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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research is to understand how cell adhesion-induced signals are transduced to negatively regulate cell growth and how this process is altered in prostate cancer. Extracellular events that regulate cell growth are transmitted by changes in tyrosine phosphorylation, which is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Cancer causing genes encode PTKs that cause uncontrolled cell growth suggesting that PTPs play a role in negative growth regulation or function as tumor suppressors. Both cell adhesion molecules and tyrosine phosphorylation regulate contact inhibition of growth, i.e. when normal cells stop growing because they contact adjacent cells. Prostate cancer cells have defects in both cell adhesion and contact inhibition of growth. The receptor PTP, PTPμ, directly interacts with E-cadherin, the major cell-cell adhesion molecule in prostate cells. Loss of components of the cadherin pathway has previously been observed in prostate cancer cells. We recently demonstrated that PTPμ is no longer expressed in prostate cancer cells. Re-expression of PTPμ restores adhesion and negatively regulates cell growth. A detailed analysis of how PTPμ alters adhesion, cell growth and signal transduction is described and provides insights into both normal cell growth as well as malignant transformation in prostate cancer.

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INTRODUCTION:

Prostate cancer is the most common malignancy in men in the United States. The exact genetic pathway that leads to prostate cancer formation has not yet been elucidated. We have found alterations in the expression of cell adhesion molecules in LNCaP prostate cancer cells. The major cell-cell adhesion molecules in differentiated epithelial cells are cadherins. Cadherins are localized at cellular communication sites called adherens junctions, which maintain the integrity of the epithelial sheet. The cytoplasmic domain of cadherins interacts with molecules termed catenins, which associate with the actin cytoskeleton. Loss of components of the cadherin/catenin pathway has been observed in many prostate cancer cells. Cadherins interact with a number of oncogenes and tumor suppressor genes suggesting an important role for this complex in tumorigenesis. Immunohistochemical studies have demonstrated reduced E-cadherin expression in a number of invasive or metastatic prostate tumors. In addition, reduction of E-cadherin protein levels and deletion of the α catenin gene have been observed in some prostate cancer cell lines. Many studies suggest that when any protein in the cadherin/catenin pathway is altered during malignant transformation it lead to decreased adhesion, invasion and metastasis. Tyrosine phosphorylation of E-cadherin and catenins has also been observed in many carcinomas and this correlates with decreased cell adhesion. However, the precise regulatory mechanism or the crucial substrates for phosphorylation are unknown. The net level of tyrosine phosphorylation is controlled by the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). We demonstrated that the phosphatase, PTPμ, directly mediates cell adhesion and interacts with cadherins, and that this complex is regulated by tyrosine phosphorylation (Brady-Kalnay et al., 1993; Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). PTPμ is endogenously expressed in normal prostate cells (Figure 3). Surprisingly, we have found that the LNCaP prostate cancer cell line does not express PTPμ (Figure 3) suggesting that loss of PTPμ contributes to tumorigenesis. The LNCaP cell line is used as a model system for prostate cancer because it is responsive to androgen and expresses prostate specific markers such as prostate specific antigen and prostatic acid phosphatase (Horoszewicz et al., 1983). LNCaP cells do express normal levels of cadherins and a number of their associated proteins (Figure 4). We have found that LNCaP cells are deficient in cadherin-dependent adhesion (Figure 8). A major goal of this study was to add normal and mutant forms of PTPμ, using a retroviral expression system, to the LNCaP cells and determine how this affects adhesion, tyrosine phosphorylation, growth and tumorigenicity of the cells. As described below, Our data suggest that there is a dramatic effect on adhesion when PTPμ is re-expressed in LNCaP cells (Figure 8). The restoration of adhesion was independent of phosphatase activity (Figures 12-13). Interestingly, re-expression of wild type PTPμ negatively regulated growth (Figure 15). However, none of the mutant forms of PTPμ affected cell growth. These data indicate that PTPμ phosphatase activity is required to negatively regulate cell growth. These data suggest that PTPμ may mediate contact inhibition of growth.

PROPOSAL BODY:

The mechanism of prostate cancer progression is not well understood but alterations in cell adhesion are often an important step towards de-regulation of cellular proliferation and de-differentiation leading to invasion and metastasis. The molecular mechanism of prostate cancer formation has not yet been elucidated. However, many specific genetic alterations have been observed during prostate cancer progression (Kallioniemi and Visakorpi, 1996). Cadherins, the major cell-cell adhesion molecules in epithelial cells, are localized at cellular communication sites called adherens junctions, which maintain the integrity of the epithelial sheet. Cadherins mediate cell-cell adhesion by forming a "zipper"-like structure that adheres two cell surfaces to one another (Takeichi, 1995). Cadherin-dependent adhesion is important for many physiological processes including establishment of cell polarity, morphogenetic movements such as epithelial/mesenchymal transitions, adherens junction formation, and cell-type sorting during development (Aberle et al., 1996; Birchmeier and Behrens, 1994; Nelson, 1996; Ranscht, 1994; Takeichi, 1995). Cadherins and their associated proteins are either tumor suppressor genes or oncogenes indicating this "pathway" is crucial for normal prostate epithelial cell growth (Birchmeier and Behrens, 1994). The cytoplasmic domain of cadherins interacts with molecules termed catenins which associate with the actin cytoskeleton (Aberle et al., 1996; Gumbiner, 1995; Vermeulen et al., 1996). The catenins include α, ß, γ/plakoglobin, *armadillo* and p120. α catenin is homologous to the cytoskeletal-associated protein vinculin. ß catenin is an "arm repeat" protein and is homologous to plakoglobin, p120, and *armadillo*. γ catenin and plakoglobin are identical (Knudsen and Wheelock, 1992; Pipenhagen and Nelson, 1993). Another cadherin-associated protein that contains "arm repeats" is called APC (Barth et al., 1997; Polakis, 1995). In addition, a number of normal tyrosine kinases and their oncogenic counterparts associate with cadherins including *met*, *src* , c-erbB2 and the EGF receptor (Aberle et al., 1996).

