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Receptor Tyrosine Phosphatase R-PTP-κ Mediates Homophilic Binding

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Receptor tyrosine phosphatases (R-PTPases) feature PTPase domains in the context of a receptor-like transmembrane topology. The R-PTPase R-PTP-κ displays an extracellular domain composed of fibronectin type III motifs, a single immunoglobulin domain, as well as a recently defined MAM domain (Y.-P. Jiang, H. Wang, P. D'Eustachio, J. M. Musacchio, J. Schlessinger, and J. Sap, Mol. Cell. Biol. 13:2942–2951, 1993). We report here that R-PTP-κ can mediate homophilic intercellular interaction. Inducible expression of the R-PTP-κ protein in heterologous cells results in formation of stable cellular aggregates strictly consisting of R-PTP-κ-expressing cells. Moreover, the purified extracellular domain of R-PTP-κ functions as a substrate for adhesion by cells expressing R-PTP-κ and induces aggregation of coated synthetic beads. R-PTP-κ-mediated intercellular adhesion does not require PTPase activity or posttranslational proteolytic cleavage of the R-PTP-κ protein and is calcium independent. The results suggest that R-PTPases may provide a link between cell-cell contact and cellular signaling events involving tyrosine phosphorylation.

The identification of a protein tyrosine phosphatase (PTPase) homology domain has recently allowed the identification of a large number of members of the PTPase family. This complexity of the PTPase family suggests that the regulation of cellular PTPase activity constitutes an intricate but hitherto poorly understood level of control of signaling mechanisms which involve tyrosine phosphorylation. A large subfamily of PTPases features a receptor-like transmembrane topology, displaying diverse but often highly conserved extracellular domains (receptor PTPases [R-PTPases]) (recently reviewed in reference 5). An important role for potential extracellular ligands for such R-PTPases can be inferred from studies on the CD45 R-PTPase. CD45 cross-linking with monoclonal antibodies can have profound effects on cellular signaling and activation pathways (13, 30, 45). Experiments with a chimeric CD45 protein in which the extracellular domain was replaced by that of the epidermal growth factor receptor led to similar conclusions (6). The identification of the in vivo ligands for R-PTPases is thus likely to lead to new insights in the field of cellular regulation.

R-PTPases with extracellular domains containing cysteine-rich clusters, highly glycosylated serine/threonine-rich regions, and carbonic anhydrase-like domains have been reported (2, 5, 27, 31). The broadest subfamily, however, is characterized by a combination of fibronectin type III repeats and immunoglobulin (Ig) domains, with members often found most highly expressed in epithelial and neuronal tissues (28, 44, 47, 51). Two other R-PTPases, R-PTP-κ, the subject of this study, and mRPTPμ, in addition contain a so-called MAM domain, so far defined solely on the basis of homology (4, 10, 22). The combination of Ig and fibronectin type III domains, as well as the posttranslational proteolytic processing observed in the case of R-PTP-κ and LAR (22, 44, 50), are features typical of cell adhesion molecules capable of mediating homophilic cell-cell binding (reviewed in references 14 and 43). In this study, we report that R-PTP-κ functions in several respects as a typical cell adhesion molecule. Its expression promotes homophilic cell aggregation, and cells expressing R-PTP-κ adhere to substrates consisting of a purified protein corresponding to the extracellular domain of R-PTP-κ. R-PTP-κ-mediated cell adhesion does not require the R-PTP-κ PTPase activity. That homophilic binding is an intrinsic property of the extracellular domain of the R-PTP-κ protein is further suggested by its ability to induce aggregation of synthetic beads (Covaspheres) coated with this domain. These data suggest that certain R-PTPases may have the potential to mediate regulatory events in response to cell-cell contact.

MATERIALS AND METHODS

Reagents, buffers, and media. The mouse R-PTP-κ cDNA and polyclonal antiserum 116 have been described previously (22). BSS buffer is 55 mM NaCl, 40 mM KCl, 15 mM $MgSO_4$, 10 mM $CaCl_2$, 20 mM glucose, 50 mM sucrose, 2 mg of bovine serum albumin (BSA) per ml, and 10 mM Tricine (pH 6.95) (42). BSA was from Intergen and heat inactivated (60°C for 30 min in phosphate-buffered saline [PBS]) before use. 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI) was from Molecular Probes, Inc. Green fluorescing Covaspheres were obtained from Duke Scientific. The monoclonal antibody against human placental alkaline phosphatase was from Medix Biotech (catalog no. A-018-02). *Drosophila melanogaster* S2 cells were propagated at room temperature in *Drosophila* Schneider medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma). Insect (*Spodoptera frugiperda*) Sf9 and High-Five cells were cultured in Grace medium supplemented with 10% heat-inactivated fetal bovine serum. L6 cells were propagated in Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum.

Expression of the R-PTP-κ protein. The entire mouse R-PTP-κ cDNA (22) was introduced in both orientations as an *Hpa*I-*Eco*RV fragment into the *Hpa*I site of a derivative

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of the pCasper expression vector containing the heat shock protein 70 (hsp70) promoter (46). A mutated cDNA failing to undergo proteolytic processing at the furin cleavage site (22) was introduced into the same vector in a similar manner. A cDNA encoding a truncated, catalytically inactive form (Δκ) of R-PTP-κ was constructed by restriction digestion with *Bsp*EI and Klenow fill-in of the wild-type cDNA. This leads to the introduction of a stop codon after amino acid residue 1083 and the generation of a protein lacking the cysteine residues essential for catalysis in the two intracellular catalytic homology domains of R-PTP-κ (18). The resulting constructs were cotransfected into *Drosophila* S2 cells with plasmid pPC4 (conferring α-amanitin resistance) (23), using calcium phosphate precipitation. Pools of stably transfected cells were selected in the presence of 5 μg of α-amanitin per ml for 3 weeks.

For expression in mammalian cells, the R-PTP-κ cDNA was introduced into the *Xho*I site of the pMJ30 vector (21) and cotransfected with plasmid pSV2neo into rat L6 cells. Clones surviving G418 selection were screened for expression by immunoblotting. This procedure did not detect endogenous R-PTP-κ protein in the parental L6 cells. The expressed protein underwent appropriate furin cleavage as described previously (22).

Aggregation assays. Transfected S2 cells were heat shocked at 37°C for 30 min to induce expression of the R-PTP-κ protein from the hsp70 promoter and allowed to recover for 2 h. Adherent cells were collected, washed twice in BSS, resuspended in BSS at a concentration of 4×10^6 cells per ml, and incubated in Coulter Counter vials on a rotary shaker for 2 h at 100 rpm at room temperature. Quantitation of aggregate formation was performed by using the Coulter Counter with the following settings: 1/amplification = 4; threshold = 10; 1/aperture current = 32.

