting from the pBS-MAMmutC36A plasmid.

pVL-FlagEx and pVL-MycEx were obtained as follows: pBS-Ex was digested with BamHI and KpnI enzymes and the cDNA fragment coding the extracellular region of PTPμ was inserted into transfer vectors pVL93MelFlag and pVL93MelMyc (19). The Bsu36I-KpnI fragment digested from pVL-MycEx was replaced with a similar restriction segment from pBS-hFl. The resulting pVL-MycPTPμ vector contains the entire human PTPμ cDNA. In a similar way, the vectors pVL-FlagExmutC36A and pVL-MycPTPμMutC36A were generated starting from pBS-ExmutC36A.

To obtain the pAc-GSTMAM baculovirus transfer vector, the cDNA coding for the MAM region 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 gp67 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 0.1% Pluronic Polyol F-68 detergent (Sigma).

Recombinant baculoviruses were made by co-transfecting Sf9 cells with BaculoGold™ viral DNA (BD Pharmingen) and the appropriate transfer vectors. The viruses were purified by plaque assay.

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_2HPO_4, 1.8 mM KH_2PO_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, Centriprep 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 protease in 1 ml of buffer. The supernatant containing both the MAM domain fragment and thrombin was supplemented with phenylmethylsulfonyl fluoride (final concentration 2 mM) and incubated for 1 h on nickel-nitrilotriacetic acid-agarose (Qiagen; 20 μl of resin per ml of supernatant). Finally, the beads were washed with 10 mM imidazole in PBS, and the MAM fragment was eluted with PBS in the presence of 300 mM imidazole. The protein was concentrated using Centricon YM-10 (Millipore).

To purify the wild-type or mutant ectodomain constructs expressed as secreted proteins, the culture medium was diluted with 2 volumes of PBS and concentrated 30-fold using Centriprep YM-30 devices. Imidazole (5 mM) and nickel-nitrilotriacetic acid-agarose (20 μl bed) were added to the clarified supernatant. After 4 h, the beads were washed 6 times with 10 volumes of ice-cold Tris-buffered saline A (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 ($10{,}000 \times g$, 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 Centriprep YM-30 devices. All steps of protein purification were carried out at 4 °C if not otherwise specified.

The protein concentration was measured with BCA reagent (Pierce). 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 glycosyl groups, 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 Coomassie Blue R-250 or transferred to polyvinylidene difluoride membrane 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 PTPμ; 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 PTPμ 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 calibration curve was established using standard globular proteins delivered by Amersham Biosciences: ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritine (440 kDa), and thyroglobulin (669 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 bis(sulfosuccinimidyl)suberate (BS^3; Sigma) in PBS (pH 7.4).

The cross-linking reaction was carried out for various periods with a 10-fold molar excess of BS^3 (0.3 mM). The reactions were quenched by addition of loading buffer, and 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.