The cadherins, α catenin, plakoglobin and APC are all tumor suppressor genes (Birchmeier and Behrens, 1994). E-cadherin is deleted in 50% of diffuse type gastric cancers while mutations have also been found in ovarian, head/neck, skin, breast, colon, prostate, bladder, thyroid, and lung cancers (Birchmeier, 1995; Vermeulen et al., 1996). Plakoglobin is deleted in some breast and ovarian cancers (Aberle et al., 1995). The adenomatous polyposis coli gene (or APC) is a tumor suppressor gene in colon carcinoma (Polakis, 1995). There are a number of mutations found in the cadherin/catenin complex in prostate cancer (Giroldi et al., 1994; Paul et al., 1997). First, mutations on chromosome 5q cluster around the APC and α catenin genes (Kallioniemi and Visakorpi, 1996). Second, deletions of 16q are commonly observed and span the region containing the E-cadherin gene (Kallioniemi and Visakorpi, 1996). Third, PC3 prostate cancer cell line has deletions in the coding region of the α catenin gene (Ewing et al., 1995; Morton et al., 1993). Fourth, the c-erbB2 (Zhau et al., 1996) and c-*met* (Pisters et al., 1995) receptor tyrosine kinases are implicated in prostate cancer progression (Kallioniemi and Visakorpi, 1996). Finally, 17q loss of heterozygosity is observed near the BRCA1 locus which is adjacent to the plakoglobin gene (Aberle et al., 1995; Kallioniemi and Visakorpi, 1996). The loss of any one of the components of the cadherin complex may cause loss of cell-cell adhesion leading to the malignant progression of prostate carcinoma.

A number of cytoplasmic and receptor protein tyrosine kinases (PTKs) including *src*, EGF receptor and *met*, (scatter factor receptor), phosphorylate components of the cadherin/catenin complex (Kemler, 1993; Shibamoto et al., 1994; Takeda et al., 1995). Tyrosine phosphorylation of components of the cadherin/catenin complex suppresses cadherin-mediated adhesion and destabilizes adherens junctions (reviewed in (Brady-Kalnay and Tonks, 1995; Takeichi, 1993). Tyrosine phosphorylation is involved in the regulation of a diverse set of cellular behaviors including growth, differentiation, survival, cytoskeletal reorganization, and migration. Often tyrosine phosphorylation is a molecular switch, turning on or off various protein-protein interactions. Phosphotyrosine levels are controlled by the balanced and competing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). A diverse family of receptor-like (RPTPs) and nontransmembrane PTPs have been identified and characterized (Neel and Tonks, 1997; Streuli, 1996). Some of the RPTPs have structural homology to cell-cell adhesion molecules such as the neural cell adhesion molecule, N-CAM (see Figure 1). N-CAM contains multiple Ig domains and fibronectin type-III (FNIII) repeats in its extracellular segment and functions as a homophilic cell-cell adhesion molecule (Brummendorf and Rathjen, 1994; Cunningham, 1995). The RPTPs have cell adhesion molecule-like extracellular segments with intracellular segments that possess tyrosine phosphatase activity (Figure 1). The RPTPs may be directly sending intracellular signals in response to extracellular changes in cell adhesion.

We demonstrated that the RPTP, PTPμ, directly mediates cell adhesion and interacts with cadherins and the complex is regulated by tyrosine phosphorylation (see Figures 1 & 2) (Brady-Kalnay et al., 1993; Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). The RPTPs (Figure 1) have one or two conserved catalytic domains in their intracellular segments while their extracellular segments are quite diverse (Brady-Kalnay, 1998; Brady-Kalnay and Tonks, 1995). This proposal focuses on PTPμ which contains a MAM domain, an Ig domain and four FNIII repeats in its extracellular segment (Gebbink et al., 1991). The combination of motifs extracellular segment of PTPμ suggested that it might function in cell-cell adhesion. In fact we (Brady-Kalnay et al., 1993), and others (Gebbink et al., 1993), demonstrated that PTPμ participates in homophilic binding interactions. Both the immunoglobulin domain (Brady-Kalnay and Tonks, 1994a) and the MAM domain play a role in cell-cell aggregation (Zondag et al., 1995).

PTPμ contains a region of homology to the conserved intracellular domain of the cadherins in its juxtamembrane segment (Brady-Kalnay and Tonks, 1994b). We speculated that PTPμ may interact with catenins (Brady-Kalnay and Tonks, 1994b). We tested whether PTPμ could associate with cadherins or catenins, immunoprecipitations from epithelial cell extracts demonstrated that PTPμ associates with a complex containing cadherins, α catenin and ß catenin (Brady-Kalnay et al., 1995). We demonstrated that greater than 80% of the total cellular cadherins associate with PTPμ in epithelial cells. PTPμ, cadherins and catenins co-localize to points of cell-cell contact in epithelial cells. We also demonstrated that the intracellular segment of PTPμ binds directly to the intracellular domain of E-cadherin but not to α catenin or ß catenin *in vitro*. Pervanadate treatment of epithelial cells, which inhibits cellular tyrosine phosphatase activity including PTPμ, resulted in the tyrosine phosphorylation of the PTPμ-associated cadherins. These results indicate that the cadherins are likely to be an endogenous substrate for the PTPμ enzyme (Figure 2). Subsequently, other PTPs were shown to interact with cadherins and catenins (Aicher et al., 1997; Balsamo et al., 1996; Cheng et al., 1997; Fuchs et al., 1996; Kypta et al., 1996). Together, this suggests it is likely that regulation of cadherin/catenin complex by PTPs will be important mechanism of control in many cell types.

We demonstrated that PTPμ specifically interacted with N-cadherin, E-cadherin and cadherin-4 (also called R-cadherin) in extracts of rat lung (Brady-Kalnay et al., 1998). Our original study demonstrated an interaction between the intracellular segment of PTPμ and the intracellular segment of E-cadherin (Brady-Kalnay et al., 1995). We used a series of WC5 rat astrocyte-like cell lines, which express PTPμ endogenously and ectopically expressed mutant forms of E-cadherin that lack various portions of the cytoplasmic segment. The WC5 cell studies indicated that the C-terminal 38 amino acids were required for the interaction with PTPμ. According to many studies, this region also contains the ß catenin binding site (Brady-Kalnay et al., 1998). However, there is another region within that sequence that regulates cell-cell adhesion independently of catenin binding (C-terminal 19AA). Deletion of the C-terminal 19 amino acids of the cadherin cytoplasmic domain had no effect on catenin binding (Finnemann et al., 1997). However this deletion had a dramatic effect on cadherin-dependent adhesion and association with the cytoskeleton. This data indicates that the cytoskeletal stabilization required for cadherin adhesion is independent of catenin binding (Finnemann et al., 1997). Since PTPμ and tyrosine phosphorylation is known to regulate cadherin-dependent adhesion, it is possible that PTPμ is the crucial protein that interacts with the C-terminal 19AA of E-cadherin.