For experiments involving the fluorescent dye diI, dye was added to the growth medium at a concentration of 3.2 μM during heat shock and washed away prior to recovery and assay. A total of 2×10^6 cells of each population were mixed and allowed to coaggregate in a total volume of 1 ml. Aggregate composition was monitored under visible and fluorescent light, using a Nikon Diaphot inverted microscope.

Expression and purification of the extracellular domain of the R-PTP-κ protein. Amino acids 1 to 639 of the R-PTP-κ proprotein (22) were fused in frame with human placental alkaline phosphatase (9) between the *Bgl*II and *Hin*dIII sites of the vector pBacBlue III (Invitrogen) by a series of appropriate cloning steps. This part of the R-PTP-κ protein corresponds to the 110-kDa cleavage product encompassing most of the extracellular domain of R-PTP-κ (the furin cleavage site occurs at amino acids 640 to 643). This leads to the production of a 180-kDa fusion protein consisting of (from N to C terminus) the R-PTP-κ signal peptide, the extracellular domain of R-PTP-κ, and human placental alkaline phosphatase. Recombinant virus was generated and expanded in Sf9 cells, using the Pharmingen Baculo-Gold transfection kit, and used to infect High-Five cells at a fivefold multiplicity of infection for production of the K2AP fusion protein. A secreted alkaline phosphatase control protein was generated in L6 myoblast cells by stable transfection with a modified version of the AP-TAG vector (9) encoding a fusion protein of alkaline phosphatase with a signal peptide. Both proteins were affinity purified by elution from an anti-alkaline phosphatase monoclonal antibody (Medix Biotech) column with 150 mM NaCl–1 mM $MgCl_2$–1 mM $CaCl_2$–100 mM diethanolamine (pH 11.5) (followed by rapid neutralization with 1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.0]) or with PBS containing 50% ethylene glycol, as indicated. The eluted protein was dialyzed against PBS and stored at 4°C. The K2AP and alkaline phosphatase proteins were approximately 90 and 50% pure, respectively, as determined by silver staining.

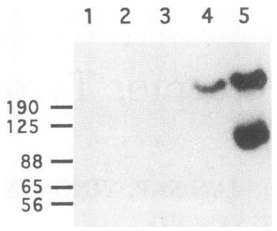

FIG. 1. Expression of the R-PTP-κ protein in transfected S2 cells. Detergent lysates were prepared from stably transfected S2 cells (2×10^6 cells per lane), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with an antiserum directed against the extracellular domain of the R-PTP-κ protein. Lanes: 1, R-PTP-κ antisense vector-transfected cells, not heat shocked; 2, antisense vector-transfected cells after heat shock; 3, sense vector-transfected cells, not heat shocked; 4, sense vector-transfected cells after heat shock; 5, lysate from COS cells transiently transfected with an R-PTP-κ expression vector (22). Molecular weight standards are indicated in kilodaltons.

Adhesion assays. For adhesion assays, 4-μl aliquots of protein samples (20 μg/ml) were spotted on 35-mm-diameter bacteriological petri dishes at equal distances from the center of the plate and allowed to bind to the plate at room temperature for 30 min. This yielded circular protein-coated areas of approximately 3-mm diameter. The solutions were removed by aspiration, and the surface of the entire plate was blocked for 60 to 90 min with 1% heat-inactivated BSA (32). The plates were incubated with a suspension of S2 cells (4×10^6/ml) in BSS with shaking (50 rpm) for 1 h at room temperature or with L6 cells in S-MEM (minimum essential medium, modified for suspension culture; GIBCO) (2×10^6/ml) without shaking at 37°C, washed three times with PBS, fixed in 3.7% formaldehyde, stained with crystal violet, and counted. Since only limited areas of the dish are coated in comparison with the number of cells available, the available surface area for adhesion is the limiting factor in these experiments.

Covasphere aggregation assays. Proteins (approximately 800 ng, 4 μg/ml) were covalently coupled to 20 μl of 0.5-μm-diameter green fluorescent Covaspheres by incubation for 1 h at 37°C and then 1 h at 4°C. After coupling, samples were sonicated for 10 s and washed with blocking buffer (10 mM [Tris pH 7.4], 1 mg of BSA per ml). After 30 min of incubation on ice in blocking buffer, the samples were resonicated and resuspended in 10 times the original Covasphere suspension volume of PBS. Aggregation of Covaspheres at this concentration was monitored by fluorescence microscopy and quantitated with a Coulter Counter (1/aperture current = 1/4; 1/amplification = 1/4; threshold = 10) after 3 h of incubation at room temperature. This setting detects particles larger than approximately 60 Covaspheres (16).

RESULTS

Stable, inducible expression of the R-PTP-κ protein in *Drosophila* S2 cells. We reasoned that analysis of adhesive