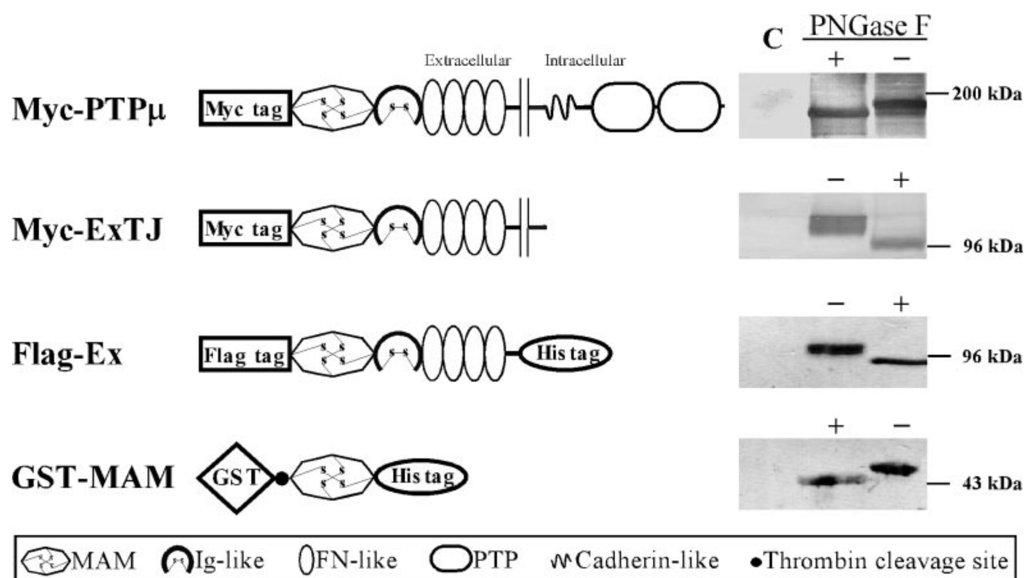

FIG. 1. **Schematic representation and expression of the recombinant forms of PTPμ.** The cell lysate (from 10^5 cells expressing Myc-PTPμ or Myc-ExTJ) and the purified, secreted proteins (1 μg of either Flag-Ex or GST-MAM) were run on 10% SDS-PAGE and the immunoblots were probed with anti-MAM antibody BK9. The protein samples were treated (+) or not (−) with PNGase F. *C* represents control experiments, where the insect cells were infected with non-recombinant baculoviruses.

In the first step, 10 μg of denatured protein was alkylated with 30 mM iodoacetic acid (IAA) at 37 °C for 20 min. The protein was precipitated with cold acetone, washed, and dissolved in PBS supplemented with 8 M 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 M urea and stained with Coomassie Blue R-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. After 36 h, cellular adhesion was examined by light microscopy (Nikon E600W Microscope) with a ×10 objective. The Sf9 cells infected with non-recombinant baculovirus were used as control.

The binding of different soluble constructs to the full-length PTPμ at the cell surface was assessed by a three-step method. First, a suspension culture of insect cells at 2×10^6 cells/ml was infected with recombinant baculoviruses Ac-FlagEx or Ac-GSTMAM 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 recovered and clarified by centrifugation (10,000 × *g*, 10 min). In the second step, the Sf9 suspension cultures (2×10^6 cells/ml) were infected with recombinant baculoviruses Ac-MycPTPμ, Ac-MycPTPμMutC36A, or Ac-MycPTPμMut5Cys and, as a control, with non-recombinant baculoviruses. At 30 h post-infection, the infected cells expressing the full-length PTPμ at the surface were recovered by centrifugation (800 × *g*, 1 min) and resuspended into the culture medium with secreted proteins obtained in the first step. After 8 h, the Sf9 cells were collected by centrifugation. In the last step, the cells were lysed by sonication in ice-cold PBS (1 ml of buffer per 20 ml of culture) and the binding of soluble proteins was tested by incubating the cell extract with anti-FLAG (M1) affinity gel or glutathione-Sepharose™ 4B (15 μl gel for each ml of sonicate). After 4 h incubation at 4 °C, the resin was extensively washed, and the bound proteins were analyzed by SDS-PAGE and immunoblotting.

Generation of Disulfide-linked Dimers—The Sf9 cells (2×10^6) were plated on the 25-cm^2 flasks and were allowed to grow for 16 h. The cells were infected with the recombinant baculoviruses: Ac-MycExTJ, Ac-MycExTJmut5Cys, or Ac-MycExTJmutC36A/5Cys. At 30 h post-infection, the monolayers were washed with PBS, and the cells were incubated in TS buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 1% SDS, pH 7.5) supplemented with benzonase (Merck, 10 units for 5×10^5 cells), for 30 min at 4 °C. After lysis, the extracts were subjected to SDS-PAGE in the presence or absence of β-mercaptoethanol, and the proteins were analyzed by immunoblotting. All buffers were supplemented with 20 mM iodoacetamide.

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, 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).

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 and Myc-ExTJ constructs, a similar experiment was performed under our experimental conditions. The formation of cellular clusters was evidenced, thus proving that these constructs mediate cell-cell adhesion (see below).

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 BS^3 (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 BS^3. Fig. 2 shows that under relatively mild cross-linking conditions the dimer can be detected after 1 min of reaction. 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.

