Homophilic Binding of PTPμ, a Receptor-Type Protein Tyrosine Phosphatase, Can Mediate Cell–Cell Aggregation

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Abstract. The receptor-like protein tyrosine phosphatase, PTPμ, displays structural similarity to cell–cell adhesion molecules of the immunoglobulin superfamily. We have investigated the ability of human PTPμ to function in such a capacity. Expression of PTPμ, with or without the PTPase domains, by recombinant baculovirus infection of Sf9 cells induced their aggregation. However, neither a chimeric form of PTPμ, containing the extracellular and transmembrane segments of the EGF receptor and the intracellular segment of PTPμ, nor the intracellular segment of PTPμ expressed as a soluble protein induced aggregation. PTPμ mediates aggregation via a homophilic mechanism, as judged by lack of incorporation of uninfected Sf9 cells into aggregates of PTPμ-expressing cells. Homophilic binding has been demonstrated between PTPμ-coated fluorescent beads (Covaspheres) and endogenously expressed PTPμ on MvLu cells. Additionally the PTPμ-coated beads specifically bound to a bacterially expressed glutathione-S-transferase fusion protein containing the extracellular segment of PTPμ (GST/PTPμ) adsorbed to petri dishes. Covaspheres coated with the GST/PTPμ fusion protein aggregated in vitro and also bound to PTPμ expressed endogenously on MvLu cells. These results suggest that the ligand for this transmembrane PTPase is another PTPμ molecule on an adjacent cell. Thus homophilic binding interactions may be an important component of the function of PTPμ in vivo.

THE reversible phosphorylation of tyrosyl residues in proteins is an essential component of the modulation of signal transduction processes involved in cell growth and differentiation. The dynamic balance of cellular phosphotyrosine levels is achieved by the opposing actions of the protein tyrosine kinases (PTKs)[1] and protein tyrosine phosphatases (PTPases). The PTKs are a structurally diverse group that includes growth factor receptors and a substantial number of oncogene products, including members of the *src* family. It is now apparent that PTPases rival the PTKs in structural diversity and complexity. PTPases have the potential to oppose the effects of kinases as illustrated by their ability to suppress the transforming potential of oncogenic PTKs (Brown-Shimer et al., 1992; Woodford-Thomas et al., 1992; Zander et al., 1993). However, the PTPases should not be viewed simply as PTK antagonists. Overexpression of PTPα leads to dephosphorylation and activation of *c-src* and induces cell transformation (Zheng et al., 1992). In addition, CD45 has been shown to play an essential role in signal transduction through the T cell receptor, also potentially exerting its effect through the dephosphorylation and activation of *src*-family PTKs such as *lck* and *fyn* (reviewed in Trowbridge, 1991). Current evidence clearly indicates an essential role for the PTPases in cellular signaling.

PTPases exist in both soluble and transmembrane, receptor-like forms, (Charbonneau and Tonks, 1992). The structural features of some of the enzymes suggest a role for subcellular localization in the regulation of activity. In the case of the receptor PTPases, similar to the receptor PTKs, there is the potential for regulating activity by the binding of ligands to the extracellular segment of the protein. Several of the transmembrane PTPases are members of the immunoglobulin superfamily and display structural motifs in their extracellular segments that are suggestive of a role in cell–cell adhesion (Streuli et al., 1989; Fischer et al., 1991).

Cell–cell adhesion molecules are grouped into two major families on the basis of homology and conditions for binding: the immunoglobulin superfamily (generally calcium-independent) and the cadherin family (calcium-dependent). N-CAM is prototypical of the immunoglobulin superfamily of adhesion molecules. It contains five immunoglobulin domains and two fibronectin type-III repeats in the extracellular segment (reviewed by Edelman and Crossin, 1991). Cadherins are calcium-dependent cell–cell adhesion molecules that associate with the actin cytoskeleton by interactions of their cytoplasmic domains with proteins termed catenins (Ozawa et al., 1989). Both N-CAM and the cadherins bind by a homophilic mechanism (Edelman et al., 1987; Hall et al., 1990; Rao et al., 1992; Takeichi, 1991).

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1. *Abbreviations used in this paper:* GST, glutathione S-transferase; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases.

The receptor-type PTPase, PTPμ, shows homology to N-CAM in that it bears an extracellular segment with an immunoglobulin domain and four fibronectin type III repeats (see Fig. 1) (Gebbink et al., 1991). This arrangement of multiple Ig-domains and fibronectin type-III repeats was first observed in LAR (Streuli et al., 1988) and has also been seen in the extracellular segments of several other transmembrane PTPases (reviewed in Charbonneau and Tonks, 1992). In this manuscript we investigate whether PTPμ can participate in homophilic binding interactions.

Introduction of potential adhesion molecules into nonadhesive *Drosophila* S2 insect cells has been used to demonstrate adhesive functions for fasciclin III, connectin and *Dtrk* molecules directly (Snow et al., 1989; Nose et al., 1992; Pulido et al., 1992). In a similar approach, we demonstrate that the full-length form of PTPμ induced aggregation, via a homophilic binding mechanism, when expressed in nonadhesive Sf9 insect cells, which are derived from the Fall armyworm *Spodoptera frugiperda*. Cells expressing mutant forms of the enzyme lacking the natural extracellular segment did not aggregate. In this system, PTPase activity and the aggregation response are not mutually dependent. We have also reconstituted the binding reaction in vitro between baculovirus-expressed PTPμ linked to beads and surfaces coated with bacterially-expressed extracellular segment of PTPμ. Finally we show that endogenously expressed PTPμ in lung cells binds homophilically to baculovirus-expressed PTPμ. These data represent the first demonstration of homophilic binding and a potential adhesive function for a member of the PTPase family of enzymes.

Materials and Methods

Cell Culture and PTPase Activity

Sf9 cells derived from the ovary of the Fall armyworm *Spodoptera frugiperda* (American Type Culture Collection [ATCC] number CRL 1711) were maintained at 27°C in Grace's Insect Medium Supplemented (GIBCO-BRL, Gaithersburg, MD) containing 10% FBS and 10 μg/ml gentamicin (GIBCO-BRL). MvLu and 3T3 cells (ATCC numbers CCL 64 and CCL 92, respectively) were grown at 37°C, 5% CO_2 in DME containing penicillin and streptomycin plus 10% FBS (GIBCO-BRL).

PTPase assays were performed as described (Flint et al., 1993). Total cell lysates were prepared in detergent containing buffer (20 mM Tris pH 7.6, 5 mM EDTA, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine, 1 mM PMSF, and 1 mM DTT). PTPase activity was measured in vitro by dephosphorylation of tyrosine-phosphorylated reduced carboxamidomethylated and maleylated (RCM) lysozyme. 1 U is defined as the amount of enzyme that catalyzes the release of 1 nmol phosphate from the substrate per min.

Plasmid and Baculovirus Construction

The soluble form (80 kD) and the full-length form (200 kD) of human PTPμ were expressed in Sf9 cells infected with the Ac-pS and Ac-pFL baculoviruses, respectively. The soluble form (Ac-pS) contains the DNA sequence coding for the intracellular domain of PTPμ (amino acids 774-1452). pS was generated by digesting bluescript TR-PTPμ (Gebbink et al., 1991) with BamHI and XbaI and ligating the fragment into pVL1392 (Invitrogen Corp., San Diego, CA). The full-length form of PTPμ was cloned out of bluescript containing the full-length cDNA of PTPμ using the NotI and XbaI sites and ligated into pVL1392 (PTPμ cDNA was provided by M. Gebbink and W. Moolenaar, Netherlands Cancer Institute). A chimeric form of PTPμ, pEGFR/PTPμ (provided by R. Beijersbergen, Netherlands Cancer Institute) was generated that contained the extracellular and transmembrane domains of the EGF receptor (673 amino acids; base pair 170-2190) and the intracellular domain of PTPμ (678 amino acids; base pair 2320-4865). These three viruses were generated by calcium phosphate mediated cotransfection of Sf9 cells with plasmid and viral DNA. Recombinant viruses were isolated as described in Summers and Smith (1987). A mutant form of PTPμ was generated that contained the extracellular domain, the transmembrane domain and 54 amino acids of the intracellular domain (Ac-PTPμ-extra). This mutant form lacks both phosphatase domains and was called PTPμ-extra. This form was cloned into the pVL1393 plasmid (Invitrogen Corp.) using a Not I site from the full length cDNA in bluescript and the Bgl II site of the PTPμ intracellular domain. The recombinant virus was made using the BaculoGold Transfection System (Invitrogen Corp.). A 2.2-kb fragment encoding the 79 kD extracellular segment of PTPμ (amino acids 21-740) was generated using oligonucleotides and PCR and ligated into the pGEX-KG vector (provided by K. Guan and J. Dixon, University of Michigan, Ann Arbor, MI) to produce a GST fusion protein. This construct was designated GST/PTPμ. The BIRK baculovirus has been described (Villalba et al., 1989). The tropomyosin baculovirus was provided by M. Pittenger and D. Helfman (Cold Spring Harbor Laboratory).