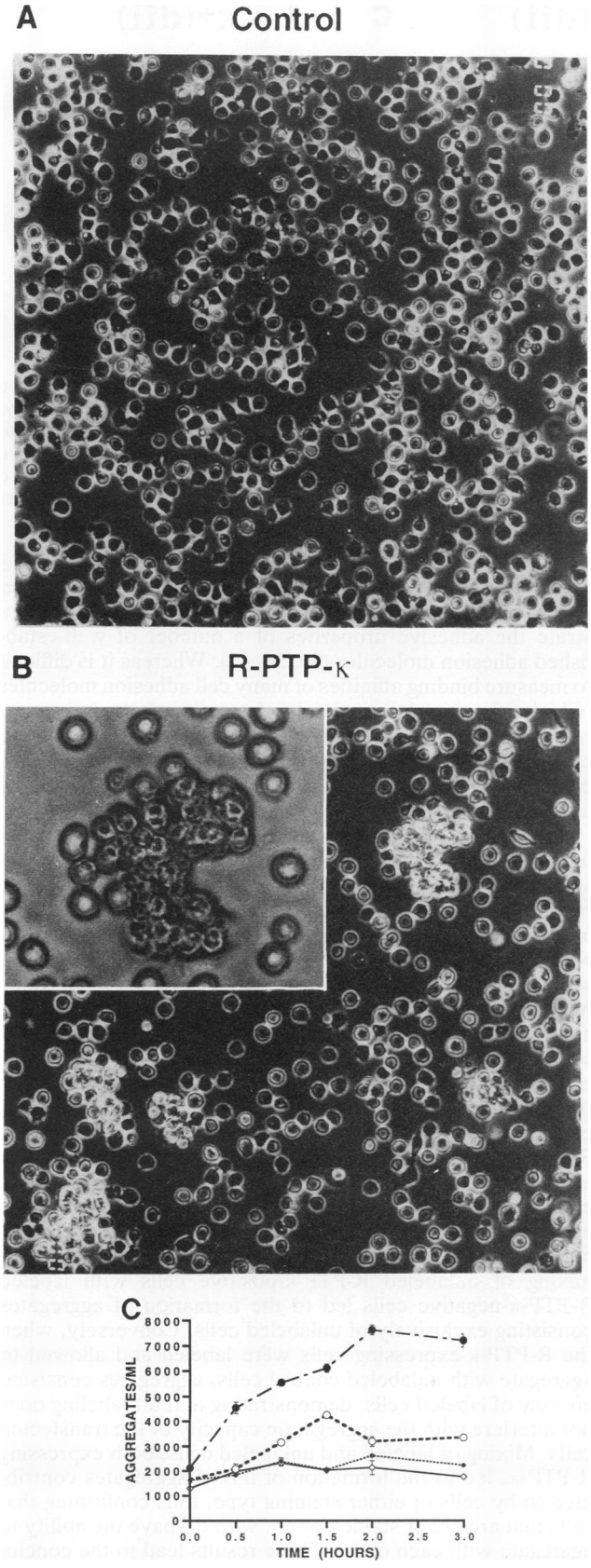

FIG. 2. S2 cell aggregation induced by R-PTP-κ expression. (A and B) Photographs of transfected S2 cell populations after heat shock induction and aggregation for 2 h. A typical experiment is shown. (A) Control (antisense vector-transfected) cells; (B) cells transfected with an expression vector carrying the R-PTP-κ cDNA in the sense orientation. Insert, higher magnification of a typical aggregate. (C) Time course and quantitation of aggregation by Coulter Counter counting of above-threshold particles. A typical experiment is shown. Open squares, antisense vector-transfected cells, uninduced; full squares, antisense vector-transfected cells, induced; open circles, cells transfected with an expression vector containing the R-PTP-κ cDNA in the sense orientation, uninduced; full circles, sense vector-transfected cells, induced. Error bars indicate standard errors (three determinations per sample and time point).

capacities conferred by R-PTP-κ would be facilitated by ectopic expression into a cell line likely to lack conserved ligands for a mammalian R-PTPase. In addition, we wished to use a recipient cell line displaying a low capacity for spontaneous aggregation or adhesion. The *Drosophila* S2 cell line meets both of these criteria and constitutes a system widely used to analyze both homophilic and heterophilic cell interactions (e.g., references 26, 36, and 42). Consequently, we introduced the wild-type R-PTP-κ cDNA (22) under the control of the hsp70 heat shock promoter into a modified version of the pCasper expression vector (46). The use of this promoter allows transcription of the introduced cDNA to be induced by brief heat treatment (30 min at 37°C, followed by a 2-h recovery at room temperature). Constructs containing the R-PTP-κ cDNA in either orientation with respect to the promoter were stably introduced into the recipient S2 cells, and the transfected cells were analyzed for inducible expression of the R-PTP-κ protein. As shown in Fig. 1 (lanes 3 and 4), in cells transfected with a construct containing the R-PTP-κ cDNA in the sense orientation with respect to the heat shock promoter, immunoblotting with an anti-R-PTP-κ antiserum revealed, upon heat induction, the appearance of a 210-kDa protein. This protein corresponds to the unprocessed form of R-PTP-κ seen in mammalian cells (22). No such protein was detected after similar treatment of S2 cells transfected with an expression vector containing the R-PTP-κ cDNA in the antisense orientation with respect to the promoter.

We previously reported that in mammalian cells, the 210-kDa R-PTP-κ precursor protein undergoes proteolytic cleavage by a furin-like protease, with both cleavage products remaining noncovalently attached (22). We have similarly observed, in R-PTP-κ-transfected S2 cells, the appearance of a minor 110-kDa species cross-reactive with anti-R-PTP-κ antiserum directed against the extracellular domain of the R-PTP-κ protein, suggesting that in S2 cells, the R-PTP-κ protein may at least partly be processed similarly as in mammalian cells through proteolytic cleavage by a furin-type endoprotease. Cleavage was also observed upon expression of the entire extracellular domain of the R-PTP-κ protein in Sf9 cells by using baculovirus vectors (not shown). A *Drosophila* furin homolog has recently been described (38). However, significant cleavage appeared to occur mainly after expression periods longer than those used in this study. Thus, most of the R-PTP-κ protein expressed in the transfected S2 cells during aggregation and adhesion assays was in the uncleaved 210-kDa precursor form.

Expression of R-PTP-κ induces cell aggregation. To study whether R-PTP-κ expression may mediate cell-cell aggregation, cells stably transfected with the R-PTP-κ cDNA in the sense orientation were subjected to an aggregation assay. Cells were induced or not by heat shock application, resuspended, subjected to rotary shaking to ensure mixing and

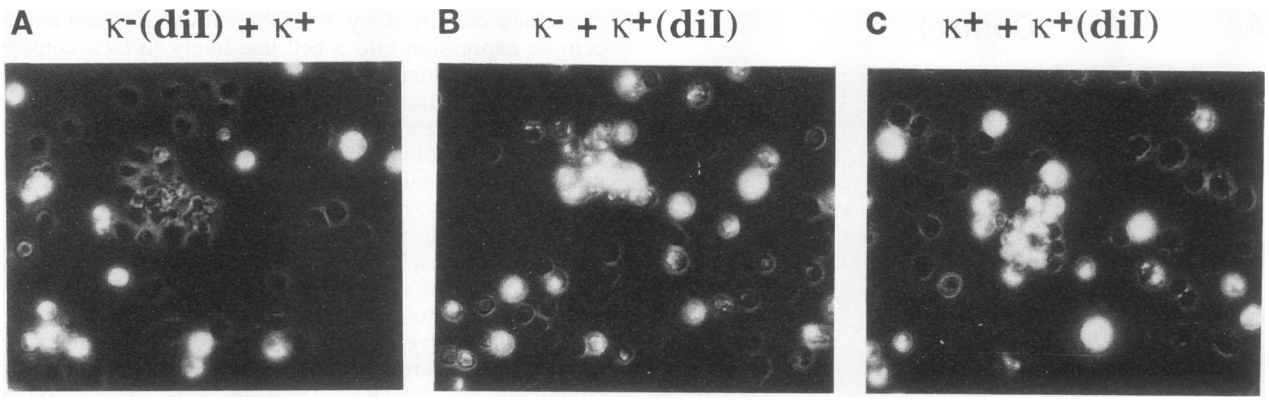

FIG. 3. Aggregates consist solely of cells expressing the R-PTP-κ protein. Two different cell populations, one of which had been labeled with the fluorescent dye diI, were allowed to coaggregate, and the resulting aggregates were inspected by visible and fluorescence microscopy. diI fluorescence is white in the photographs. (A) A pool of R-PTP-κ-expressing cells (sense vector transfected, heat induced) was allowed to aggregate in the presence of an equal number of diI-stained R-PTP-κ-negative (antisense vector-transfected, heat-induced) cells. (B) R-PTP-κ expressing cells were stained with diI and allowed to aggregate in the presence of unstained R-PTP-κ-negative cells. (C) Mixture of stained and unstained R-PTP-κ-positive cells. Representative aggregates are shown. In each case, 10 aggregates were randomly localized under visible light only. Subsequent inspection under UV light consistently showed the staining pattern exemplified in the photographs.