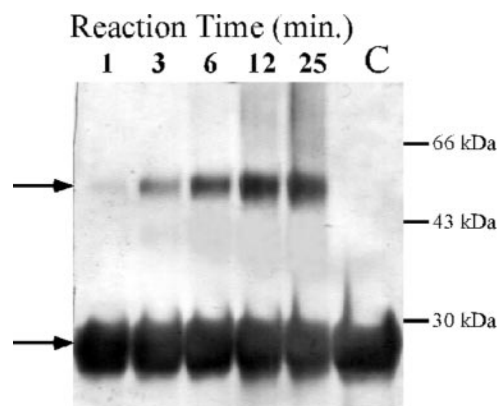

FIG. 2. **The MAM domain interacts homophilically in solution.** The purified protein at 0.7 mg/ml was incubated with 0.3 mM BS^3 at 25 °C. After different periods of reaction, 10 μg of the cross-linked protein were recovered and subjected to 10% SDS-PAGE. This panel is an immunoblot using anti-poly-His antibody. The *arrows* indicate the oligomeric and monomeric forms of the cross-linked protein. The control experiment (*C*) was performed under similar conditions except BS^3 was not added.

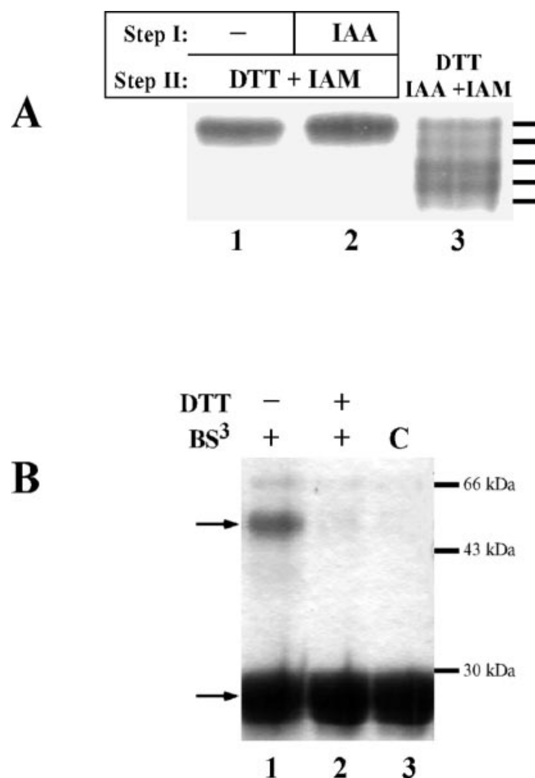

FIG. 3. **The binding capacity of the MAM domain requires the preservation of intramolecular disulfide bridges.** *Panel A*, the samples (5 μg) treated (*lane 2*) or not (*lane 1*) with IAA have the same electrophoretic mobility. The fully reduced sample (10 μg) treated with a mixture of alkylating reagents, IAA and IAM (*lane 3*), migrates as five electrophoretic bands (labeled by *bars*) corresponding to the introduced number of IAA carboxyls (0, 1, 2, 3, and 4 molecules). The proteins in *lanes 1* and *2* migrate as the *upper band* of *lane 3* confirming that there is no IAA reagent in these molecules. The bands were stained with Coomassie Blue R-250. The entire procedure was performed in the presence of 8 M urea. *Panel B*, the covalent dimerization of the MAM domain fragment in the presence of BS^3 cross-linker (*lane 1*) is not possible when the protein is first treated with DTT (*lane 2*). This panel is a 12.5% SDS-PAGE and the bands were stained with Coomassie Blue R-250. The *arrows* indicate the monomer and the dimer.

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 sulfhydryl groups but iodoacetic 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. 3*A* (*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. 3*A*, *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 BS^3, the dimer form was not still observed on SDS-PAGE gel (Fig. 3*B*, *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-