Antibodies

For isolation of the soluble (80 kD) form of PTPμ, Sf9 cells were infected with Ac-pS recombinant baculovirus for 4 d. Soluble PTPμ was purified from the cytoplasmic fraction of Sf9 cell lysates by sequential application to fast flow Q Sepharose, Mono Q, and Mono S as described (Brady-Kalnay and Tonks, 1993). The purified protein was used to generate six mAbs. Each of the mAbs was characterized in terms of binding to PTPμ; none recognized any proteins in extracts from uninfected Sf9 cells (manuscript in preparation). All of the monoclonals recognized the full-length and soluble PTPμ proteins from baculovirus-infected Sf9 cells and COS cells transiently transfected with PTPμ. One of the mAbs (SBK-15) was used for coupling PTPμ to Covaspheres and another antibody (SBK-10) was used in immunoblotting. A rabbit polyclonal antipeptide antibody to the amino terminus of PTPμ (residues 42-57, provided by M. Gebbink) was used for detection of PTPμ in immunoblots, coating of the MvLu cells and Fab' production. Fab' fragments of this antibody were generated according to Brackenbury et al. (1977). Monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology Inc. (catalog no. 05-321; Lake Placid, NY) and used at 1:1,000 dilution.

Electrophoresis and Immunoblotting

Sf9 cells were infected with Ac-pS, Ac-pFL, Ac-pEGFR/PTPμ, or Ac-PTPμ-extra recombinant baculoviruses to express the various forms of PTPμ. For analysis of expression of the various proteins, cells were harvested 42 h post-infection by centrifugation for 5 min at 3,000 *g*, and lysed in 1 ml of buffer (20 mM Tris pH 7.6, 5 mM EDTA, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine, 1 mM PMSF, 200 μM phenyl arsine oxide, 1 mM vanadate and 0.1 mM molybdate). After incubation on ice for 30 min, the lysate was centrifuged at 10,000 *g* for 10 min and the supernatant was used for immunoblotting. The amount of protein was determined by the Bradford method using BSA as a standard (Bradford, 1976). 4× sample buffer was added to the lysates and they were separated by electrophoresis on 8% SDS polyacrylamide gels (Laemmli, 1970). Proteins were transferred to nitrocellulose (Towbin, 1979), blocked in 5% nonfat dry milk in TTBS, incubated with mAb (SBK-10) or polyclonal amino-terminal anti-peptide antibody against PTPμ. A secondary antibody conjugated to HRP was added and the bound antibody was visualized by enhanced chemiluminescence (ECL, Amersham Corp., Arlington Heights, IL).

Cell surface expression of the various forms of PTPμ was verified by examining their protease sensitivity on intact cells. Sf9 cells were removed from a T25 flask in 2 mls Grace's medium in the presence or absence of 0.05% trypsin (wt/vol) (Worthington Biochemical Corp., Freehold, NJ) and incubated for 15 min at room temperature. The cells were centrifuged, extracted and immunoblotted as described above except that lima bean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ) was added to the lysis buffer (60 μg/ml).

Phosphotyrosine levels were compared by reactivity to an anti-phosphotyrosine antibody on immunoblots. Co-infections were performed by simultaneously adding both viruses to Sf9 cells and incubating for 42 h. The immunoblots were performed as described above.

Cell Aggregation Assays

Sf9 adhesion assays were similar to those performed in Snow et al. (1989).

The quantitation of the extent of aggregation followed the procedure of Brackenbury et al. (1977). Sf9 cells were harvested 2 or 3 d post-infection. 1×10^6 cells were added to 2 mls Grace's medium containing 150 μg/ml DNase I in a glass scintillation vial (Fisher Scientific, Pittsburgh, PA) and incubated at 25°C at 90–100 rpm in a gyratory shaker. Aliquots were diluted 50-fold and the number of particles was determined using a Coulter Counter. The Coulter Counter settings were: lower threshold–5.0, upper threshold–99.9, current–500 mA, full scale–1, polarity–auto, attenuation–16, and preset gain–1. The percent aggregation was calculated by subtracting the particle number after the 30 min or 1 h time point (N_t) from the initial particle number (N_o) and dividing by the initial number $\{[(N_o - N_t)/N_o] \times 100\}$. Aggregates were visualized with a phase contrast microscope using the 10× objective.

Fluorescent labeling of cells using Di I was performed according to the protocol supplied by Molecular Probes Inc. (Eugene, Oregon). Sf9 cells were incubated with 20 μM Di I for 15 min at room temperature, then washed two times in Grace's medium. Di I labeled, uninfected Sf9 cells were mixed with an equal number of cells expressing full-length PTPμ and aggregation assays were performed as described above. Aliquots were spotted onto microscope slides and Di I labeled cells were observed with a Zeiss Axiophot microscope equipped for epifluorescence using a 20× lens.

In a subset of assays, a chelating resin, iminodiacetic acid cross-linked to polystyrene (Sigma Chemical Co., St. Louis, MO), was used to remove divalent cations from the insect cell medium. One ml swollen resin was used to deplete 3 mls media by mixing for 1 h at room temperature. The effect of this procedure was assessed in aggregation assays as described above.

Preparation of Covaspheres

MX Covaspheres (0.9 μm) were purchased from Duke Scientific (Palo Alto, CA). After brief sonication 100 μl of Covaspheres were added to 100 μg of mAb-SBK15. The protein and the Covaspheres were incubated for 75 min at room temperature then centrifuged at 10,000 *g* for 2 min. Any unbound sites were blocked by incubation for 15 min in 20 mM Tris pH 7.3 containing 1% BSA. The beads were washed with PBS and briefly sonicated before use in immunoprecipitation of full length PTPμ or the EGFR/PTPμ chimera. A triton soluble lysate from full length PTPμ or EGFR/PTPμ chimera-expressing Sf9 cells was added to the SBK15-Covaspheres, incubated at 4°C for 1 h, washed with lysis buffer three times, resuspended in PBS and used in binding assays. Successful immunoprecipitation by the antibody-linked Covaspheres was verified by depletion of the PTPμ reactivity upon immunoblotting. This antibody has been used to purify full-length PTPμ from Sf9 cells (Brady-Kalnay and Tonks, 1993).

The in vitro binding assays also utilized a purified fusion protein, comprising glutathione S-transferase linked to the NH_2-terminus of the extracellular segment of PTPμ (GST/PTPμ). The GST/PTPμ fusion protein was expressed in *E. coli* and purified according to Guan and Dixon (1991). The GST protein alone (27 kD) was used as a negative control. These GST proteins were coupled directly to Covaspheres by adding 3.9 μg protein per μl of Covaspheres as described above.

Covasphere Binding Assays

The binding assay was performed as described in Mauro et al. (1992). Briefly, 10 μg of purified GST proteins were adsorbed to 35-mm petri dishes for 30 min, then remaining unbound sites were blocked with 2% BSA in PBS. PTPμ-linked Covaspheres (50 μl) were added to the dishes in a final volume of 1 ml PBS. Bound Covaspheres were visualized with a Zeiss Axiophot microscope equipped for epifluorescence using a 20× lens.

The ability of GST/PTPμ-linked Covaspheres to self-aggregate was assessed by adding 30 μl of the Covasphere preparation to 2 ml of PBS followed by rotation for 30 min under low shear conditions. Controls utilizing Covaspheres coated with GST alone were performed in parallel. Aggregates were visualized with a Zeiss Axiophot microscope as above.

Various protein-coated Covaspheres described above were also used in assays to assess binding to the surface of MvLu mink lung cells or 3T3 cells. Covaspheres (50 μl) were added in DME plus 10% FBS to confluent 35-mm plates of MvLu cells, then incubated with the cells for 30 min at room temperature with rocking. The plates were washed 3 times with DME and visualized as described above. All antibody competition experiments were performed using the anti-peptide antibody to the amino-terminus of the extracellular segment of PTPμ. In experiments in which binding was blocked with Fab' fragments the Covaspheres were preincubated for 30 min at room temperature in 0.6 mg pre-immune or immune Fab' per 50 μl Covaspheres in a final volume of 500 μl. The Fab'/Covasphere solution was added to the cells and incubated for 30 min. Alternatively, the MvLu cells were incubated for 30 min with pre-immune or immune serum to PTPμ, diluted 1:500 in culture media, before the addition of 50 μl of full-length PTPμ-linked Covaspheres.