We tested whether alteration of tyrosine phosphorylation affects PTPμ/cadherin interactions. We used the WC5 rat cerebellar cell line which is transformed with a temperature-sensitive form of the Rous Sarcoma Virus (RSV) (Brady-Kalnay et al., 1998). The mutant RSV is temperature sensitive for pp60*src* tyrosine kinase activity. The data suggested that increased tyrosine phosphorylation induced by *src,* or its "downstream" tyrosine kinases, results in decreased association between PTPμ and E-cadherin. This decreased association correlates with increased tyrosine phosphorylation of E-cadherin. In addition, preliminary experiments indicate that the major site of tyrosine phosphorylation is in the C-terminal 38 amino acids of E-cadherin. This data indicates that tyrosine phosphorylation of that site may disrupt the interaction with PTPμ. Therefore, adhesive function may be controlled, by reversible tyrosine phosphorylation, through altering the presence of PTPs, which normally maintain cadherin in the functional dephosphorylated state. The association of the cadherins with both kinases and phosphatases indicates a critical role for dynamic phosphorylation in their function. Therefore, alterations in PTK or PTP expression may result in changes in cadherin function.

PTPμ is endogenously expressed in normal prostate cells (Figure 3). Surprisingly, we have found that the LNCaP prostate cancer cell line no longer expresses PTPμ (Figure 3) compared to normal prostate cells. LNCaP cells do express normal levels of cadherins and a number of their associated proteins (Figure 4). However, we have found that LNCaP cells are deficient in cadherin-dependent adhesion (Figure 8). The LNCaP cell line is a good model system for prostate cancer because it is androgen responsive, expresses prostate specific antigen and prostatic acid phosphatase (Horoszewicz et al., 1983). A major goal of this study was to add normal and mutant forms of PTPμ, using a retroviral expression system, to the LNCaP cells and determine how this affects adhesion, tyrosine phosphorylation, growth and tumorigenicity of the cells. As I will describe below, our data suggest that there is a dramatic effect on both cell growth and adhesion when PTPμ expression is restored in LNCaP cells. In addition, we tested whether this is a general phenomenon, i.e. are there changes in PTPμ expression in other prostate cancer cell lines and/or human prostate tumors. Loss of PTPμ may ultimately trigger the same cellular defects as loss of cadherins or catenins. In addition, since PTPμ is a cell surface protein normally expressed in prostate, loss of PTPμ expression could be a useful marker as a common step along the path to malignant progression in prostate cancer.

We tested the hypothesis that changes in PTPμ expression during prostate cancer progression result in modulations in cell-cell adhesion and signaling that culminate in alterations in contact inhibition of growth. We have a unique handle on a central question that plagues tumor cell biology: How does a prostate cancer cell evade normal growth control mechanisms such as contact inhibition of growth? Growth inhibitory signals are transmitted by cell-cell contact as normal cells reach confluence. In cancer such "contact inhibition of growth" is abrogated. Contact inhibition of growth has been popular a concept for decades but the molecular mechanisms are not understood to this day. The ability of normal prostate cells to exhibit contact inhibition of growth is likely to be due to cell-cell adhesion induced signaling events. Loss of contact inhibition of growth and adhesion-based signal transduction is crucial for the development of the transformed state. This proposal investigates one adhesion molecule, PTPμ that is ideally suited to control signals involved in the regulation of cell-cell adhesion. The association of PTPμ with E-cadherin suggests that this phosphatase may be the crucial cadherin-associated protein that regulates cytoskeletal association and adhesion. To investigate the role of PTPμ association with cadherins during prostate cancer progression, we identified a prostate cancer cell line in which PTPμ expression is lost. As shown in Figure 3, PTPμ is not expressed in the prostate carcinoma cell line (LNCaP) compared to normal prostate cells. This system allowed us to re-express normal and mutant forms of PTPμ. Interestingly, there are no changes in E-cadherin expression or the expression of catenins but the cells are defective in cadherin-dependent adhesion (Figures 4, 5, 8).

RESULTS FROM THE TECHNICAL OBJECTIVES:

I. Determination of the function of the PTPμ phosphatase in the cadherin complex

This aim directly tests the hypothesis that PTPμ plays a crucial role in adhesion and signaling in LNCaP cells. The research outlined in this aim relates the biochemical properties of the PTPμ enzyme to its ability to alter the phenotype of the LNCaP human prostate cancer cells. The LNCaP cell line is a good model system for prostate cancer because it is androgen responsive (Horoszewicz et al., 1983). In addition, these cells express prostate specific antigen and prostatic acid phosphatase (Horoszewicz et al., 1983). PTPμ expression was compared by immunoblotting the prostate carcinoma cell line (LNCaP) and normal prostate cells (Clonetics Inc.). Normal prostate expresses full length PTPμ (200kDa) and normal proteolytically processed fragments (105 &100kDa) (Brady-Kalnay and Tonks, 1994a). PTPμ is not expressed in LNCaP cells (Figure 3). Previously, LNCaP cells were shown to have the normal expression and interaction of E-cadherin, α catenin as well as ß and γ catenin (Morton et al., 1993). We observed normal expression patterns of E-cadherin, N-cadherin, α catenin, β catenin, γ catenin/plakoglobin, p120 and vinculin (Figure 4) under all conditions tested in this proposal. In addition, the cadherin complex contained α, ß, γ catenin and p120 (Figure 5) as expected. However, LNCaP cells are defective in cadherin-dependent adhesion (Figure 8). In this aim, we determined the effect that re-expression of PTPμ in LNCaP cells had on cadherin-dependent adhesion and overall cellular behavior.

A. Re-expression of PTPμ

Long-term over-expression of PTPμ results in cell death. Therefore, we needed a high level of transient expression or a repressible expression system. Both of these criteria are fulfilled in a new retroviral/tetracycline-repressible system (Paulus et al., 1996) that we used to make retroviruses that express PTPμ (Figure 6). We constructed a full length PTPμ protein tagged with the green fluorescence protein (GFP). The LNCaP cells infected with a vector only virus did not show any fluorescence (Figure 7A-B). However, when the PTPμ-GFP retrovirus was used to infect LNCaP cells, PTPμ-GFP was expressed in most cells (Figure 7C-D) and localized to points of cell-cell contact as expected. In addition, the PTPμ-GFP expression is negatively regulated by the presence of tetracycline (Figure 7E-F).