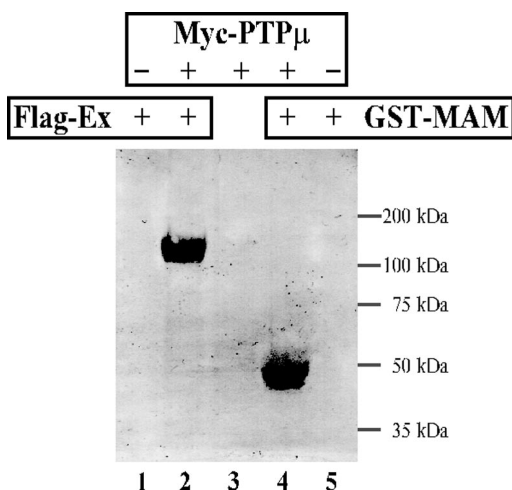

FIG. 4. **The secreted MAM-containing proteins bind to PTP*μ* at the insect cell surface.** The insect cells expressing Myc-PTP*μ* at their surface or the non-recombinant baculovirus-infected cells were incubated in medium containing the secreted proteins Flag-Ex or GST-MAM. The cell extracts were incubated with the appropriate affinity beads and the bound proteins were analyzed by 10% SDS-PAGE and immunoblotting with anti-poly-His antibody. The bound proteins Flag-Ex and GST-MAM were evidenced only in the case of cells expressing full-length PTP*μ* (*lanes 2* and *4*, respectively), but not the control cells (*lanes 1* and *5*, respectively). The anti-poly-His antibody probing was specific because no electrophoretic bands were detected in the absence of the secreted proteins (*lane 3*). For each lane, the samples were prepared starting from 10 ml of suspension culture. The same results were obtained in four independent experiments.

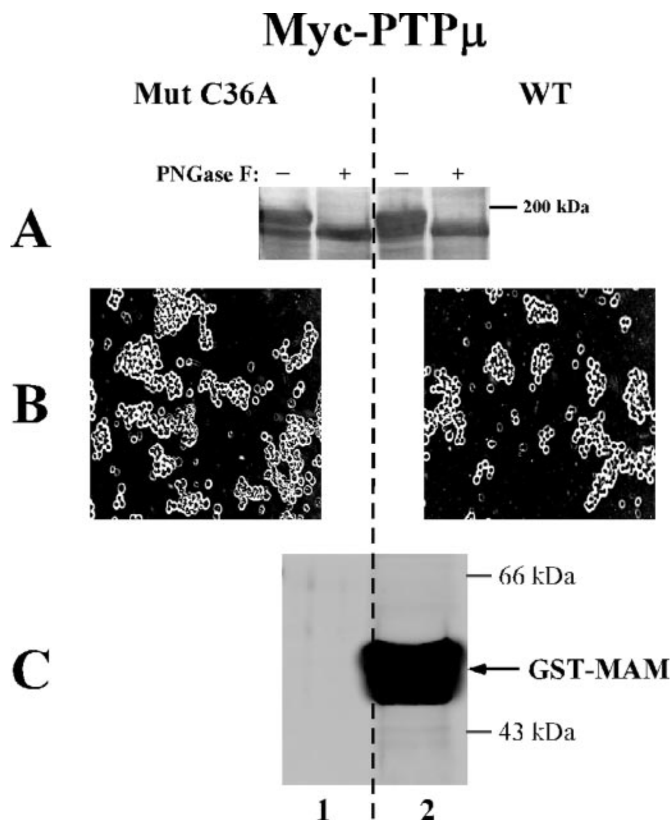

FIG. 5. **A mutant form of PTP*μ* can mediate the cellular adhesion but cannot bind the MAM domain expressed as a secreted protein.** *A*, both wild-type (WT, *right half*) and mutant (MutC36A, *left half*) proteins migrate at the same molecular weight. Treatment with PNGase F indicates that both proteins are glycosylated. On each lane, the lysates of 10^5 infected Sf9 cells were applied and the immunoblot was probed with anti-myc antibody. *B*, micrographs show that the mutant form (MutC36A) of full-length PTP*μ* is able to induce cellular aggregation (*left panel*) as the wild-type protein (*right panel*). In each case, a suspension culture with 10^6 cells/ml was monitored. *C*, the mutant PTP*μ* is not able to bind the secreted GST-MAM protein (*lane 1*), whereas the WT construct can interact with the fusion protein (*lane 2*). The secreted protein bound by PTP*μ*-expressing cells (from 10 ml of culture medium) was applied onto 10% SDS-PAGE and stained with Coomassie Blue R-250.

tion can occur in conditions when the self-binding capacity of MAM region is abrogated.

Previously, it was reported that the mutation of the second conserved cysteine residue in the MAM domain of meprin decreases the capacity of this domain to make homophilic interactions (21). To test whether a similar conclusion is valid in the case of PTP*μ*, the equivalent cysteine residue of the PTP*μ* MAM region was replaced with Ala. This mutation was introduced in the full-length construct Myc-PTP*μ*MutC36A. The mutant protein is expressed at the expected molecular weight and is also glycosylated, like the wild-type protein (Fig 5*A*). Insect cells infected with baculoviruses carrying this mutant still displayed the capacity to form cellular aggregates (Fig. 5*B*). This result indicates that the mutant full-length PTP*μ* 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-PTP*μ*MutC36A at the cell surface. Fig. 5*C* evidences the lack of interaction between the soluble protein and the PTP*μ* 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. 3*B*), this result indicates that the PTP*μ* ectodomain-MAM domain interaction is based on MAM-to-MAM binding. Interestingly, these results indicate that the cellular adhesion driven by the *trans* interactions of the PTP*μ* takes place even in the absence of MAM-to-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 PTP*μ* 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 PTP*μ* ectodomain.