Results

PTPμ Is Expressed at the Cell Surface

Human PTPμ was expressed in Sf9 insect cells by infection with recombinant baculoviruses. Three forms of human PTPμ were expressed: (*a*) a full-length form (200 kD); (*b*) a soluble form, comprising only the intracellular segment

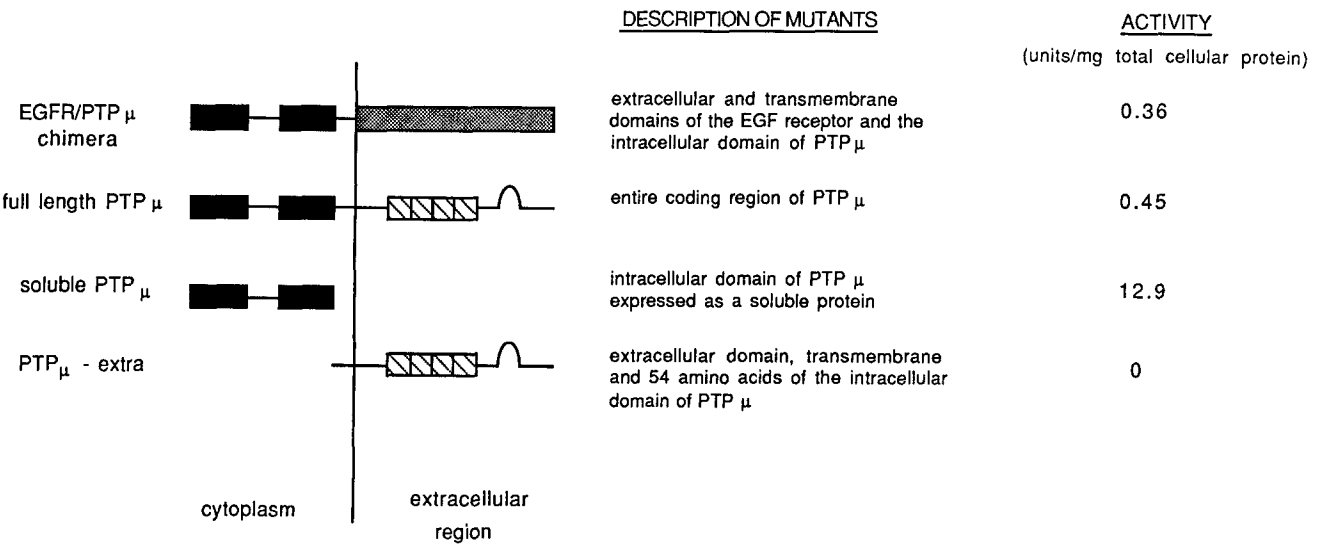

Figure 1. Structure and PTPase activity of the various baculovirus-expressed recombinant forms of PTPμ. The solid black boxes represent PTPase domains. The hatched boxes are fibronectin type-III repeats and the loop represents the immunoglobulin-like domain. The stippled box represents the extracellular domain of the EGF receptor. The PTPase activity is expressed as units per mg of total cellular protein above the PTPase activity of lysates from uninfected Sf9 cells which was 0.09 U/mg.

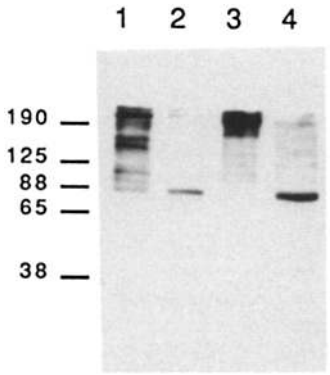

Figure 2. Expression of PTPμ and the EGFR/PTPμ chimera at the cell surface. This figure is an immunoblot of whole cell lysates using mAb SBK-10 generated against the intracellular segment of PTPμ. Lane *1* is from cells expressing full-length PTPμ, Lane *2* is from PTPμ-expressing cells that were subjected to limited trypsin treatment. Extracts from EGFR/PTPμ chimera-infected cells without or with trypsin treatment are shown in lanes *3* and *4*, respectively. Each lane contains 5 μg total protein.

containing the PTPase domains (80 kD); and (*c*) a chimera containing the extracellular and transmembrane domains of the EGF receptor and the intracellular PTPase domains of PTPμ (195 kD) (see Fig. 1). The activity of these PTPases in lysates of infected cells was measured using tyrosine-phosphorylated RCM lysozyme as a substrate (Fig. 1). The uninfected Sf9 cells displayed low levels of endogenous PTPase activity (0.09 U/mg total protein). The soluble form of PTPμ was expressed at much higher levels (~140-fold over background) than either of the transmembrane forms (~fivefold over the endogenous activity) (see Fig. 1). The expression of the various forms of PTPμ was also assessed by immunoblotting with antibodies to its intracellular segment. Fig. 2 shows that the full-length form of PTPμ (lane *1*) and the EGFR/PTPμ chimera (lane *3*) are expressed at similar levels. Immunoblotting of Sf9 cell lysates expressing the

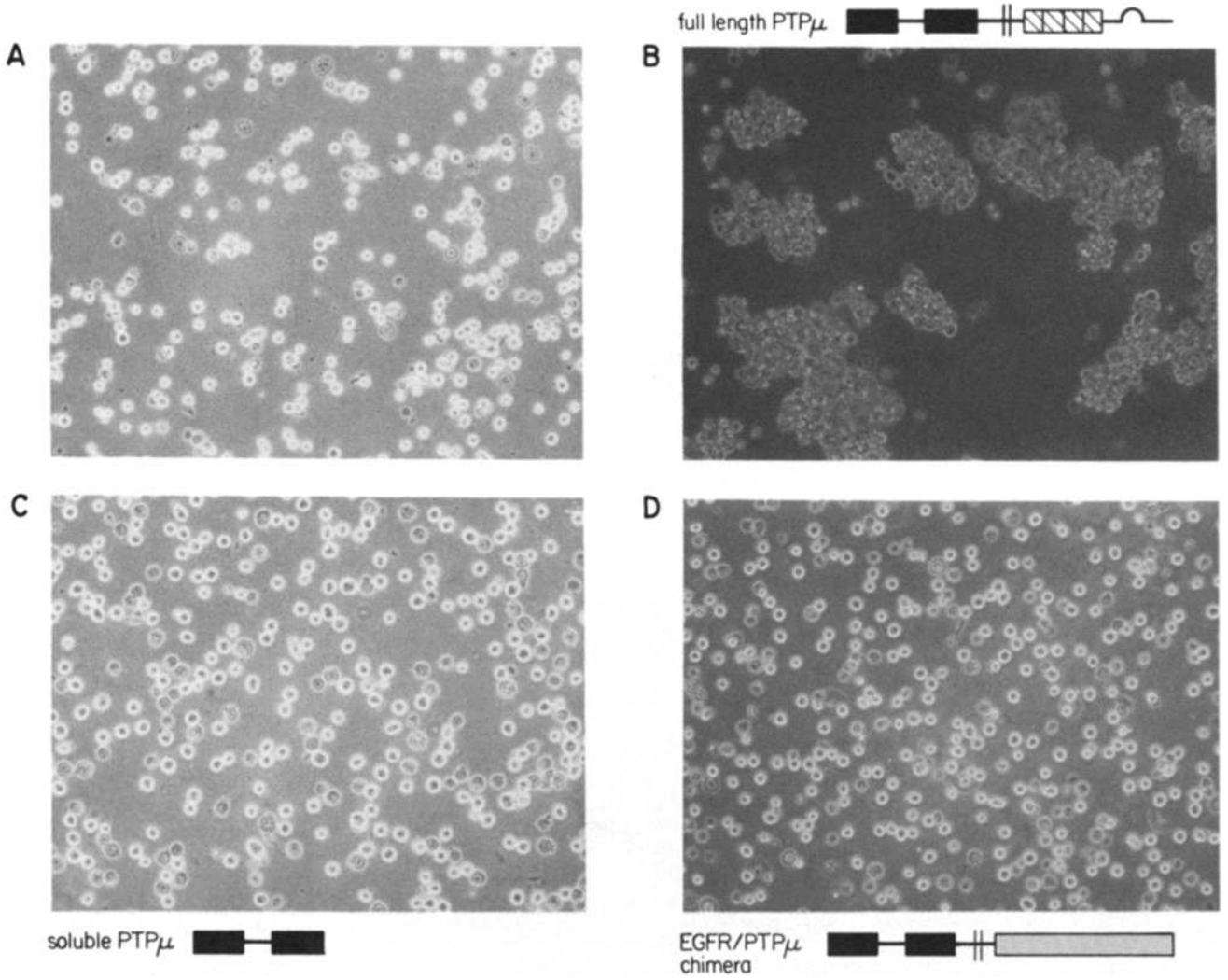

Figure 3. Aggregation of baculovirus-infected Sf9 cells. This figure shows phase contrast micrographs of uninfected or infected Sf9 cells allowed to aggregate under low shear conditions for 1 h. *a* shows uninfected cells which did not aggregate while the full-length PTPμ infected cells (*b*), formed large aggregates. Cells infected with recombinant baculoviruses expressing soluble PTPμ (*c*) or EGFR/PTPμ chimera (*d*) did not aggregate.