B. Analysis of cell-cell adhesion

The LNCaP cells express E-cadherin, N-cadherin and the laminin receptor(s). To test whether these proteins were functional, we developed an *in vitro* adhesion assay that tests the ability of cells to bind to purified proteins coated on a dish. First, the proteins were purified as previously described (Burden-Gulley and Lemmon, 1996). A drop of purified protein is added to the petri dish, the protein binds to the dish, and nonspecific adhesion is blocked by bovine serum albumin. Cells are added to the dish, unbound cells are washed off and adhesion of cells to the purified protein is visualized by dark field microscopy. The purified proteins that were used include laminin, E-cadherin, N-cadherin and PTPμ.

The LNCaP cells both pre- and post-infection with PTPμ-GFP were tested for their ability to bind to coverslips coated with: 1) the extracellular matrix protein laminin; 2) the extracellular domain of PTPμ; 3) the extracellular domain of E-cadherin; or 4) the extracellular domain of N-cadherin. The vector infected LNCaP cells only bound to the laminin coated spots (Figure 8 B) but not to PTPμ, E-cadherin or N-cadherin (Figure 8A,C, D). We then infected LNCaP cells with a PTPμ-GFP retrovirus, no change was observed in the ability of PTPμ-GFP infected cells to bind laminin (panel F). However, the PTPμ-GFP infected cells bound to PTPμ, E-cadherin and N-cadherin substrates (Figure 8E,G, H). This result is quite exciting, since it suggests that PTPμ is regulating the ability of cadherins to mediate cell adhesion (Hellberg et al., 2000a; Hellberg et al., 2000b). This is quite exciting, it suggests that PTPμ may be regulating the ability of cadherins to mediate cell adhesion presumably through some type of protein-protein interaction or signaling event.

C. Analysis of protein-protein interactions

We investigated whether the cadherin complex is altered in LNCaP cells. Specifically, we performed immunoprecipitation/immunoblot analyses on cadherins and catenins. The vector only or PTPμ-GFP infected cells were compared to determine if there are changes in the composition of the cadherin complex (Hellberg et al., 2000b). The traditional components of the cadherin/catenin complex (α catenin, β catenin, plakoglobin and p120) were expressed in uninfected, vector-infected and PTPμ-infected LNCaP cells (Figure 4). In addition, the complex contained α, ß, γ catenin an p120 even in the absence of PTPμ (Figure 5). The phosphorylation of the proteins in the cadherin complex in LNCaP cells are also being compared by immunoprecipitation with cadherin or catenin antibodies followed by immunoblotting with antiphosphotyrosine antibodies. We are still in the process of examining changes in any of the cadherin-associated proteins phosphorylation state.

D. Examine changes in cellular behavior

Tyrosine phosphorylation has been proposed to affect many aspects of cell behavior such as transformation, loss of growth regulation, invasion, changes in cell adhesion or cytoskeletal association. When PTPμ is reintroduced into LNCaP cells, we observed changes in both PTPμ, E-cadherin and N-cadherin-dependent cell adhesion. We hypothesized that this change in adhesion is likely to result in other changes in LNCaP cell behavior. We investigated whether reintroduction of PTPμ results in changes in particular aspects of cell behavior including:

1) cell growth analyses (growth curves-doubling time and saturation density measurements)
   We observed that re-expression of PTPμ negatively regulated cell growth (Hellberg and Brady-Kalnay, 2000). (see aim II and Figure 15).

2) ability of the cells to undergo apoptosis (FACS, anoikis assay)
   Cell-cell adhesion may also be involved in the prevention of apoptosis (programmed cell death). Cell cycle analysis using fluorescent activated cell sorting of propidium iodide stained cells was used to determine if there was an apoptotic peak of cells. No change in the number of apoptotic cells was observed when wild type PTPμ was added to LNCaP cells. In addition, an anoikis assay was performed. Anoikis is a type of cell death that results from the loss of adhesion to the extracellular matrix. The number of floating, or non-adherent, cells are counted. No difference in the number of non-adherent cells was observed.

3) alterations in the ability to form cellular junctions (morphology, changes in subcellular localization)
   E-cadherin is involved in the establishment and regulation of both the adherens junction and tight junctions (Marrs et al., 1995; McNeill et al., 1990). We performed immunocytochemistry to analyze whether the localization of cadherins or catenins changed in response to PTPμ re-expression. Most components of the

cadherin/catenin complex were localized at cell-cell contact sites in LNCaP cells even in the absence of PTPμ. There was a slight increase in the amount of E-cadherin at cell-cell contact sites in cells expressing PTPμ (Hellberg et al., 2000b). We were planning on doing transepithelial resistance measurements to look at cell junction formation. Unfortunately, LNCaP cells do not form a tight monolayer even in the presence of PTPμ so transepithelial resistance measurements could not be performed.

II. Assess the requirement for phosphatase activity in the regulation of adhesion and signal transduction

This aim tests the hypothesis that the PTPμ enzyme directly mediates a signal that regulates the tyrosine phosphorylation state of key cellular substrates. Both normal and mutant forms of PTPμ will be expressed in LNCaP cells. The mutant forms of PTPμ include catalytically dead, substrate trapping and dominant negative versions of the phosphatase which are useful for determining physiological substrates and functions of these enzymes (Flint et al., 1997; Garton et al., 1996). The PTP enzymes share a catalytic domain with ~30% amino acid identity. The catalytic domain is characterized by a unique sequence motif {(I/V)HCXAGXXR(S/T)G}, which forms the phosphate-binding pocket. The cysteine residue sits at the base of the active site cleft and is essential for catalysis (Denu et al., 1996). The structural data on the PTP catalytic domain suggested mutations that could be made to alter either the affinity for substrate (K_m) or the rate of catalysis (V_{max}). A diagram of these residues in the active site of PTP1B is provided (Figure 9). The "substrate trapping" mutant (D-A) retain normal affinity for their substrate but catalytic activity is markedly reduced resulting in irreversible binding of the substrate (Neel and Tonks, 1997). Mutations of the conserved cysteine residue (C-S), or a conserved aspartate (D-A) residue create a substrate trap by affecting only V_{max} and not K_m. While mutation of a conserved arginine (R-M) residue decreases both K_m and V_{max} of the enzyme which may act as a "dominant negative" (Neel and Tonks, 1997). These three mutant forms of PTPμ have been generated. The different forms of the enzymes were added to LNCaP cells using the retroviral system and analyzed as in section I. The LNCaP model system allows us to add mutants of PTPμ without the complication of endogenous PTPμ being present. As anticipated this aim separated the role PTPμ phosphatase activity performs from the role played by the domains involved in adhesion and protein-protein interaction.