To answer this question we performed the protein-protein binding assay, in which the interaction between GST-MAM and the PTP*μ* 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 PTP*μ* is abolished (Fig. 6*A*), the MAM domain can still interact with the ectodomain (Fig. 6*B*). Therefore, MAM-to-MAM binding can take place under conditions in which the *trans* interaction of the PTP*μ* 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 PTP*μ*.

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 PTP*μ* ectodomain by a different approach: the whole extracellular region of PTP*μ* 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

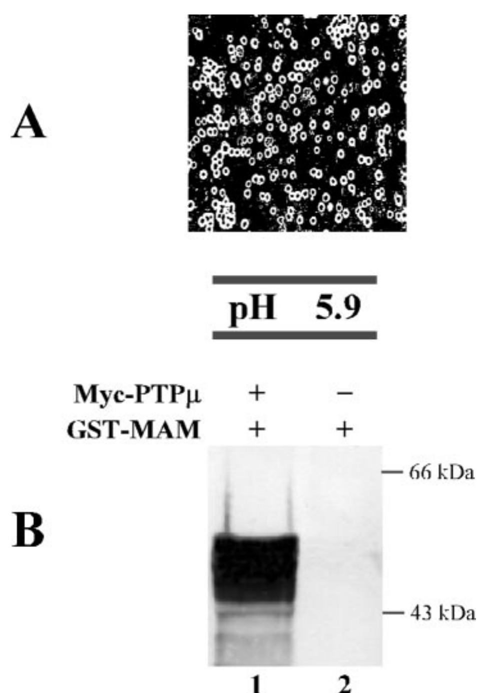

FIG. 6. **The MAM domain expressed as a secreted protein can interact with PTPμ even in pH conditions when the *trans* interaction is blocked.** *Panel A*, at pH below 6 the homophilic *trans* interactions of full-length PTPμ are blocked because the protein is not able to mediate cell-cell aggregation when the pH of the culture medium is set at 5.9. The cells (10^6 cells/ml) were infected with the appropriate recombinant baculovirus and were maintained is suspension during the protein expression. *Panel B*, at the same pH of the medium, the PTPμ expressed at the cell surface is still able to bind the secreted GST-MAM protein (*lane 1*). The interaction is specific because no fusion protein was recovered from Sf9 cells infected with non-recombinant baculovirus (*lane 2*). In both situations, the cells from a 10-ml suspension culture were lysed and incubated with glutathione-Sepharose 4B. The bound protein was probed with anti-GST antibody.