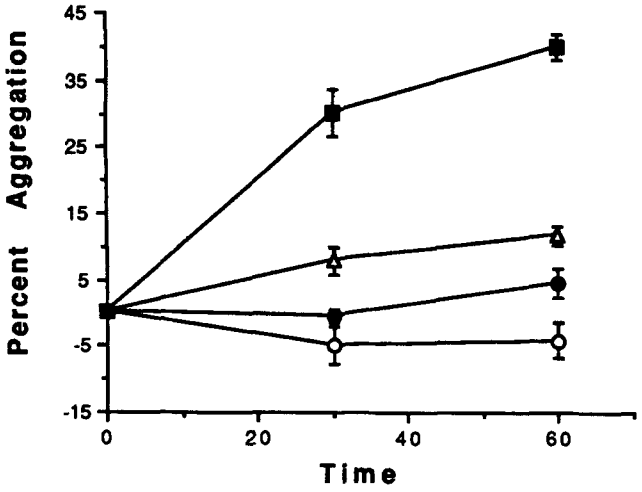

Figure 4. Quantitation of aggregation. A Coulter counter is used to measure a decrease in particle number as single cells are incorporated into aggregates (*percent aggregation*). Full-length PTPμ-infected cells are represented by the solid squares. Soluble PTPμ (*solid circles*) or EGFR/PTPμ chimera (*open circles*)-expressing cells showed even lower levels of aggregation than the uninfected cells (*open triangles*). Error bars indicate SEM from at least five determinations.

soluble form of PTPμ confirmed that it is present at much higher levels than the transmembrane forms.

To verify that PTPμ and the EGFR/PTPμ chimera were expressed at the cell surface, trypsin sensitivity assays were performed (Brady-Kalnay et al., 1993). This assay involves incubation of intact cells with a 0.05% (wt/vol) trypsin solution which allows the protease to act only on the portion of molecules expressed at the cell surface. Full-length forms are converted into smaller fragments still associated with the cell, i.e., possessing their transmembrane and cytoplasmic segments. The viability and integrity of the trypsinized cells was confirmed by trypan blue dye exclusion. Lysates of treated cells were immunoblotted with monoclonal antibody SBK-10 directed against the carboxy-terminal domain of PTPμ, which thus reacts with both full-length PTPμ and the EGFR/PTPμ chimera. Fig. 2 shows ~90% of full-length PTPμ (lane *2*) and the EGFR/PTPμ chimera (lane *4*) were degraded after trypsinization, thus suggesting that these proteins are expressed predominantly on the cell surface.

Full Length PTPμ Expressed in Sf9 Cells Mediates Aggregation

We have used the nonadhesive Sf9 insect cells and the baculovirus expression system to demonstrate that the protein tyrosine phosphatase PTPμ functions in cell–cell aggregation. The infected cells were tested in aggregation assays which involve the rotation of cells under low shear conditions (Brackenbury et al., 1977). Samples are counted at various time points to measure the incorporation of single cells into aggregates.

Sf9 cells were infected with recombinant baculoviruses, harvested and assayed for aggregation. The results of a visual inspection of the cells is illustrated in the phase contrast micrographs in Fig. 3. While uninfected cells do not aggregate (*a*) expression of the full-length form of PTPμ, shown in *b*, induces the formation of large aggregates. Cells expressing the soluble PTPμ (*c*) or the EGFR/PTPμ chimera (*d*) also did not aggregate suggesting that increased phosphatase activity alone was not sufficient for this response. Neither overexpression of a nonspecific transmembrane protein nor the localization of PTPase activity at the membrane was sufficient to induce aggregation.

Quantitative analysis of the aggregation of Sf9 cells is displayed in Fig. 4. The quantitation, measured using a Coulter Counter, is expressed as percent aggregation which is a function of decrease in particle number as single cells are incorporated into multicellular aggregates. Uninfected cells display low levels of aggregation (mean = 11.7%) whereas full-length PTPμ-expressing cells showed high levels of aggregation (mean = 39.8%). Cells expressing soluble PTPμ (mean = 4.6%) or the EGFR/PTPμ chimera (mean = −4.1%) showed low levels of aggregation. The negative number indicates that the final cell count was higher than the initial cell count due to the dissociation of cells during the aggregation assay. Control infected cells showed lower levels of aggregation than uninfected Sf9 cells.

Adhesion molecules are classified by their dependence on divalent cations, primarily calcium (Takeichi, 1977). The aggregation assay described above was routinely performed in Grace's insect cell medium which contains 7 mM calcium chloride. To test whether divalent cations were important for aggregation, we used a chelating resin to remove them from the medium prior to performing the assay. The full-length PTPμ-expressing cells aggregated equally well with or without divalent cations (data not shown) suggesting that, like

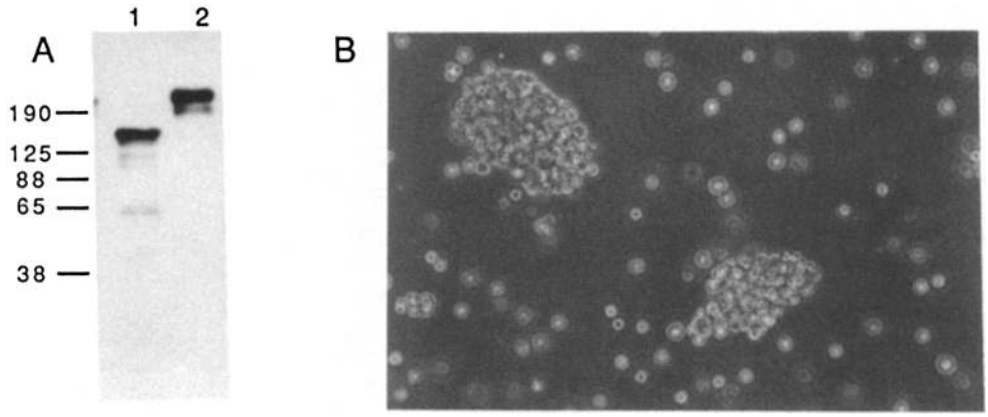

Figure 5. Aggregation of PTPμ-extra expressing cells. In *A*, the immunoblot shows that PTPμ-extra (lane *1*) and full length PTPμ (lane *2*) are expressed at similar levels. The anti-peptide antibody used in this immunoblot recognizes a sequence (residues 42–57) at the amino-terminus of PTPμ. Each lane contains 5 μg protein. *B* is a phase contrast micrograph of PTPμ-extra expressing cells after a 1 h aggregation assay.

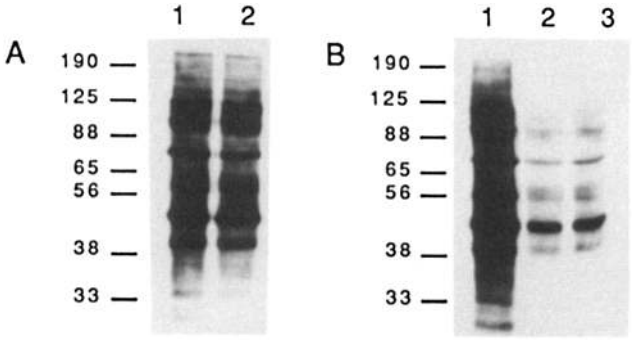

Figure 6. Analysis of phosphotyrosine levels of infected Sf9 cells. The BIRK (tyrosine kinase)-infected (*a*, lane *1*, and *b*, lane *1*) or BIRK-tropomyosin co-infected (*a*, lane *2*) cells showed high levels of anti-phosphotyrosine immunoreactivity. Co-infection of BIRK and full length PTPμ produced a reduction in signal that was unchanged before (*b*, lane *2*) and after aggregation of the cells (*b*, lane *3*). The same amount of recombinant PTPμ baculovirus was added in single or co-infection experiments. Each lane contains 30 μg protein.

other members of the Ig-superfamily of adhesion molecules, it functions in a divalent cation-independent manner.