We used the retroviral system (Figure 6) to express wild-type, D-A, C-S and R-M forms of PTPμ in LNCaP cells (Figure 10). All mutants were localized at the cell surface similar to wild-type PTPμ (Figure 11). The LNCaP cells expressing wild type or mutant forms of PTPμ-GFP were tested for their ability to bind to laminin, PTPμ, E-cadherin or N-cadherin. LNCaP cells infected with control virus only bound to laminin (Figure 12 B) but not to PTPμ, E-cadherin or N-cadherin (Figure 12 A, C, D). No change was observed in the ability of any of the PTPμ-GFP infected cells to bind to laminin. However, both the wild type and mutant PTPμ-GFP infected cells bound to the PTPμ, E-cadherin and N-cadherin substrate (Figure 12 E-T). These results are quantitated in Figure 13. These data indicate that the presence of the PTPμ protein restores cadherin-dependent adhesion but phosphatase activity is not necessarily required (Hellberg et al., 2000a; Hellberg et al., 2000b). Our hypothesis is that PTPμ may be recruiting proteins to the cadherin complex that somehow restore cadherin-dependent adhesion.

To test this hypothesis, we generated a new mutant form that contained the entire extracellular and transmembrane domains of PTPμ but lacked the intracellular phosphatase domains (PTPμ-extra-see Figure 10). This mutant form induced adhesion to PTPμ as expected (Figure 14A). Importantly, PTPμ-extra did not restore adhesion to E-cadherin (Figure 14C). These data indicate that the presence of the phosphatase domains is required to restore cadherin-dependent adhesion (Hellberg et al., 2000a; Hellberg et al., 2000b).

Next, growth rates of the cells infected with wild type or mutant forms of PTPμ were analyzed. Surprisingly, expression of wild type PTPμ negatively regulated the growth of LNCaP cells (Figure 15). However, the mutant forms of PTPμ with altered catalytic activity did not affect cell growth. **These data suggest that PTPμ phosphatase activity is required to negatively regulate cell growth** (Hellberg and Brady-Kalnay, 2000). Wild type PTPμ appeared to induce a saturation density effect on the cells. Interestingly, preliminary data suggests that it may affect the G1 to Go transition of the cell cycle. This is intriguing because the mutants have allowed us to separate the effect of PTPμ on cell growth and cadherin-dependent cell adhesion. Specifically, we have determined that cell growth is regulated by enzymatic activity while effects on cadherin-dependent adhesion are regulated by the presence of the intracellular domain presumably by protein-protein interactions.

III. Analyze alterations in PTPμ expression in prostate cancer xenografts and tumors

This aim tests the hypothesis that changes in PTPμ expression are commonly observed in human prostate cancer. Interestingly, reduced E-cadherin expression has been observed in a number of invasive or metastatic prostate tumors (Cheng et al., 1996; Murant et al., 1997; Umbas et al., 1994) and reviewed in (Cohen et al., 1997; Giroldi et al., 1994; Paul et al., 1997). The ability of transformed cells to invade *in vitro* is prevented if these cells are transfected with cDNA for E-cadherin. In this case, E-cadherin altered the differentiation of the cells (from a fibroblastic to an epithelial phenotype) and negatively regulated cell motility (Chen and Obrink, 1991; Frixen et al., 1991; Vleminckx et al., 1991). Reduction of E-cadherin protein levels and deletion of the α catenin gene have been observed in some prostate cancer cell lines (Morton et al., 1993). In addition, poor patient survival correlates with aberrant E-cadherin and α catenin expression in prostate cancer (Richmond et al., 1997). PC3 prostate cancer cells have a deletion in the α catenin gene (Morton et al., 1993). Restoration of α catenin expression in the PC3 cell line results in decreased tumorigenicity (Ewing et al., 1995). Loss of PTPμ may ultimately trigger the same cellular defects as loss of cadherins or catenins.

To determine if loss of PTPμ is detectable in other prostate cancer cell lines or tissues, we performed immunohistochemistry and immunoblotting experiments. Prostate cancer tissue sections are being analyzed by immunohistochemistry for changes in PTPμ expression. We have initiated a project that is still in progress, with histology core facility here at CWRU, to perform immunohistochemical analyses of PTPμ expression in human prostate tumors. Immunohistochemical analyses are being performed using cancerous tissues of different grades to determine if there are changes in PTPμ expression during malignant progression. Analysis of the staining pattern will be performed by a prostate pathologist, Dr. Tom Pretlow. Our preliminary data suggests that there may be changes in PTPμ expression in Gleason Pattern 3 prostate cancers.

We analyzed some prostate cancer cell lines and xenografts for PTPμ expression by immunoblot. Changes in PTPμ expression were only observed in LNCaP and CWRU xenograft prostate cancer cells (Figure 16). The xenograft tumor cell system developed at CWRU by Dr. Tom Pretlow is a useful tool because it is serially passaged primary human prostate tumor cells (Wainstein et al., 1994). We observed no PTPμ expression in both the androgen-dependent (CWR22) and relapsed (androgen-insensitive) cell lines (CWR22R). Therefore, changes in PTPμ expression may be relevant to a subset of prostate cancer and not simply a phenomenon observed in LNCaP cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. Comparison of PTPμ, cadherin and catenin protein expression in normal prostate cells (Clonetics) and LNCaP cells
2. Analysis of LNCaP adhesion to various cell adhesion molecules
3. Generation of wild type and mutant forms of PTPμ tagged with the green fluorescence protein by site directed mutagenesis
4. Construction of retroviral constructs containing wild type and mutant PTPμ-GFP using pBSTR1
5. Packaging and production of viral stocks for wild type and mutant PTPμ-GFP
6. Infection of LNCaP cells and visualization of expression of wild type and mutant PTPμ-GFP
7. Performance of adhesion assays on retrovirally-infected LNCaP cells
8. Analysis of cell growth for retrovirally-infected LNCaP cells
9. Major accomplishments include: determination of the requirement for the presence of PTPμ protein for proper cadherin-dependent adhesion as well as the requirement for catalytically active PTPμ phosphatase for the negative regulation of cell growth

REPORTABLE OUTCOMES:

Publications:

1. Mourton, T., Hellberg, C., Burden-Gulley, S., Hinman, J., Rhee, A. and **Brady-Kalnay, S.** The protein tyrosine phosphatase, PTPμ, binds and recruits RACK1 to points of cell-cell contact. Submitted to the *Journal of Cell Biology.*

2. Hellberg, C.B., Burden-Gulley, S.M., Pietz, G.E., and **Brady-Kalnay, S.** Expression of the receptor protein tyrosine phosphatase PTPμ restores E-cadherin-dependent adhesion in human prostate carcinoma cells. Submitted to the *Journal of Cell Biology.*

3. Hellberg, C.B., Burden-Gulley, S.M., and **Brady-Kalnay, S.** N-cadherin-dependent adhesion is regulated by the receptor protein tyrosine phosphatase PTPμ. In preparation.