avoid adhesion to the vessel, and then assayed for aggregate formation. The extent of aggregate formation was compared with that displayed by uninduced (not heat-shocked) cells or similarly treated control cells transfected with the vector containing the R-PTP-κ cDNA in the antisense orientation. In heat-shocked sense cDNA-expressing cells, we observed the formation of a large number of aggregates consisting of more than 10 and up to approximately 100 cells. By contrast, control cells (not heat shocked or antisense vector transfected) remained primarily as a single cell suspension (Fig. 2A). This property of the transfected cells was stable and reproducible over more than 2 months of continuous culture of the transfected cells and was consistently observed in several independent stable and transient expression experiments (not shown) of S2 cells. The fact that aggregation was incomplete, with a proportion of R-PTP-κ-transfected cells remaining as single cells throughout the assay period, may be due to the fact that the transfected cell population consisted of an uncloned pool of cells presumably differing in their levels of R-PTP-κ expression or to a loss of protein expression or cell viability during the aggregation assay. However, the requirement for R-PTP-κ expression in all cells within aggregates was proven by mixing experiments using dye-labeled cells (see below).

To quantitate objectively the extent of cell aggregation, we performed a determination of superthreshold particles with a Coulter Counter (Fig. 2C). This type of counting underestimates the real extent of cell aggregation, since only the largest aggregates formed are scored by this procedure, as determined by comparison with visual inspection and counting of the aggregates formed (not shown). This procedure revealed no induction of aggregation by cells transfected with a vector expressing the R-PTP-κ cDNA in the antisense orientation. By contrast, sense vector-transfected cells showed a high degree of time-dependent aggregate formation after heat shock induction. Aggregation reached a plateau value after approximately 2 h of incubation. A low but reproducible degree of aggregation was seen in sense vector-transfected cells in the absence of heat shock induction. This may be due to a degree of leakiness of the heat shock promoter under conditions of the assay (25°C) or because of induction of the heat shock promoter by stress conditions due to performance of the assay itself.

Notably, the conditions of the assay (medium, time scale, and speed of shaking) are similar to those used to demonstrate the adhesive properties of a number of well-established adhesion molecules (26, 36, 42). Whereas it is difficult to measure binding affinities of many cell adhesion molecules (which often are low and rely on cooperativity (see, e.g., reference 8), this similarity suggests that the strength of cell-cell interaction conferred by expression of R-PTP-κ is comparable to that previously described for conventional adhesion molecules.

Aggregation of R-PTP-κ-expressing cells is strictly homophilic. The observed aggregation correlating with expression of R-PTP-κ could be accounted for by a homophilic binding mechanism, in which cell-cell binding is mediated by interaction between R-PTP-κ proteins on different cells within aggregates, by binding of the R-PTP-κ protein to a second cell surface ligand intrinsic to the parental transfected cells (heterophilic mechanism), or by a non-cell-autonomous mechanism, e.g., one involving a secreted soluble factor. We were able to distinguish between these possibilities by marking different populations of cells with the fluorescent lipophilic dye diI (39) and testing for their coaggregation. In these experiments, cells expressing R-PTP-κ or not were labeled with the dye, mixed with unlabeled cells of either type, and subjected to aggregating conditions. The presence of cells of either population in the aggregates formed was then monitored by fluorescence microscopy. The results are illustrated in Fig. 3. Strikingly, mixing of unlabeled R-PTP-κ-positive cells with labeled R-PTP-κ-negative cells led to the formation of aggregates consisting exclusively of unlabeled cells. Conversely, when the R-PTP-κ-expressing cells were labeled and allowed to aggregate with unlabeled control cells, aggregates consisted entirely of labeled cells, demonstrating that diI labeling does not interfere with the aggregation capacity of the transfected cells. Mixing of labeled and unlabeled cells, both expressing R-PTP-κ, led to the formation of mixed aggregates contributed to by cells of either staining type, thus confirming that cells that are either stained or not with diI have the ability to aggregate with each other. These results lead to the conclusion that aggregation of the R-PTP-κ-transfected cells requires the presence of the protein on all cells within the aggregate, implying a homophilic binding mechanism. How-

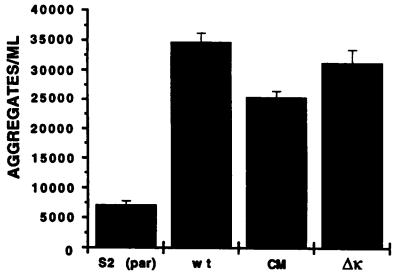

FIG. 4. R-PTP-κ-induced S2 cell aggregation does not require the intracellular catalytic domain of the R-PTP-κ protein or post-translational proteolytic processing at the furin cleavage site. Parental S2 [S2 (par)] cells were stably transfected with expression vectors encoding an R-PTP-κ cDNA in which the furin cleavage site had been mutated (CM) (22), a cDNA encoding a catalytically inactive deletion mutant of R-PTP-κ lacking most of the intracellular (PTPase) domain (Δκ), or a wild-type R-PTP-κ cDNA (wt). Cells were heat induced and subjected to aggregating conditions for 2 h, and above-threshold aggregates were counted with a Coulter Counter. Error bars indicate standard errors (three determinations per sample). The apparent differences in aggregation intensity between the different forms of R-PTP-κ may reflect small differences in protein expression levels (not shown). The numbers provided by use of the Coulter Counter actually provide an underestimation of the amount of aggregation as determined by visual inspection and counting of aggregates, since only large particles above a certain threshold size are scored by the Coulter Counter.

ever, these experiments do not rule out the possibility of heterophilic ligands in other types of cells found in vivo.