Phosphatase Activity Is not Required for Aggregation

To test whether phosphatase activity is required for the adhesive function, we generated a mutant form of PTPμ containing the entire extracellular domain, transmembrane domain and 54 amino acids of the intracellular domain, which we termed PTPμ-extra. This protein lacks both phosphatase domains. As expected, in lysates of cells expressing PTPμ-extra, no PTPase actvity above that found in uninfected cells was detected. An immunoblot using an antipeptide antibody directed against the NH_2-terminal extracellular segment of PTPμ (Fig. 5 *a*) illustrates that PTPμ-extra (lane *1*) is expressed at similar levels to the full-length protein (lane *2*). The ability of PTPμ-extra expressing cells to aggregate is shown in Fig. 5 *b*. Quantitative analysis of the assays indicated a percent aggregation of the PTPμ-extra infected cells of 30.3% $\pm$ 4.3 (mean $\pm$ SEM, $n = 8$), compared to 38.2% $\pm$ 1.9 (mean $\pm$ SEM, $n = 6$) for full-length PTPμ-infected cells. Thus, the adhesive function of PTPμ and its capacity to dephosphorylate proteins are not mutually dependent.

A common feature of receptor-like molecules is that the enzymatic activity of their intracellular domains is regulated by binding of ligands to their extracellular segments. We thus sought to examine whether aggregation might affect the activity of PTPμ. To this end, the levels of total cellular phosphotyrosine were analyzed. Sf9 cells contain very low levels of phosphotyrosine-containing proteins. To increase the amount of cellular phosphotyrosine and the variety of potential substrates, we infected the cells with a recombinant baculovirus expressing BIRK, a soluble protein comprising the catalytic domain of the mammalian insulin receptor tyrosine kinase (Villalba et al., 1989). There are many examples of direct protein:protein and enzyme:substrate interactions that have been investigated by co-infection of Sf9 cells with distinct recombinant baculoviruses (Kaplan et al., 1990; Parker et al., 1991; Kato et al., 1993). In light of these reports, we co-infected two batches of Sf9 cells. One batch was co-infected with baculoviruses expressing BIRK and a nonphosphatase control (tropomyosin) and the other batch with BIRK and full-length PTPμ. Cell lysates were then analyzed for changes in phosphotyrosine levels. Fig. 6 is an immunoblot, using anti-phosphotyrosine antibodies, of extracts from cells infected with BIRK (Fig. 6 *a*, lane *1*) or co-infected with BIRK and tropomyosin (*a*, lane *2*). The co-infection with the non-phosphatase control did not alter the BIRK-induced increase in phosphotyrosine levels. The presence of tropomyosin protein was confirmed by immunoblotting (data not shown). Fig. 6 *b* shows that while high levels of phosphotyrosine containing proteins were seen in BIRK-infected cells (lane *1*) co-infection with BIRK and full-length PTPμ (lane *2*) resulted in a dramatic decrease in phosphotyrosine levels, presumably due to high basal activity of the PTPase. Immunoblotting verified that full-length PTPμ was expressed at similar levels in the single or co-infected Sf9 cell lysates. The overall levels and pattern of anti-phosphotyrosine immunoreactive proteins were unchanged by aggregation. BIRK/full-length PTPμ expressing cells before (lane *2*) and after (lane *3*) aggregation are shown in Fig. 6 *b*. Co-infection of BIRK and the soluble PTPμ, which is expressed at ~25-fold higher levels than the transmembrane forms, as determined by activity assays, contained even lower levels of anti-phosphotyrosine reactivity, suggesting that the immunoreactive bands were indeed tyrosine phosphorylated proteins. In addition to this in vivo assay, total cell lysates of full-length PTPμ-infected cells were assayed for PTPase activity with RCM lysozyme as a substrate, before and after aggregation. The activity was essentially unchanged after aggregation.

PTPμ Mediates Homophilic Aggregation

We have tested whether the aggregation of Sf9 cells expressing PTPμ is mediated via a homophilic mechanism in which molecules of PTPμ on different cells interact with one another or by a heterophilic mechanism in which the insect cells express a molecule that binds to human PTPμ. Uninfected nonadhesive Sf9 cells were labeled with the fluorescent lipophilic dye, Di I (Snow et al., 1989) and mixed with unlabeled full-length PTPμ-infected cells. After aggregation the cells were visualized using an epifluorescence microscope. The Di I labeled cells did not aggregate and did not contribute to the aggregates formed by the full-length PTPμ-infected cells. Fig. 7 is a representative example of 32 aggregates examined in three independent experiments; no Di I-positive cells were seen in any of the aggregates. These results strongly suggest that PTPμ binds in a homophilic fashion.

Reconstitution of PTPμ-mediated Homophilic Binding In Vitro

We have examined the ability of baculovirus-expressed PTPμ to bind to surfaces coated with bacterially-expressed extracellular segment of PTPμ in vitro. A mAb to the intracellular domain of PTPμ was covalently linked to Covaspheres (fluorescent beads). These antibody-linked Covaspheres were used to immunoprecipitate either the full length PTPμ or the EGFR/PTPμ chimera from lysates of infected cells. The entire extracellular domain of PTPμ was expressed as a glutathione S-transferase (GST) fusion protein

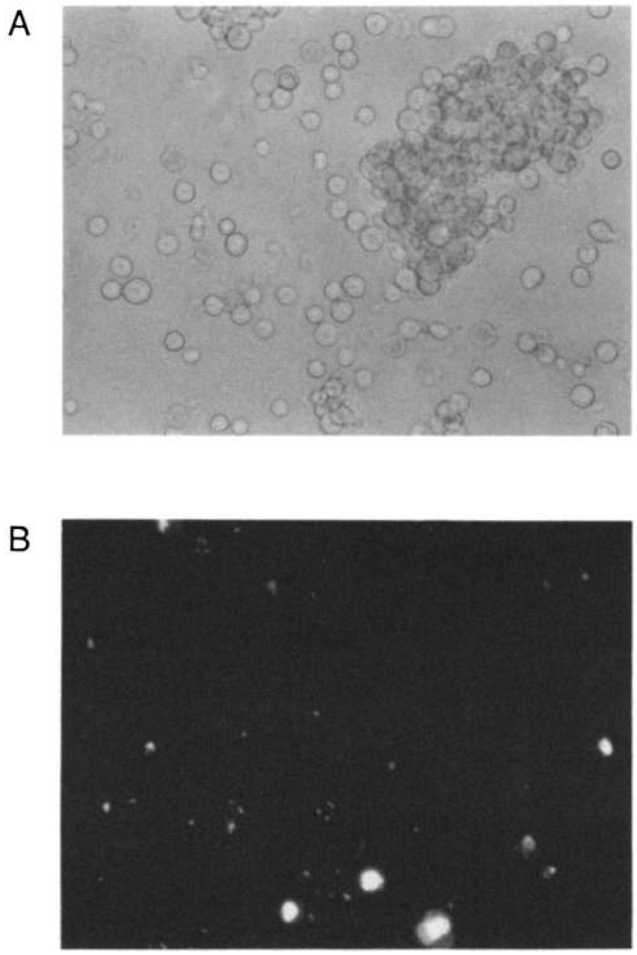

Figure 7. Aggregation of Sf9 cells expressing PTPμ occurs through homophilic binding. *a* is a representative phase contrast micrograph of an aggregation experiment in which Di I labeled uninfected Sf9 cells were mixed with full-length PTPμ-expressing cells before aggregation. *b* shows the same field as *a* photographed using an epifluorescence microscope to visualize the Di I. The Di I labeled uninfected cells did not contribute to the large aggregate. This is a representative picture of 32 such aggregates examined in three independent experiments.

and purified from *E. coli* using a glutathione Sepharose affinity column. This purified protein was adsorbed onto petri dishes and the binding of PTPμ or EGFR/PTPμ chimera-coated Covaspheres was visualized using an epifluorescence microscope after washing the dishes to remove nonadherent Covaspheres. There was no significant binding of the full-length PTPμ Covaspheres to GST alone [29 $\pm$ 0.6 Covaspheres per field (mean $\pm$ SEM: $n = 6$) shown in Fig. 8 *a*]. Similarly, Fig. 8 *b* shows that there was no significant binding of EGFR/PTPμ chimera-coated Covaspheres to the GST/PTPμ extracellular segment (28 $\pm$ 3.9 Covaspheres per field: $n = 8$). However, Fig. 8 *c* demonstrates that the full-length PTPμ-coated Covaspheres bound specifically to the GST/PTPμ extracellular segment fusion protein (764 $\pm$ 41 Covaspheres per field: $n = 5$). These data establish that the homophilic binding in vitro, which was performed in PBS, was independent of any added divalent cations. Also because the GST/PTPμ protein was isolated from bacteria, glycosylation of the extracellular domain is unlikely to contribute to the binding reaction. Similar observations were made for Nr-CAM (Mauro et al., 1992).