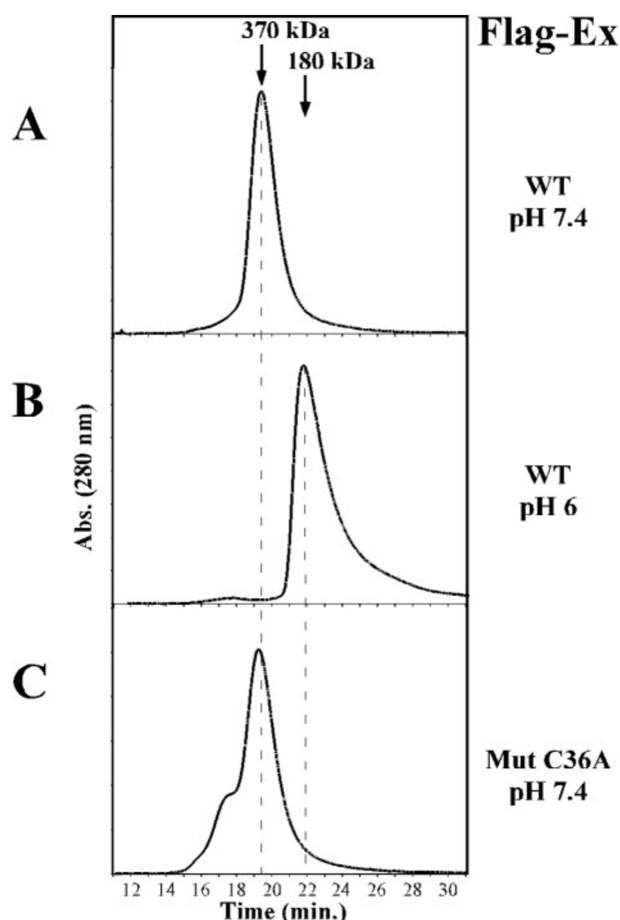

FIG. 7. **Homophilic binding of PTPμ ectodomain in solution.** Size exclusion profiles of purified wild-type (*WT*) and mutant (MutC36A) PTPμ ectodomain are shown. Equal amounts (100 μg) of samples at 0.5 mg/ml were applied on a Superdex 200 HR 10/30 column and eluted with PBS adjusted to different pH values. *Panel A* indicates that the PTPμ ectodomain is mainly an oligomer in solution at physiological pH. The peak of the oligomer is shifted to a lower molecular form at a lower value of pH, as shown in *panel B*. The mutation C36A introduced into the MAM region did not affect significantly the stability of the oligomeric form of the PTPμ ectodomain (*panel C*). The fractions corresponding to the peaks were collected, concentrated, and applied again, confirming the reproducibility of experiments.

contain an oligomeric form having an apparent molecular mass of 375 kDa (probably the dimer; Fig. 7*A*). 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 longer elution time, corresponding to an apparent molecular mass of 180 kDa (Fig. 7*B*). Thus, the dissociation of the oligomeric form was induced by decreasing the pH from 7.2 to 6.

Because the cellular adhesion mediated by PTPμ can be blocked in a pH-dependent manner (Ref. 11 and Fig. 6*A*), 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 PTPμ. 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. 7*C*). 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 PTPμ ectodomain.

MAM Domain Can Promote Lateral (cis) Dimerization of PTPμ—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 PTPμ ectodomain. Consequently, a question can be raised whether the MAM domain of PTPμ 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 PTPμ 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 Pro^{61} and Trp^{62} of the MAM domain of PTPμ (Fig. 8*A*). The insertion was placed at this position based on the sequence alignment between the MAM regions of meprin and PTPμ (Fig. 8*A*). Structure prediction for the MAM domain of PTPμ displays the lack of secondary structural elements in this region, suggesting