R-PTP-κ-mediated cell aggregation does not require phosphatase activity, furin proteolytic cleavage of the R-PTP-κ proprotein, or calcium. The experiments described above were performed with a full-length R-PTP-κ cDNA, leaving unclear whether the PTPase activity of the intracellular domain is required to confer adhesive properties. In several instances, an intact intracellular domain of cell adhesion molecules has in fact been shown to be required for certain aspects of cell-cell interaction (20, 34). To test this issue, we constructed a cDNA encoding a truncated protein lacking most of the intracellular, catalytic domain of R-PTP-κ (Δκ). Figure 4 shows that cells expressing the Δκ protein displayed aggregation properties similar to those of cells expressing the catalytically active wild-type R-PTP-κ protein. We also tested the role of the furin cleavage site in the extracellular domain of R-PTP-κ. Mutation of this site also left the adhesion behavior intact, suggesting that cleavage of the R-PTP-κ proprotein (22) is not required for induction of cell aggregation.

Cell adhesion molecules which either do (e.g., cadherin family members and integrins) or do not (e.g., N-CAM and Ng-CAM) require the presence of Ca^{2+} have been described (12, 14, 19). Our experiments in Fig. 2 were performed in the presence of 10 mM Ca^{2+} in the aggregating cell suspension. A similar experiment performed in the absence of calcium ions and in the presence of 1 mM EGTA revealed no calcium requirement for R-PTP-κ-mediated cellular aggregation under the conditions of the assay (data not shown). This calcium-independent type of cell-cell binding is thus similar to that described for other cell adhesion molecules containing Ig and fibronectin type III repeats.

The purified extracellular domain of the R-PTP-κ protein functions as a specific substrate for adhesion by R-PTP-κ-expressing cells. We next wished to determine whether the extracellular domain of R-PTP-κ was able to function by itself as a substrate for attachment for cells expressing the R-PTP-κ protein without the requirement for other factors to assist in the adhesion process. A baculovirus expression system was used to produce a soluble recombinant protein consisting of virtually the entire extracellular domain of the R-PTP-κ protein, fused at its C terminus to placental alkaline phosphatase to serve as a tag for purification and detection (9). Fusion between the two protein moieties was designed to occur precisely before the furin proteolytic cleavage signal in the fourth fibronectin type III repeat in R-PTP-κ. The recombinant protein (K2AP) was purified from the conditioned medium of baculovirus-infected cells by affinity chromatography and used to coat small areas of bacteriological petri dishes along with a number of control proteins. Subsequently, the petri dishes were briefly incubated with R-PTP-κ-expressing or control cells under conditions of moderate shear force, washed, and fixed, and the number of adherent S2 cells was counted. Significantly, only induced, R-PTP-κ-expressing cells adhered to the K2AP-coated surface area (Fig. 5; Table 1). Uninduced or antisense vector-transfected cells did not adhere to the K2AP-coated surface areas. In addition, there was no attachment of induced, sense vector-transfected cells to control coated surfaces, including surfaces coated with the alkaline phosphatase tag moiety or recombinant extracellular domain of human epidermal growth factor receptor also purified by affinity chromatography from a baculovirus expression system (29).

Whereas, for technical reasons, all the experiments described above were performed in the context of insect cells, we also tested the effect of R-PTP-κ expression in mammalian cells in a similar cell-to-substrate adhesion assay. Stable R-PTP-κ-overexpressing cell lines were generated from transfected rat L6 cells. The resulting clones expressed appropriately cleaved and processed R-PTP-κ protein, as reported previously (22). In contrast to the situation for transfected and heat shock-induced S2 cells, virtually all of the R-PTP-κ protein present in the transfected cells was present in the mature cleaved form (not shown). The cell line used as a recipient for R-PTP-κ overexpression, rat L6 myoblast cells, already shows a low level of spontaneous adhesion to a K2AP protein-coated surface, although we were unable to detect endogenous R-PTP-κ protein in these cells by immunoblotting (data not shown). However, stable overexpression of an R-PTP-κ cDNA in these cells led to a significant and reproducible (2.7-fold ± 1.0; $n = 3$) increase in adhesive capacity to a surface coated with the recombinant soluble extracellular domain of the R-PTP-κ protein (Fig. 5; Table 2).

The purified extracellular domain of R-PTP-κ can mediate aggregation of coated synthetic beads. Further evidence that the extracellular domain of the R-PTP-κ protein can bind homophilically in the absence of accessory mechanisms was gained from experiments with synthetic beads. The purified recombinant extracellular domain of R-PTP-κ (K2AP, fused to an alkaline phosphatase tag) was covalently coupled to synthetic 0.5-μm-diameter fluorescing beads (Covaspheres). Such coated beads rapidly self-aggregated to form large particles which eventually precipitated out of the suspension. No such behavior was observed for beads coated with the alkaline phosphatase tag protein alone or with a control protein (BSA). Particle formation was quantified by use of a Coulter Counter (Fig. 6). Measurement of superthreshold aggregates showed dramatic aggregation of beads coated with the K2AP protein but not of beads coated with control protein. In similar experiments, we attempted inhibiting