Baculovirus Expressed PTPμ Binds by a Homophilic Mechanism to PTPμ that Is Normally Expressed in MvLu Cells

We have examined the ability of PTPμ to bind homophilically when expressed at physiological levels. At this time, the only cell line we have found that expresses PTPμ is MvLu mink lung cells. This cell line expressed low but detectable levels of the enzyme as determined by immunoblotting with intracellular segment-directed monoclonal anti-PTPμ antibodies (data not shown). The ability of PTPμ-coated Covaspheres to bind to these cells was tested. As shown in Fig. 9, *a* and *b*, only low levels of the EGFR/PTPμ chimera-coated Covaspheres bound nonspecifically to the cells while high levels of binding of PTPμ-coated Covaspheres were observed (*c* and *d*). This binding was inhibited by pre-incubating the MvLu cells with antibodies to the extracellular domain of PTPμ (*e* and *f*). Pre-immune IgG did not affect the binding of PTPμ-coated Covaspheres (Table I). This "sided" experiment selectively blocks PTPμ on the MvLu cells, thus illustrating that binding occurs through a homophilic interaction. In a separate experiment, Fab' fragments of antibodies to PTPμ were pre-incubated with the PTPμ-coated Covaspheres, which also prevented binding. Incubation with Fab' fragments derived from pre-immune serum did not affect the binding. A quantitative summary of the results of Covasphere binding are presented in Table I. These data demonstrate that purified PTPμ was capable of binding homophilically to PTPμ expressed on the surface of MvLu cells.

GST/PTPμ-coated Covaspheres Aggregate In Vitro and Bind to MvLu Cells

In light of the preceding data, one might anticipate that Covaspheres coated with PTPμ would self-aggregate. To test this possibility the GST/PTPμ fusion protein was covalently coupled directly to Covaspheres to ensure a high protein:bead ratio. The Covaspheres were then rotated under low shear conditions to allow any aggregation to occur. As shown in Fig. 10, the Covaspheres coated with GST alone did not aggregate (*a*) whereas the GST/PTPμ-coated Covaspheres formed aggregates (*b*). These Covaspheres were also examined for their ability to bind to the surface of MvLu cells (Table II). While there was no significant binding of control GST Covaspheres, GST/PTPμ Covaspheres bound at high levels to MvLu cells. Furthermore, the GST/PTPμ Covaspheres did not bind to 3T3 cells which do not express detectable amounts of immunoreactive PTPμ.

Discussion

The cloning of receptor-type PTPases has led to the convergence of the fields of cell adhesion and protein tyrosine dephosphorylation. The structural similarity of some of the receptor-type PTPases to the immunoglobulin superfamily of cell adhesion molecules suggests that these phosphatases may participate in homophilic binding interactions, i.e., the "ligand" for one of these PTPases may be a molecule of the same enzyme expressed on an adjacent cell.

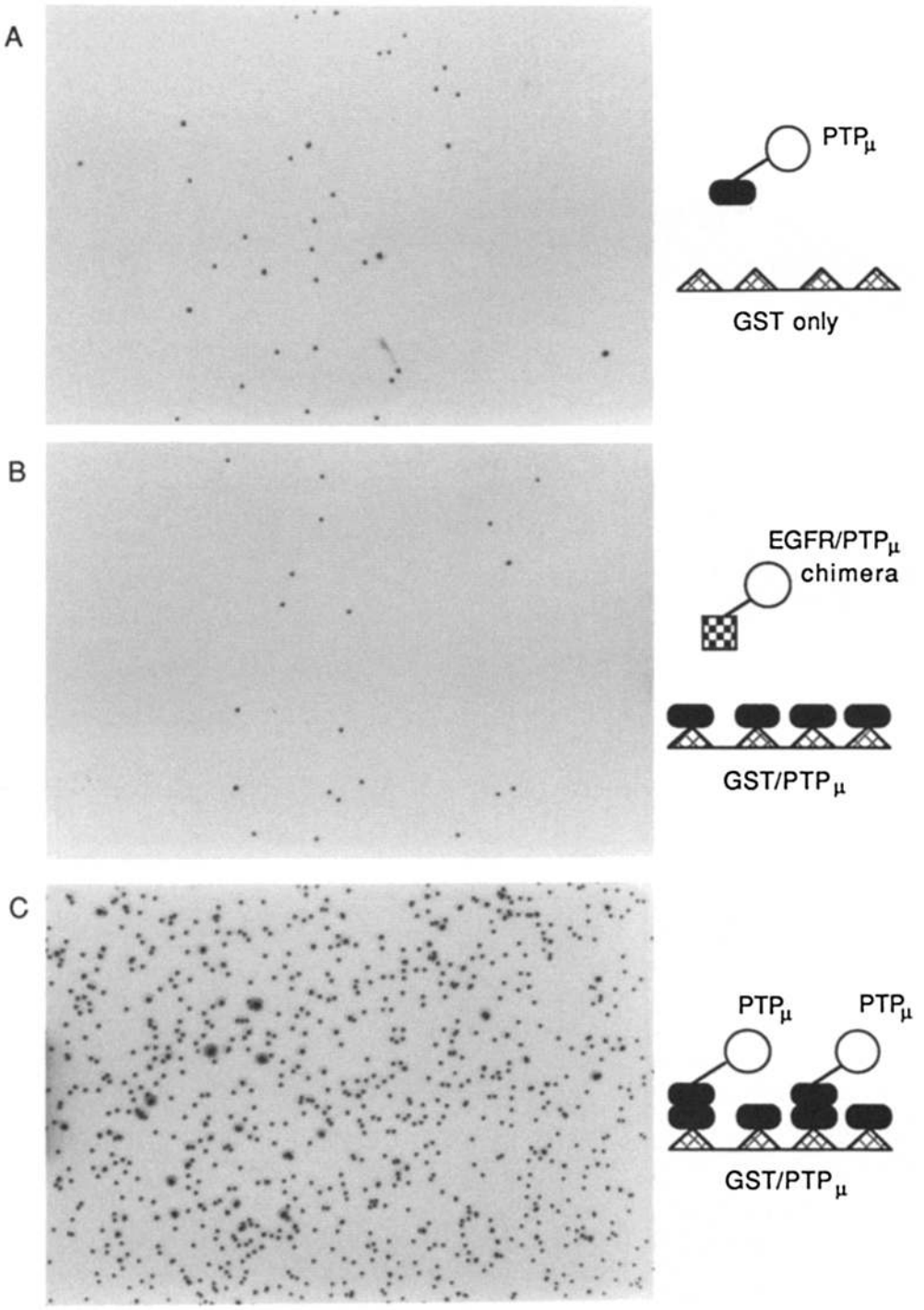

Figure 8. Binding of baculovirus-expressed PTPμ-linked Covaspheres to surfaces coated with purified fusion protein containing the extracellular segment of PTPμ (*GST/PTPμ*). *a* shows that there were only low levels of nonspecific binding of full-length PTPμ-coated Covaspheres to petri dishes coated with GST protein alone. Similarly, only low levels of nonspecific binding were seen when the EGFR/PTPμ chimera-coated Covaspheres were added to GST/PTPμ fusion protein-coated dishes (*b*). However, numerous full length PTPμ-coated Covaspheres bound to the GST/PTPμ fusion protein-coated dishes (*c*).