4. Hellberg, C.B., and **Brady-Kalnay, S.** The receptor protein tyrosine phosphatase PTPμ negatively regulates cell growth which is dependent upon phosphatase activity. In preparation.

Abstracts:

1. Hellberg, C., Burden-Gulley, S., Pietz, G. and **Brady-Kalnay, S.** Expression of the Receptor Protein Tyrosine Phosphatase, PTPμ, is necessary for Cadherin-mediated adhesion in Prostate carcinoma cells. Gordon Conference on Cell Adhesion. 1999.

2. Hellberg, C., Burden-Gulley, S., Pietz, G. and Brady-Kalnay, S. Cadherin-mediated adhesion is dependent on the expression of the receptor protein tyrosine phosphatase PTPμ in prostate carcinoma cells. UICC Advanced Course in Cell Signaling and Cancer, Tammsvik, Sweden. 1999.

CONCLUSIONS:

Many studies suggest that when any protein in the cadherin/catenin pathway is altered during malignant transformation it lead to decreased adhesion, invasion and metastasis. Tyrosine phosphorylation of E-cadherin and catenins has also been observed in many carcinomas and this correlates with decreased cell adhesion. However, the precise regulatory mechanism or the crucial substrates for phosphorylation are unknown. The net level of tyrosine phosphorylation is controlled by the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). We recently demonstrated that the receptor PTP, PTPμ, directly interacts with E-cadherin. PTPμ is endogenously expressed in normal prostate cells. Surprisingly, we have found that the LNCaP prostate cancer cell line does not express PTPμ. LNCaP cells do express E-cadherin and the catenins although the LNCaP cells are deficient in cadherin-dependent adhesion. A major goal of this study was to re-introduce PTPμ into LNCaP prostate cancer cells and determine how this affects adhesion, tyrosine phosphorylation, growth and tumorigenicity of the prostate cancer cells.

A new retroviral/tetracycline-repressible system (Paulus et al., 1996) was used to make retroviruses that express PTPμ-GFP (Figure 6). The retroviruses were used to perform transient assays at high levels of infection. Both normal and mutant forms of PTPμ will be expressed in LNCaP cells (Figures 9-11). The mutant forms of PTPμ include catalytically dead, substrate trapping and dominant negative versions of the phosphatase (Figure 9) which are useful for determining physiological substrates and functions of these enzymes (Flint et al., 1997; Garton et al., 1996). The LNCaP cells infected with a vector only virus did not show any fluorescence (Figure 11). However, when the wild type or mutant PTPμ-GFP containing retrovirus were used to infect LNCaP cells, all forms of PTPμ-GFP were expressed (Figures 10-11). The LNCaP cells both pre- and post-infection with wild type or mutant forms of PTPμ-GFP were tested for their ability to bind to coverslips coated with four different spots. The spots contained either: 1) the extracellular matrix protein laminin; 2) the extracellular domain of PTPμ; 3) the extracellular domain of E-cadherin; or 4) the extracellular domain of N-cadherin. The vector only infected LNCaP cells only bound to the laminin coated spots but not to PTPμ, E-cadherin or N-cadherin (Figure 12). We infected LNCaP cells with wild type or mutant PTPμ-GFP containing retroviruses and tested the adhesivity of the infected cells (Figure 12). No change in the ability to bind laminin was observed in any of the PTPμ-GFP infected cells (Figure 12). However, both the wild type and mutant PTPμ-GFP infected cells bound to the PTPμ, E-cadherin and N-cadherin (Figure 12). Quantitation of the data gave similar results (Figure 13). This data indicates that the presence of the PTPμ protein is required for cadherin-dependent adhesion but phosphatase activity is not necessarily required.

Growth rates of the cells infected with wild type or mutant forms of PTPμ were analyzed (Figure 15). Surprisingly, expression of wild type PTPμ negatively regulated the growth of LNCaP cells (Figure 15). However, the mutant forms of PTPμ with altered catalytic activity did not effect growth of LNCaP cells (Figure 15). These results are very intriguing because these mutants have allowed us to separate the effect of PTPμ on cell growth and cell adhesion. We will be following up on these results by trying to determine the interacting proteins and crucial substrates of the wild type PTPμ enzyme as outlined above. The study of this coordinated regulation of adhesion and tyrosine phosphorylation by PTPμ will be important for understanding both the loss of adhesion and loss of growth control seen upon malignant transformation in prostate cancer.

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Hellberg, C.B., S.M. Burden-Gulley, G.E. Pietz, and S.M. Brady-Kalnay. 2000b. Expression of the receptor tyrosine phosphatase PTPμ restores E-cadherin-dependent adhesion in human prostate carcinoma cells. *Submitted to J. Cell Biol.*

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APPENDICES:

Figures 1-16

Four preprints of manuscripts

Two abstracts from meetings

FINAL REPORT INFORMATION:

Publications:

1. Mourton, T., Hellberg, C., Burden-Gulley, S., Hinman, J., Rhee, A. and **Brady-Kalnay, S.** The protein tyrosine phosphatase, PTPμ, binds and recruits RACK1 to points of cell-cell contact. Submitted to the *Journal of Cell Biology.*

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Tracy Mourton (Research Assistant)
Rachna Dave (Undergraduate Researcher)
Dan Davis (Undergraduate Researcher)
Jason Hinman (Undergraduate Researcher)