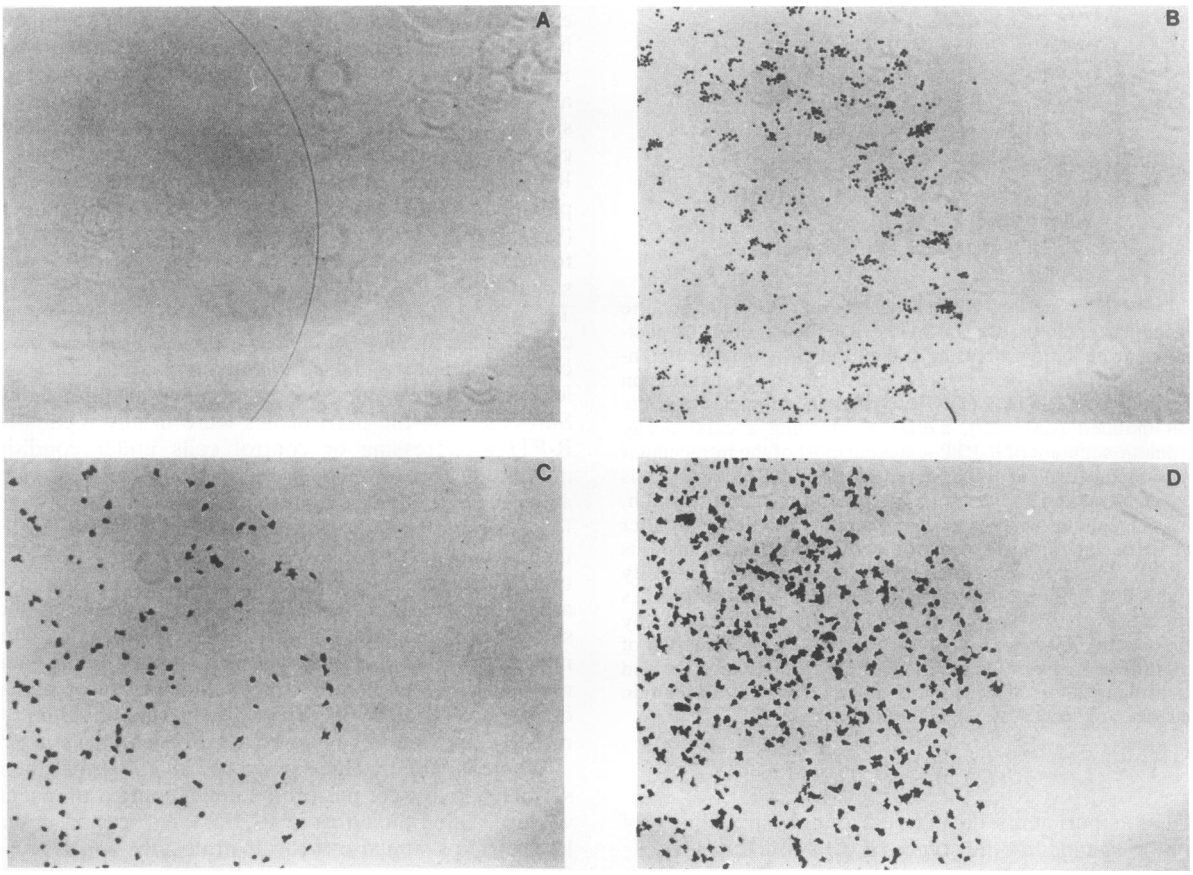

FIG. 5. Adhesion of R-PTP-κ-transfected cells to a surface coated with recombinant purified R-PTP-κ extracellular domain (K2AP) protein. A representative of several experiments is shown. R-PTP-κ-negative (antisense vector-transfected, induced) (A) and -positive (sense vector-transfected, induced) (B) S2 cells and R-PTP-κ-negative (C) and -positive (D) L6 cells were incubated with a surface partially coated with the K2AP protein (circle), and the adherent cells were fixed and stained (see also Tables 1 and 2).

aggregation by using antiserum 116, directed against a peptide corresponding to amino acids 60 to 76 of the R-PTP-κ protein (22). No inhibition could be observed with use of this antiserum (Fab fragments at a final concentration of 1 mg/ml; data not shown).

DISCUSSION

The data presented demonstrate that R-PTP-κ, an R-PTPase related to the LAR-like subfamily (containing a combination of Ig and fibronectin type III domains), is capable of mediating homophilic binding between cells, thus indicating a function for the extracellular domain of an R-PTPase. In this respect, the R-PTP-κ protein behaves similarly to members of the N-CAM family of cell adhesion molecules. Our observation makes it likely that homophilic binding may be a more general phenomenon among R-PTPases of the LAR family. In fact, during and after submission of this report, two reports describing cell aggregation mediated by the R-PTPase mRPTPμ appeared (4, 11). This phosphatase displays 54% homology at the amino acid level to mRPTPμ in its extracellular domain (10). Whereas our experiments identify one ligand for the extracellular domain of the R-PTP-κ protein, however, they clearly do not rule out the possibility of additional, as yet to be identified heterophilic ligands. In fact, many cell adhesion molecules of the N-CAM family, for instance, appear to display heterophilic

TABLE 1. Adhesion of control and R-PTP-κ-expressing S2 cells to surfaces coated with the recombinant, purified extracellular domain of the R-PTP-κ protein[a]

Protein[b]	No. of adherent cells/protein-coated area			
	S2 control		S2-R-PTP-κ	
	Uninduced	Induced	Uninduced	Induced
K2AP-a	0	0	0	1,367 ± 545 (n = 3)
AP	0	0	0	0
HER	0	0	0	0
BSA	0	0	0	0
Fibronectin	~4,000	~4,000	~4,000	~4,000

[a] Different populations of S2 cells were incubated in bacterial petri dishes containing a set of 3-mm-diameter areas coated with the different proteins tested. No variation was observed between experiments in the extent of adhesion by the various cell types to the positive control fibronectin or polylysine substrates.

[b] K2AP-a, K2AP protein purified by elution from an affinity column at alkaline pH (comparable data were obtained with K2AP protein purified by elution with 50% ethylene glycol). AP, alkaline phosphatase control protein (9), corresponding to the tag portion of the K2AP fusion protein. HER, human epidermal growth factor receptor extracellular domain affinity purified from a baculovirus expression system (29).

TABLE 2. Effect of R-PTP-κ expression on adhesion of rat L6 cells to surfaces coated with the recombinant, purified extracellular domain of the R-PTP-κ protein[a]

Protein[c]	No. of adherent cells/protein-coated area		Fold increase
	L6	L6-R-PTP-κ[b]	
K2AP-a			
Expt 1	145	540	3.7
Expt 2	296	525	1.8
Expt 3	87	238	2.7
Avg			2.7 ± 1.0
AP	0	0	
HER	0	0	
BSA	0	0	
Polylysine	~4,000	~4,000	

[a] The experiments were performed as described in Table 1, footnote *a*.
[b] A representative clone of L6 cells stably expressing the transfected R-PTP-κ cDNA.
[c] See Table 1, footnote *b*.

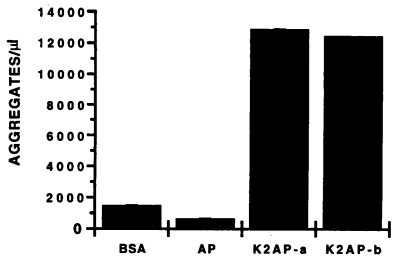

FIG. 6. Aggregation of synthetic beads (Covaspheres) coated with a purified recombinant protein (K2AP) corresponding to the extracellular domain (amino acids 1 to 639) of R-PTP-κ, fused to an alkaline phosphatase tag. Beads were covalently coated with the K2AP protein or with control proteins (alkaline phosphatase tag moiety alone [AP] or BSA), sonicated to generate a single bead suspension, and allowed to aggregate for 3 h at room temperature. The extent of aggregation was monitored with a Coulter Counter. At the settings used, the detected particle size corresponds approximately to 60 beads (16). K2AP-a, protein eluted from the affinity column at alkaline pH; K2AP-b, protein eluted from affinity column with 50% ethylene glycol. Error bars indicate standard errors.

in addition to homophilic binding activity (15–17, 25, 37, 43). Further mapping of the homophilic binding capacity to a particular subdomain of the extracellular moiety of R-PTP-κ may help to focus the search for putative additional, heterophilic ligands.