In this manuscript we report experiments which demonstrate homophilic binding between molecules of PTPμ by several criteria: (*a*) Expression of full-length PTPμ, or mutant forms with an intact extracellular segment, in the presence or absence of the PTPase domains, caused Sf9 cells to aggregate. Neither a chimeric phosphatase bearing the extracellular segment of the EGF-receptor and the intracellular segment of PTPμ nor the PTPase domains of PTPμ expressed as a soluble protein induced aggregation. When uninfected, fluorescently labeled Sf9 cells were mixed with unlabeled PTPμ-expressing cells, the labeled cells were excluded from the aggregates. Furthermore, no immunoreactive species were detected after immunoblot analysis of lysates of uninfected Sf9 cells using a variety of mAbs to PTPμ. These data indicate that the counter-receptor for PTPμ was only found on the surfaces of other PTPμ-expressing cells. (*b*) The binding reaction has been reconstituted in vitro. First, fluorescent Covaspheres linked to baculovirus expressed PTPμ will bind to petri dishes coated with a GST fusion protein (GST/PTPμ) containing the ex-

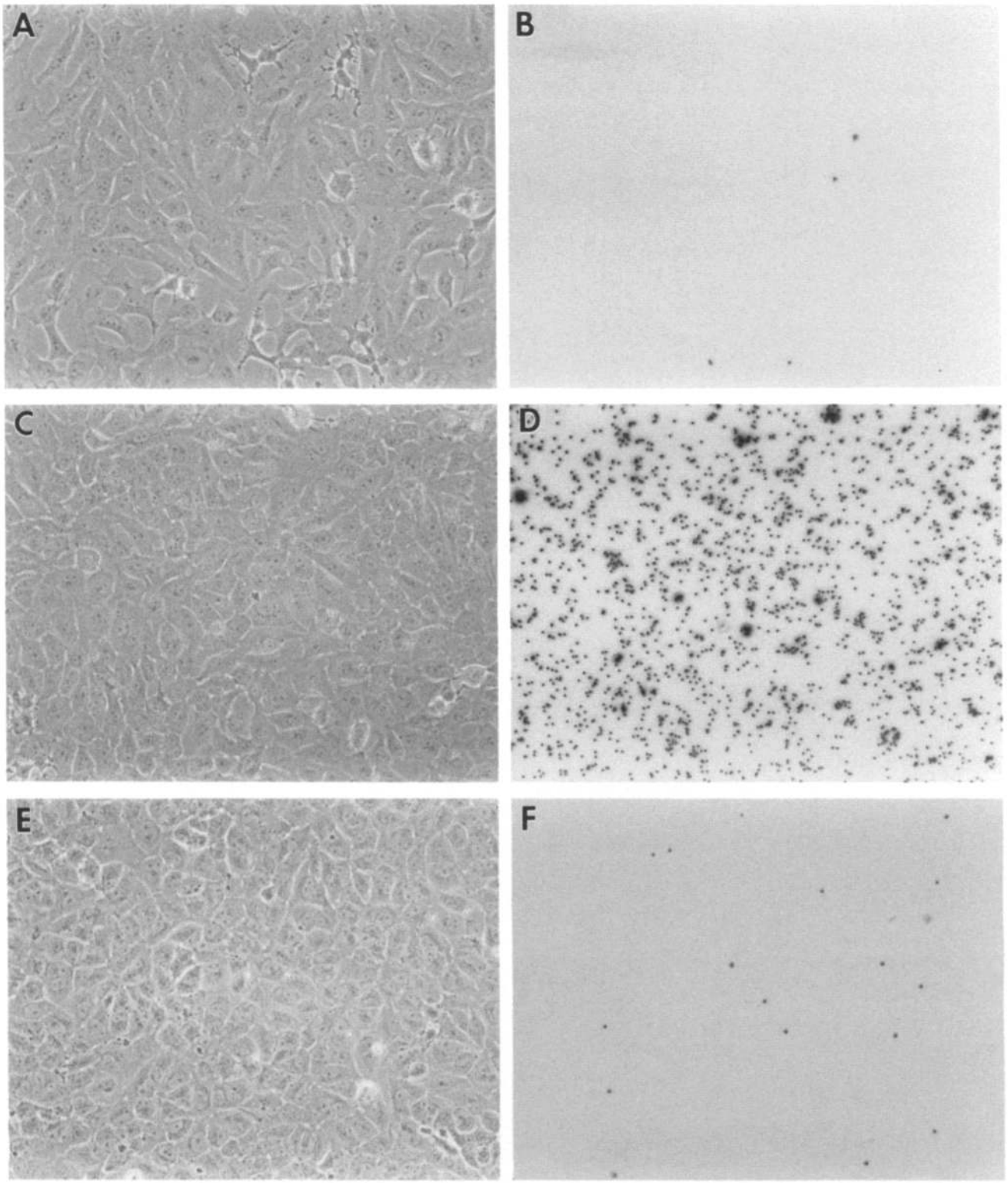

Figure 9. Homophilic binding of baculovirus-expressed PTPμ-coated Covaspheres to endogenous PTPμ in MvLu cells. Phase contrast micrographs of MvLu cells (*a*, *c*, and *e*) and micrographs of fluorescent Covasphere binding (*b*, *d*, and *f*) are shown. *a* and *b* show the low level of nonspecific binding when EGFR/PTPμ chimera-coated Covaspheres are applied to the cells. *c* and *d*, show full-length PTPμ-coated Covaspheres bound to the cells. In *e* and *f*, the MvLu cells were preincubated with an anti-peptide antibody to the amino-terminus of PTPμ before addition of the PTPμ-bound Covaspheres.

Table I. Quantitation of the Binding of PTPμ-coated Covaspheres to MvLu Cells

	Number bound/field*
PTPμ-Covaspheres	539 (± 94)
EGFR/PTPμ chimera-Covaspheres	9 (± 2)
PTPμ-Covaspheres pretreated with preimmune Fab'	475 (± 39)
PTPμ-Covaspheres pretreated with anti-PTPμ Fab'	15 (± 3)
PTPμ-Covaspheres added to MvLu cells pretreated with pre-immune antibody	401 (± 10)
PTPμ-Covaspheres added to MvLu cells pretreated with anti-PTPμ antibody	19 (± 3)

* The number of Covaspheres bound is represented as the mean of at least three fields. The field is 0.09 mm². The number in parentheses indicates SEM. Some of these results are illustrated in Fig. 9.

Table II. Quantitation of Covasphere Binding to Cell Surfaces

	Number bound/field*
GST-Covaspheres added to MvLu cells	12 (± 2)
GST/PTPμ-Covaspheres added to MvLu cells	1,006 (± 78)
GST/PTPμ-Covaspheres added to 3T3 cells	9 (± 2)

* The number of Covaspheres bound is represented as the mean of at least six fields. The field is 0.09 mm² and contained approximately equal numbers of MvLu and 3T3 cells. The number in parentheses indicates SEM.

tracellular segment of PTPμ purified from *E. coli*, but will not bind to GST alone. Furthermore, Covaspheres linked to the EGFR/PTPμ chimera bind neither GST nor GST/PTPμ. Secondly, Covaspheres coated with GST/PTPμ fusion protein self-aggregate, whereas those coated with GST alone do not. (*c*) We show that PTPμ, as it is expressed endogenously on MvLu cells, retains the capacity to interact with baculovirus-expressed PTPμ linked to Covaspheres. While PTPμ-coated Covaspheres bind to MvLu cells, EGFR/PTPμ Covaspheres do not. Binding of PTPμ Covaspheres is blocked by pretreating the cells with antibody to the extracellular segment of PTPμ but not by a control pre-immune antibody. Also if the PTPμ Covaspheres are pretreated with Fab' fragments from the anti-PTPμ antibody, binding to MvLu cells is blocked whereas control Fab's are without effect. Collectively, these data establish homophilic binding between PTPμ molecules in a heterologous expression system, in in vitro binding assays and in cells in which the molecule is normally expressed.