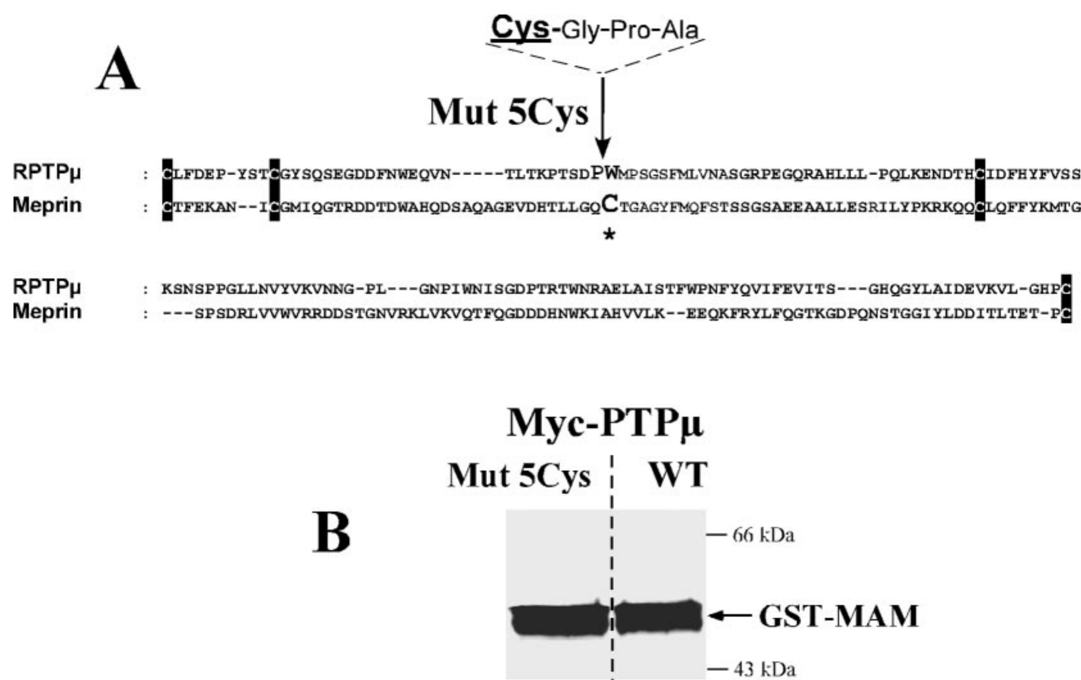

FIG. 8. **The insertion of an additional cysteine into the MAM domain did not block its self-binding capacity.** *A*, schematic representation of the 5Cys mutation within the MAM domain. The Cys residue involved in formation of the intermolecular disulfide bridge of meprin is marked by an *asterisk*. 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 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. 8*A*). 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. 8*B* 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. 9*A* 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).)

Electrophoretic analysis under non-reducing conditions of the Myc-ExTJmut5Cys protein evidenced the presence of two distinct bands corresponding to ~140 and 280 kDa, respectively (Fig. 9*B*). The wild-type protein (Myc-ExTJ) migrated under similar conditions as a single band corresponding to 140 kDa. In contrast, under reducing conditions, the SDS-PAGE for the mutant protein evidenced the absence of the electrophoretic band at 280 kDa. These results suggest that the 280-kDa band corresponds to the disulfide-linked dimer.

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.

To test whether these dimers are generated as a consequence of a MAM-to-MAM interaction, the formation of disulfide-linked dimers was analyzed when the second conserved cysteine of the MAM domain was mutated to alanine. Previously, we provided evidence that mutation of this conserved residue abolishes the MAM-to-MAM interaction (Fig. 5*C*). Fig. 9*B* (*right panel*) proves that in the case of this double mutant (second conserved Cys missing, fifth Cys inserted) the covalent dimer is not formed anymore, indicating that the self-binding capacity of the MAM domain is essential for lateral dimerization of PTPμ. In conclusion, these results show that the MAM domain can promote *cis* binding of the PTPμ ectodomain by its capacity to self-interact.

DISCUSSION

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 IIB 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 DAlk (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.