While contact inhibition of cell growth has been a long-known phenomenon, several potential links between the adhesive capacities of cells and signal transduction pathways have recently been emerging (24). Adherens junctions correspond to sites of locally increased tyrosine phosphorylation and are subject to its control (49). Furthermore, reagents that bind cell adhesion molecules are known to activate a number of second messenger signals (7, 41). In the case of integrins or extracellular domains of established cell adhesion molecules, ligand binding or cross-linking has been reported to induce changes in cellular tyrosine phosphorylation (1, 24). However, the nature of the intracellular domains of most cell adhesion molecules yields little information as to how such signals might be generated. By contrast, the observation that an R-PTPase displays properties of a cell adhesion molecule readily suggests possible mechanisms for the generation of certain intracellular signals in response to cell-cell contact. For instance, direct cell-cell contact between R-PTPases on adjacent cells could modify R-PTPase oligomerization and thus, analogously to receptor tyrosine kinases, lead to changes in catalytic activity (40). Alternatively, cell-cell adhesion-mediated relocalization of R-PTPases could modulate substrate accessibility. Moreover, the similar structural properties of the extracellular domains of R-PTPases and cell adhesion molecules prompts the speculation that R-PTPases may, in addition to self-interaction, also be capable of interacting heterophilically with other molecules involved in cell adhesion, whether in *cis* or in *trans*, thus further integrating cell-cell contact with signal transduction processes (14–17, 24, 25, 37, 43).

Preliminary experiments have so far failed to reveal unambiguous changes in protein tyrosine phosphorylation or PTPase activity in response to R-PTP-κ-mediated aggregation events (data not shown). This may be due to a number of reasons. R-PTPases may display a high degree of substrate specificity (48). Furthermore, only limited knowledge is available concerning specific in vivo substrates for R-PTPases or the specific signaling pathways in which they are involved (5, 33, 35, 52). Specific substrates for R-PTP-κ are so far unidentified or may be lacking in the cell lines investigated. The situation is thus different from that for tyrosine kinases, in which case autophosphorylation can serve as a marker for activity. Finally, since changes in activity may be local, individual cell recording assays may be required to detect cellular responses. The identification of a ligand for a typical R-PTPase now provides the way further to address this question.

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REFERENCES

1. **Atashi, J. R., S. G. Klinz, C. A. Ingraham, W. T. Matten, M. Schachner, and P. F. Maness.** 1992. Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. Neuron **8**:831–842.
2. **Barnea, G., O. Silvennoinen, B. Shaanan, A. M. Honegger, P. D. Canoll, P. D'Eustachio, B. Morse, J. B. Levy, S. LaForgia, K. Huebner, J. M. Musacchio, J. Sap, and J. Schlessinger.** 1993. Identification of a carbonic anhydrase-like domain in the extracellular region of RPTPγ defines a new subfamily of receptor tyrosine phosphatases. Mol. Cell. Biol. **13**:1497–1506.
3. **Beckmann, G., and P. Bork.** 1993. An adhesive domain detected in functionally diverse receptors. Trends Biochem. Sci. **18**:40–41.
4. **Brady-Kalnay, S. M., A. J. Flint, and N. K. Tonks.** 1993. Homophilic binding of PTPμ, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation. J. Cell Biol. **122**:961–972.
5. **Charbonneau, H., and N. K. Tonks.** 1992. 1002 protein phosphatases? Annu. Rev. Cell Biol. **8**:463–493.
6. **Desai, D. M., J. Sap, J. Schlessinger, and A. Weiss.** 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. Cell **73**:541–554.
7. **Doherty, P., S. Ashton, S. E. Moore, and F. S. Walsh.** 1991. Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G-protein dependent activation of L- and N-type neuronal Ca^{2+} channels. Cell **67**:21–33.
8. **Doherty, P., M. Fruns, P. Seaton, G. Dickson, C. H. Barton,**