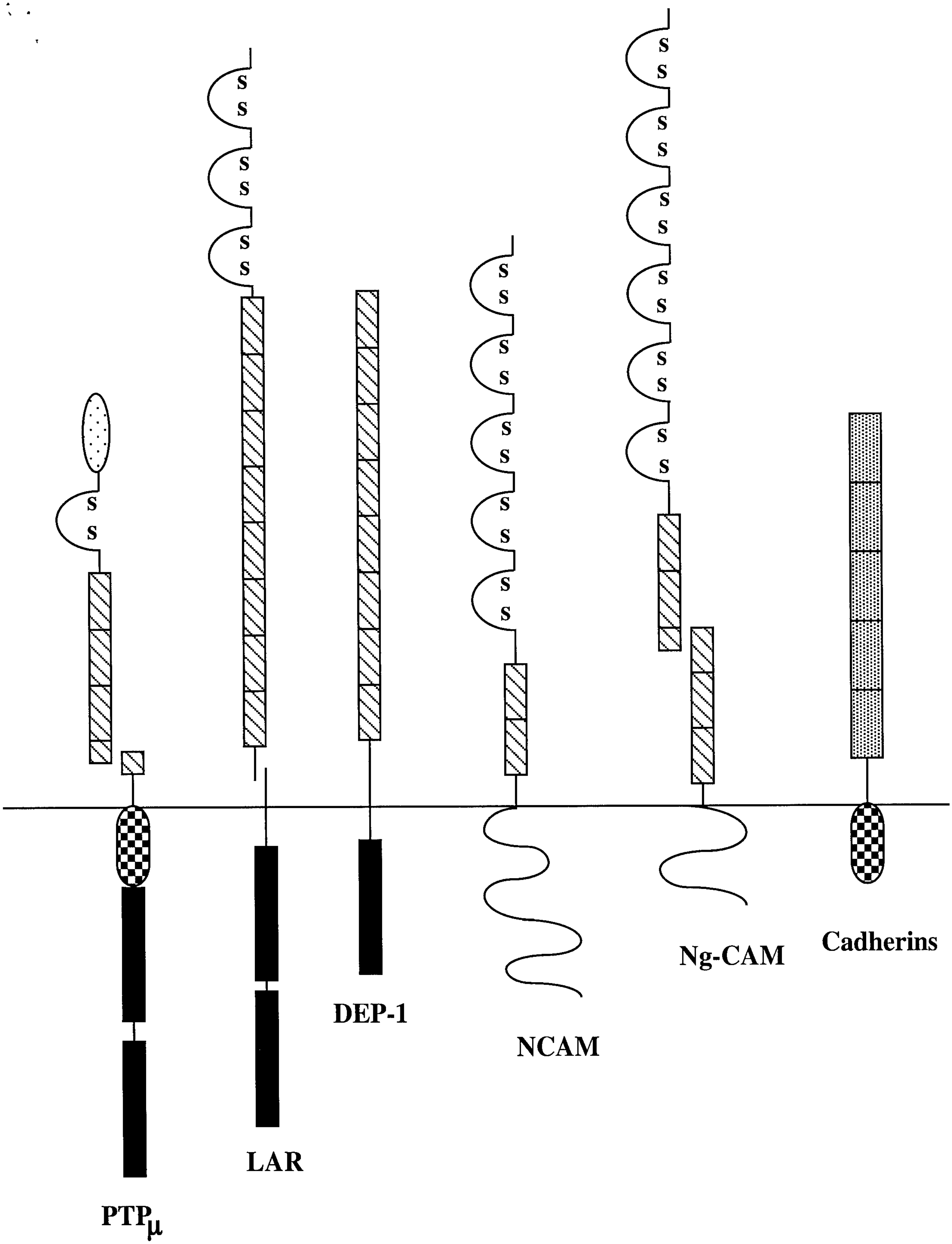


Figure 1. The receptor PTPs (PTP , LAR and DEP-1) and other cell-cell adhesion molecules including NCAM, Ng-CAM and Cadherins are shown diagramatically.