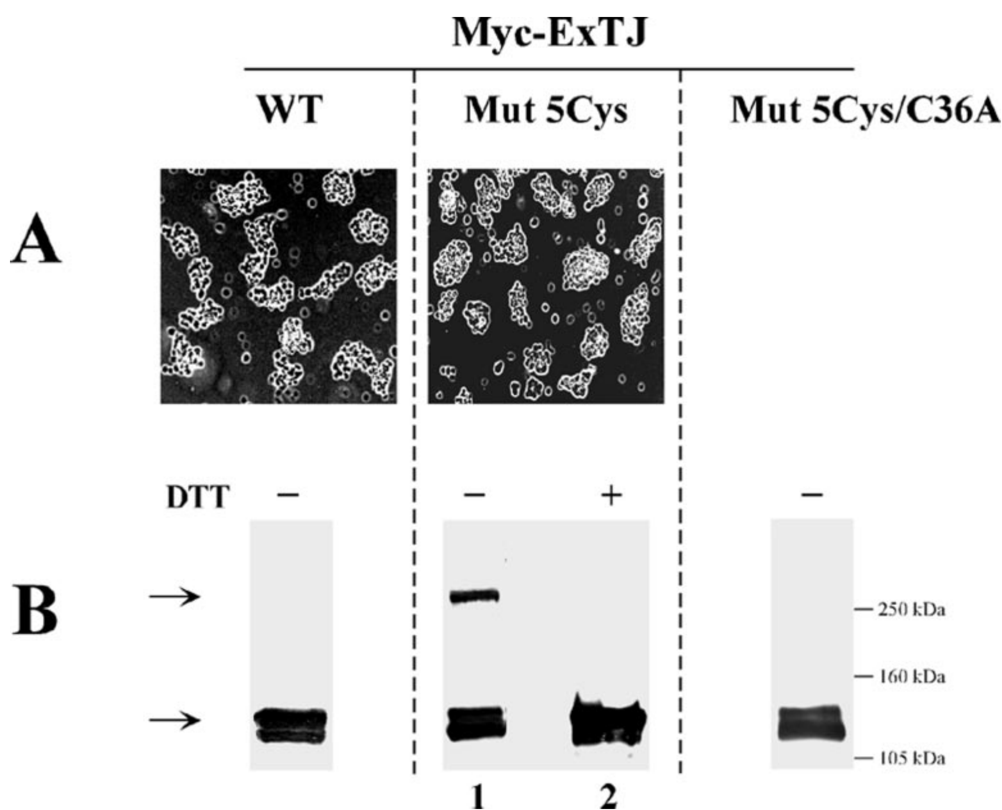

FIG. 9. **The MAM domain promotes lateral binding of PTPμ ectodomain.** *A*, the Sf9 cells expressing the mutant protein Myc-ExTJmut5Cys (*left*) aggregates like those expressing the wild-type protein (*middle*). The suspension cultures (10^6 cells/ml) were monitored by light microscopy. *B*, under non-reducing conditions, the Myc-ExTJmut5Cys protein runs as 140- and 280-kDa electrophoretic bands (indicated by *arrows*), corresponding to monomer and disulfide-linked dimer, respectively (*lane 1*). Under the same conditions, the *upper band* is absent as in the case of the wild-type construct (*left panel*), as well as in the case when both mutations (MutC36A and Mut5Cys) are present the same molecule (*right panel*). Under reducing conditions, the upper band is not visible (*lane 2*). The proteins were probed with anti-myc antibody.

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 PTPμ 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 PTPμ 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 PTPμ homophilic binding.

Moreover, our results suggest that the homophilic binding capacity of the MAM domain does not require prior formation of the *trans* PTPμ 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 PTPμ 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 PTPμ 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 PTPμ *cis* interactions. Thus, insertion of a supplementary Cys residue into the MAM sequence yielded PTPμ dimers stabilized by intermolecular disulfide bridges. Because the experiment was conducted in such a way as to avoid *trans* interactions, the disulfide-linked PTPμ 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 PTPμ could not be detected. Consequently, the MAM-to-MAM interaction is essential for the PTPμ *cis* dimerization. In addition, the results reported here support the idea that the *cis* and *trans* interactions of PTPμ 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 PTPμ 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

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 PTPμ. Under our experimental conditions, the homophilic binding property of this domain is not required for the cellular aggregation induced by PTPμ. However, we cannot rule out the hypothesis that the MAM domain could indirectly contribute to the PTPμ capacity of promoting cellular clustering. In this respect, cell surface expression of a PTPμ-truncated construct, lacking the MAM domain, was found to be unable to induce cell-cell aggregation (16).[2] 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 PTPμ has the capacity to self-interact. Meprin, neuropilin, and PTPμ 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 PTPμ might be involved in the signal transduction mechanism. The current opinion about PTPμ is that this transmembrane protein plays a role in signaling, in response to cell-cell adhesion. Although the signaling pathway downstream of PTPμ is still unclear, the interaction of its intracellular region with specific ligands like cadherins (27), p120^{ctn} (28), and the scaffold protein RACK1 (29) is well established. In addition, PTPμ 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 PTPμ is accumulated at the cell-cell contact sites (30). At high cell density, the PTPμ-RACK1 interaction is increased and RACK1 is recruited at the intercellular contacts (29). Therefore, it could be speculated that the high PTPμ 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 PTPμ might also be regulated by the induced dimerization. Thus, Feiken *et al.* (31) demonstrated that the juxtamembrane region of PTPμ 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 PTPμ 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 PTPμ. This model, in combination with the hypothesis of cell-density controlled *cis*-dimerization, suggests a potential link to the cadherin-dependent adhesion. Indeed, p120^{ctn} has been proved to be dephosphorylated both *in vitro* and in intact cells by PTPμ (28). On the other hand, p120^{ctn} plays a key role in maintaining normal levels of cadherins in mammalian cells (33). Thus, modification of the PTPμ catalytic activity against p120^{ctn}, as driven by increased cell-density (via *cis* interaction of extracellular regions of PTPμ), 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 PTPμ and LAR intracellular domains did not show dimers like in the case of PTPα (44, 45). Data reported here support the hypothesis that PTPμ 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 PTPμ 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 PTPμ 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 PTPμ. Further studies are necessary to identify the physiological consequences of PTPμ *cis* interaction as well as its specific role in signal transduction mechanisms.

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