T. A. Sears, and F. S. Walsh. 1990. A threshold effect of the major isoforms of NCAM on neurite outgrowth. Nature (London) 343:464–466.
9. Flanagan, J. G., and P. Leder. 1990. The *Kit* ligand: a cell surface molecule altered in *Steel* mutant fibroblasts. Cell 63: 185–194.
10. Gebbink, M. F. B. G., I. van Etten, G. Hateboer, R. Suijkerbuijk, R. L. Beijersbergen, A. Geurts van Kessel, and W. Moolenaar. 1991. Cloning, expression and chromosomal localization of a new putative receptor-like protein tyrosine phosphatase. FEBS Lett. 290:123–130.
11. Gebbink, M. F. B. G., G. C. M. Zondag, R. W. Wubbolts, R. L. Beijersbergen, I. van Etten, and W. H. Moolenaar. 1993. Cell-cell adhesion mediated by a receptor-like protein tyrosine phosphatase. J. Biol. Chem. 268:16101–16104.
12. Geiger, B., and O. Ayalon. 1992. Cadherins. Annu. Rev. Cell Biol. 8:307–332.
13. Goldman, S. J., S. Uniyal, L. M. Ferguson, D. Golan, S. J. Burakoff, and P. A. Kiener. 1992. Differential activation of phosphotyrosine protein phosphatase activity in a murine T cell hybridoma by monoclonal antibodies to CD45. J. Biol. Chem. 267:6197–6204.
14. Grumet, M. 1991. Cell adhesion molecules and their subgroups in the nervous system. Curr. Opin. Neurobiol. 1:370–376.
15. Grumet, M. 1992. Structure, expression, and function of Ng-CAM, a member of the immunoglobulin superfamily involved in neuron-neuron and neuron-glia adhesion. J. Neurosci. Res. 31:1–13.
16. Grumet, M., and G. M. Edelman. 1988. Neuron-glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. J. Cell Biol. 106:487–503.
17. Grumet, M., A. Flaccus, and R. U. Margolis. 1993. Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules. J. Cell Biol. 120:815–824.
18. Guan, K. L., and J. E. Dixon. 1991. Evidence for protein-tyrosine phosphatase catalysis proceeding via a cystein-phosphate intermediate. J. Biol. Chem. 266:17026–17030.
19. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.
20. Jaffe, S. H., D. R. Friedlander, F. Matsuzaki, K. L. Crossin, B. Cunningham, and G. Edelman. 1990. Differential effects of the cytoplasmic domains of cell adhesion molecules on cell aggregation and sorting out. Proc. Natl. Acad. Sci. USA 87:3589–3593.
21. Jaye, M., R. M. Lyall, R. Mudd, J. Schlessinger, and N. Sarver. 1988. Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. EMBO J. 7:963–969.
22. Jiang, Y.-P., H. Wang, P. D'Eustachio, J. M. Musacchio, J. Schlessinger, and J. Sap. 1993. Cloning and characterization of R-PTP-κ, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved extracellular adhesion molecule-like extracellular region. Mol. Cell. Biol. 13: 2942–2951.
23. Jokers, R. S., J. R. Weeks, W. A. Zehring, and A. L. Greenleaf. 1989. Analysis of the gene encoding the largest subunit of RNA polymerase II in *Drosophila*. Mol. Gen. Genet. 215:266–275.
24. Juliano, R. L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. J. Cell Biol. 120:577–585.
25. Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990. The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. J. Cell Biol. 110:193–208.
26. Krämer, H., R. L. Cagan, and S. L. Zipursky. 1991. Interaction of *bride of sevenless* membrane-bound ligand and the *sevenless* tyrosine kinase receptor. Nature (London) 352:207–212.
27. Krueger, N. X., and H. Saito. 1992. A human transmembrane protein-tyrosine-phosphatase, PTPzeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. Proc. Natl. Acad. Sci. USA 89:7417–7421.
28. Krueger, N. X., M. Streuli, and H. Saito. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. EMBO J. 9:3241–3252.
29. Lax, I., A. K. Mitra, C. Ravera, D. R. Hurwitz, M. Rubinstein, A. Ullrich, R. M. Stroud, and J. Schlessinger. 1991. Epidermal growth factor (EGF) induces oligomerization of soluble, extracellular, ligand-binding domain of EGF receptor. A low resolution projection structure of the ligand-binding domain. J. Biol. Chem. 266:13828–13833.
30. Ledbetter, J. A., N. K. Tonks, E. H. Fischer, and E. A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. Proc. Natl. Acad. Sci. USA 85:8628–8632.
31. Levy, J. B., P. D. Canoll, O. Silvennoinen, G. Barnea, B. Morse, A. M. Honegger, J.-T. Huang, L. A. Cannizzaro, S.-H. Park, T. Druck, K. Huebner, J. Sap, M. Ehrlich, J. M. Musacchio, and J. Schlessinger. 1993. The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system. J. Biol. Chem. 268:10573–10581.
32. Mauro, V. P., L. A. Krushel, B. A. Cunningham, and G. M. Edelman. 1992. Homophilic and heterophilic binding activities of Nr-CAM, a nervous system cell adhesion molecule. J. Cell Biol. 119:191–202.
33. Mustelin, T., and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase pp56^{lck} by the leucocyte common antigen (CD45). Oncogene 5:809–813.
34. Nagafuchi, A., and M. Takeichi. 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J. 7:3679–3684.
35. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the *lck*-encoded tyrosine kinase in murine lymphoma T-cell lines. Proc. Natl. Acad. Sci. USA 86:8959–8963.
36. Pulido, D., S. Campuzano, T. Koda, J. Modolell, and M. Barbacid. 1992. D*trk*, a *Drosophila* gene related to the *trk* family of neurotrophin receptors, encodes a novel class of neural cell adhesion molecule. EMBO J. 11:391–404.
37. Reyes, A. A., R. Akeson, L. Brezina, and G. J. Cole. 1990. Structural requirements for neural cell adhesion molecule-heparin interaction. Cell Regul. 1:567–576.
38. Roebroek, A. J. M., J. W. M. Creemers, I. G. L. Pauli, T. Bogaert, and W. J. M. Van de Ven. 1993. Generation of structural and functional diversity in furin-like proteins in *Drosophila melanogaster* by alternative splicing of the *Dfur1* gene. EMBO J. 12:1853–1870.
39. Schlessinger, J., D. Axelrod, D. E. Koppel, W. W. Webb, and E. L. Elson. 1977. Lateral transport of a lipid probe and labeled proteins on a cell membrane. Science 195:307–309.
40. Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. Neuron 9:1–20.
41. Schuch, U., M. J. Lohse, and M. Schachner. 1989. Neural cell adhesion molecules influence second messenger systems. Neuron 3:13–20.
42. Snow, P. M., A. J. Bieber, and C. S. Goodman. 1989. Fasciclin III: a novel homophilic adhesion molecule in *Drosophila*. Cell 59:313–323.
43. Sonderegger, P., and F. G. Rathjen. 1992. Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily. J. Cell Biol. 119:1387–1394.
44. Streuli, M., N. X. Krueger, P. D. Ariniello, M. Tang, J. M. Munro, W. A. Blattler, D. A. Adler, C. M. Disteche, and H. Saito. 1992. Expression of the receptor-linked protein tyrosine phosphatase LAR: proteolytic cleavage and shedding of the CAM-like extracellular region. EMBO J. 11:897–907.
45. Thomas, M. L. 1989. The leukocyte common antigen family. Annu. Rev. Immunol. 7:339–369.
46. Thummel, C. S., A. M. Boulet, and H. D. Lipshitz. 1988. Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. Gene 74:445–456.
47. Tian, S. S., P. Tsoulfas, and K. Zinn. 1991. Three receptor-linked protein-tyrosine phosphatases are selectively expressed on central nervous system axons in the *Drosophila* embryo. Cell 67:675–685.
48. Tonks, N. K., C. D. Diltz, and E. H. Fisher. 1990. CD45, an

integral membrane protein tyrosine phosphatase. J. Biol. Chem. **265**:10674–10680.

49. **Volberg, T., Y. Zick, R. Dror, I. Sabany, C. Gilon, A. Levitzki, and B. Geiger.** 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. EMBO J. **11**:1733–1742.
50. **Yu, Q., T. Lenardo, and R. A. Weinberg.** 1992. The N-terminal and C-terminal domains of a receptor tyrosine phosphatase are associated by non-covalent linkage. Oncogene **7**:1051–1057.
51. **Yang, X., K. T. Seow, S. M. Bahri, S. H. Oon, and W. Chia.** 1991. Two *Drosophila* receptor-like tyrosine phosphatase genes are expressed in a subset of developing axons and pioneer neurons in the embryonic CNS. Cell **67**:661–673.
52. **Zheng, X. M., Y. Wang, and C. J. Pallen.** 1992. Cell transformation and activation of pp60$^{c\text{-}src}$ by overexpression of a protein tyrosine phosphatase. Nature (London) **359**:336–339.