For immunoglobulin superfamily-type adhesion molecules, such as N-CAM, aggregation is highly dependent upon the concentration of the molecule at the cell surface (Hoffman and Edelman, 1983). Therefore, to assess the aggregation potential of PTPμ a model system had to be used that could generate high level expression of the protein at the cell surface. Introduction of potential adhesion molecules into nonadhesive cells has been used to demonstrate directly their adhesive functions. Transfection for determination of an adhesive function was first described by Takeichi and colleagues for E-cadherin (Nagafuchi et al., 1987). Goodman

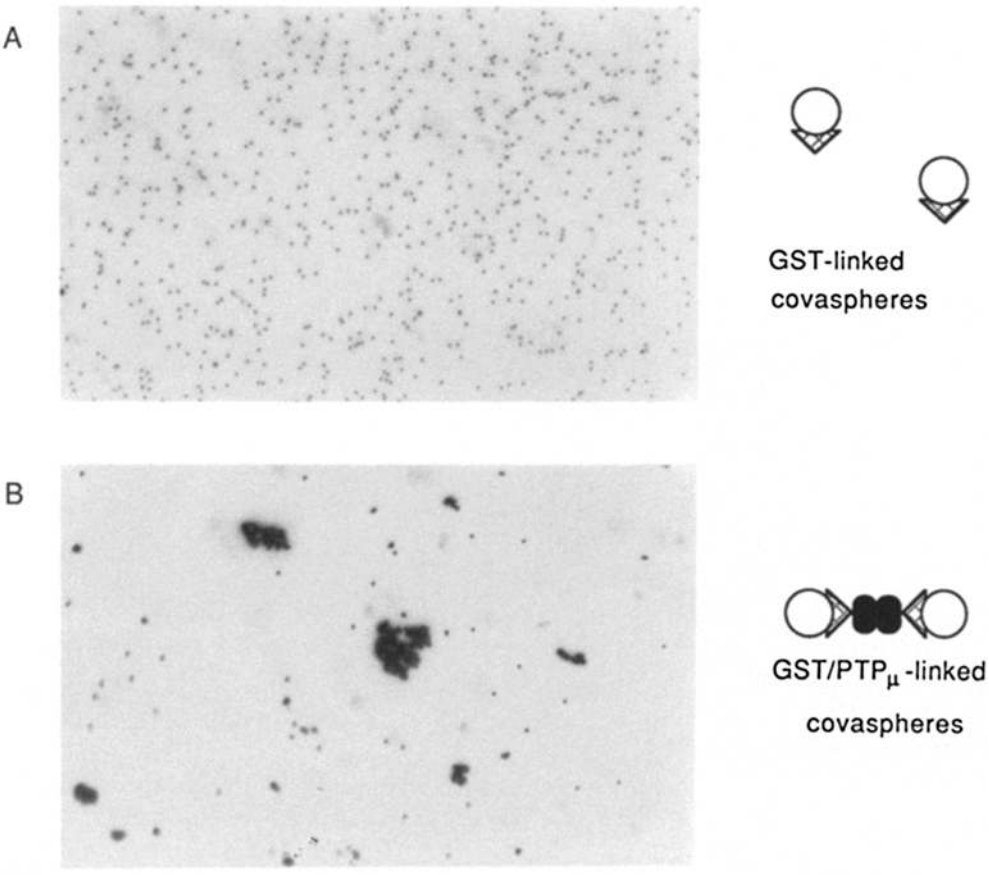

Figure 10. GST/PTPμ-coated Covaspheres aggregate in vitro. Covaspheres coated with GST alone or GST/PTPμ were used in an in vitro aggregation assay and visualized by an epifluorescence microscope. GST Covaspheres did not aggregate (*a*) whereas GST/PTPμ Covaspheres formed aggregates (*b*).

and co-workers introduced fasciclin III and connectin molecules into nonadhesive *Drosophila* S2 cells to demonstrate that these molecules mediated homophilic adhesion (Snow et al., 1989; Nose et al., 1992). Similarly, Pulido et al. (1992) have used *Drosophila* S2 cells to demonstrate that the receptor tyrosine kinase, *Dtrk*, may function as an adhesion molecule. We chose to use Sf9 insect cells and the baculovirus expression system (Matsuura et al., 1987; Summers and Smith, 1987) to express PTPμ. This system has been used previously to express high levels of glycosylated, biologically active haemagglutinin (Kuroda et al., 1986) and tissue plasminogen activator (Jarvis and Summers, 1989). An additional requirement for use in adhesion studies is a baculovirus-susceptible cell line with low levels of endogenous aggregation. Sf9 cells fulfill this condition in that they do not aggregate well in an in vitro assay.

The Sf9 cells infected with full length PTPμ showed high levels of aggregation (39%) compared to uninfected cells (11.9%), and the even lower levels of aggregation of cells infected with soluble PTPμ (4.6%) or the EGFR/PTPμ chimera (−4.1%). The level of aggregation induced by PTPμ was similar to that induced by other known adhesion molecules in various model systems. For example, transfection of mouse L-cells with N-CAM resulted in a difference in aggregation of 30% (Edelman et al., 1987) whereas N-CAM expression increased aggregation of an RSV-transformed neuronal cell line from 20 to 40% (Brady-Kalnay et al., 1993). Expression of Nr-CAM in L-cells increased aggregation to 14% (Mauro et al., 1992) while Nose et al. (1989) detected a 50% difference in aggregation of fasciclin III-transfected versus untransfected S2 cells. Therefore, PTPμ promotes similar levels of aggregation to those detected in response to well established cell–cell adhesion molecules. These data suggest that the Sf9 cell/baculovirus expression system may represent a useful tool for revealing potential adhesive functions for other molecules.

Whether or not PTPμ has the ability to perform this adhesive function in its normal environment remains to be determined. Nevertheless, we have shown that PTPμ expressed endogenously on the surface of MvLu cells retains the capacity for homophilic binding. However, MvLu cells do not aggregate in an aggregation assay. The low levels of the PTPase expressed in these cells may preclude the successful application of this assay. We have observed PTPμ protein to be expressed abundantly in lung, however at present we have not found a cell line that reflects that high level of expression. Thus, the ultimate resolution of the issue of whether, as suggested by the data presented in this manuscript, PTPμ can function as an adhesion molecule in vivo may require the use of primary cell systems.

High local concentrations in a specific area of the membrane could allow PTPμ to bind homophilically and function in an adhesive role even though it may be expressed at low overall levels in a particular cell. One novel feature of PTPμ that may be interesting in this regard is that its intracellular juxtamembrane segment is 70 amino acids longer than the equivalent segment in other receptor PTPases. Using the ALIGN program, we have shown that this portion of PTPμ displays sequence similarity to the intracellular segment of members of the cadherin family (Tonks et al., 1992). This homology is interesting because no other receptor type PTPases have such similarity to the cadherins, nor do any other members of the immunoglobulin superfamily. The intracellular domain of the cadherins is the most highly conserved segment (~90% identity) among members of this family (reviewed by Takeichi, 1991) and interacts indirectly with the actin cytoskeleton (Ozawa et al., 1989). The cadherins are found at areas of cell contact, for example adherens-type cell junctions (Tsukita et al., 1991). Such cell junctions are areas of rapid phosphotyrosine turnover and locations at which tyrosine kinases including *src* and pp125^{FAK} are concentrated in normal and transformed cells (Volberg et al., 1991; Guan and Shalloway, 1992). In fact, changes in tyrosine phosphorylation may be involved in controlling the structural integrity of these junctions (Volberg et al., 1992). In light of this homology to the cadherins it is possible that PTPμ may also associate with the cytoskeleton through an interaction with accessory molecules at points of cytoskeletal-membrane association such as intercellular junctions.

PTPμ displays phosphatase activity after expression in Sf9 cells. To address the possibility that phosphatase activity was a requirement for cell–cell aggregation, we made a mutant form of PTPμ, PTPμ-extra, that lacked both phosphatase domains. This form of PTPμ induced aggregation with similar efficiency to the full-length form indicating that phosphatase activity and the aggregation response were not mutually dependent. Furthermore, the fact that the bacterially-expressed fusion protein GST/PTPμ containing only the extracellular segment of the PTPase was able to induce binding in vitro also suggested that aggregation was mediated solely by the extracellular domain. Aggregation did not result in dramatic changes in cellular phosphotyrosine levels as determined by anti-phosphotyrosine immunoblots. These data raise the possibility that, unlike the growth factor receptor PTKs where binding of the ligand to the extracellular segment triggers the kinase activity of the intracellular domain, PTPμ activity may not be strictly dependent upon ligand binding. However, this should be considered in light of the caveat that it is unlikely that Sf9 insect cells contain the normal substrates for human PTPμ. Changes in activity or affinity for a particular substrate may be seen when two molecules of PTPμ contact one another in their normal environment. Nevertheless, one could also propose a tethering role for homophilic binding interactions among PTPμ extracellular segments. Thus, the spatial distribution of the enzyme on the cell surface may be restricted, for example to intercellular junctions, with concommitant restriction of the spectrum of substrates or regulatory proteins with which it can interact.

PTPμ offers a unique, potentially direct link between cell adhesion phenomena and the triggering of signal transduction pathways. It remains possible that PTPμ itself may not directly promote cell–cell adhesion in vivo. However, in the context of adhesion driven by PTPμ-independent binding mechanisms, homophilic interactions between apposing PTPμ molecules may alter their phosphatase activity. Thus the homology of PTPμ to the cadherins, N-CAM-like molecules and PTPases predicts an interesting role in cell–cell communication and continuing studies of PTPμ should yield important new insights into the control of signal transduction processes.

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