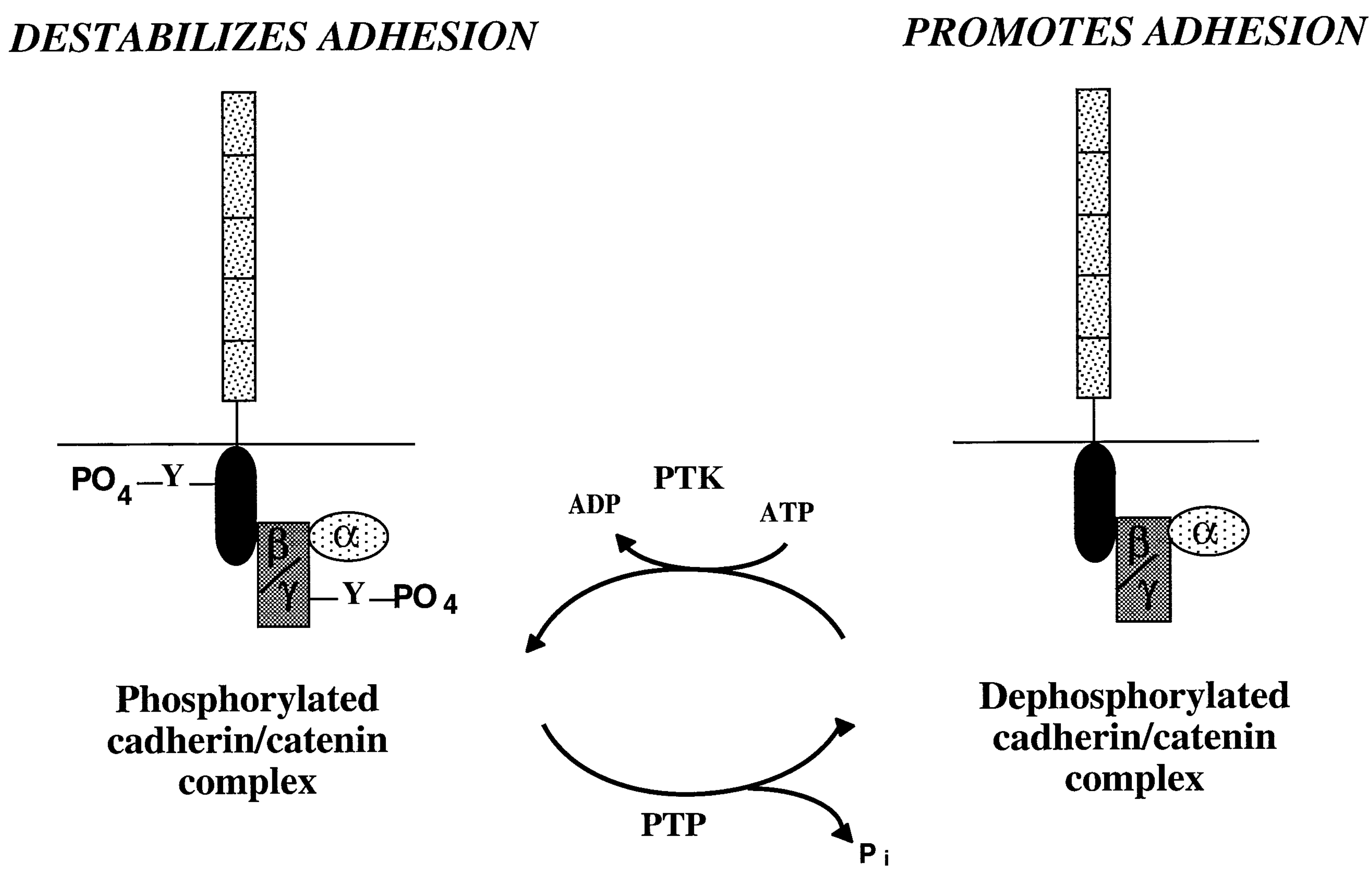


Figure 2. The hypothetical view of how PTP may regulate the function of the cadherin/catenin complex in cells.

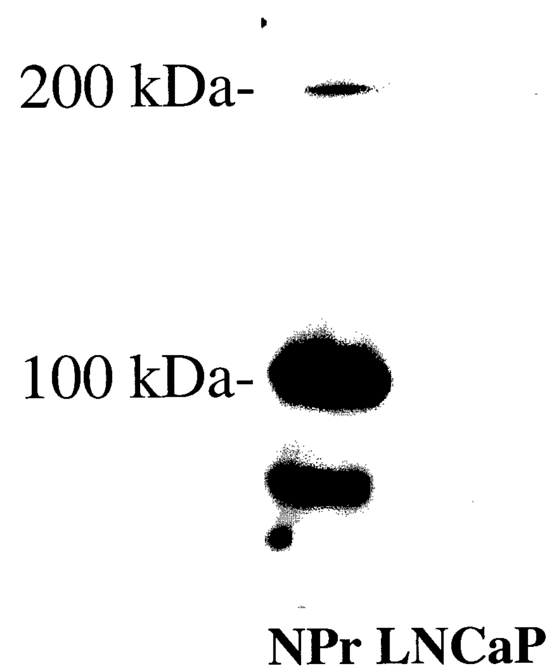


Figure 3. Normal prostate cells (NPr) express PTPμ but LNCaP prostate carcinoma cells do not

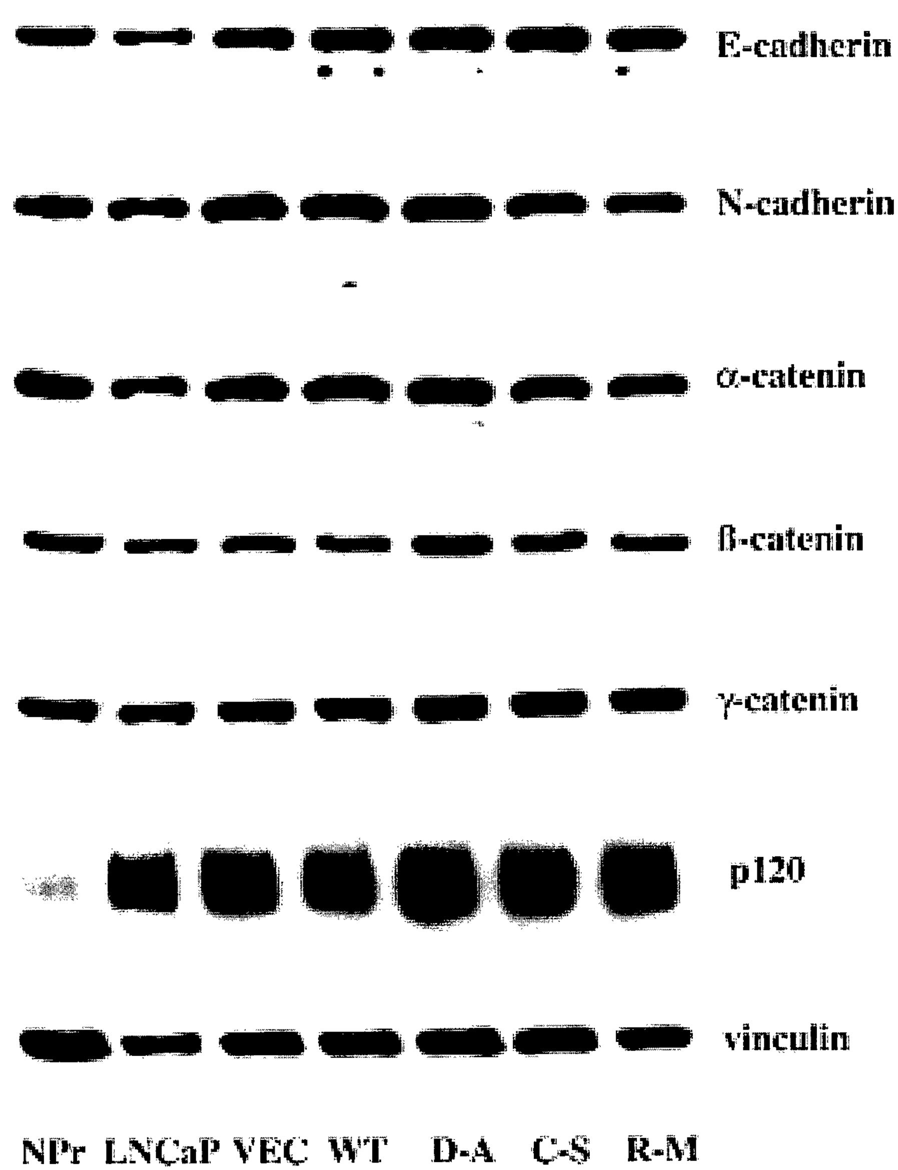


Figure 4. Expression of cadherins and catenins in Normal prostate cells (NPr) and LNCaP cells infected with vector (vec), wild-type PTPμ or three mutant forms of PTPμ (D-A, C-S and R-M)

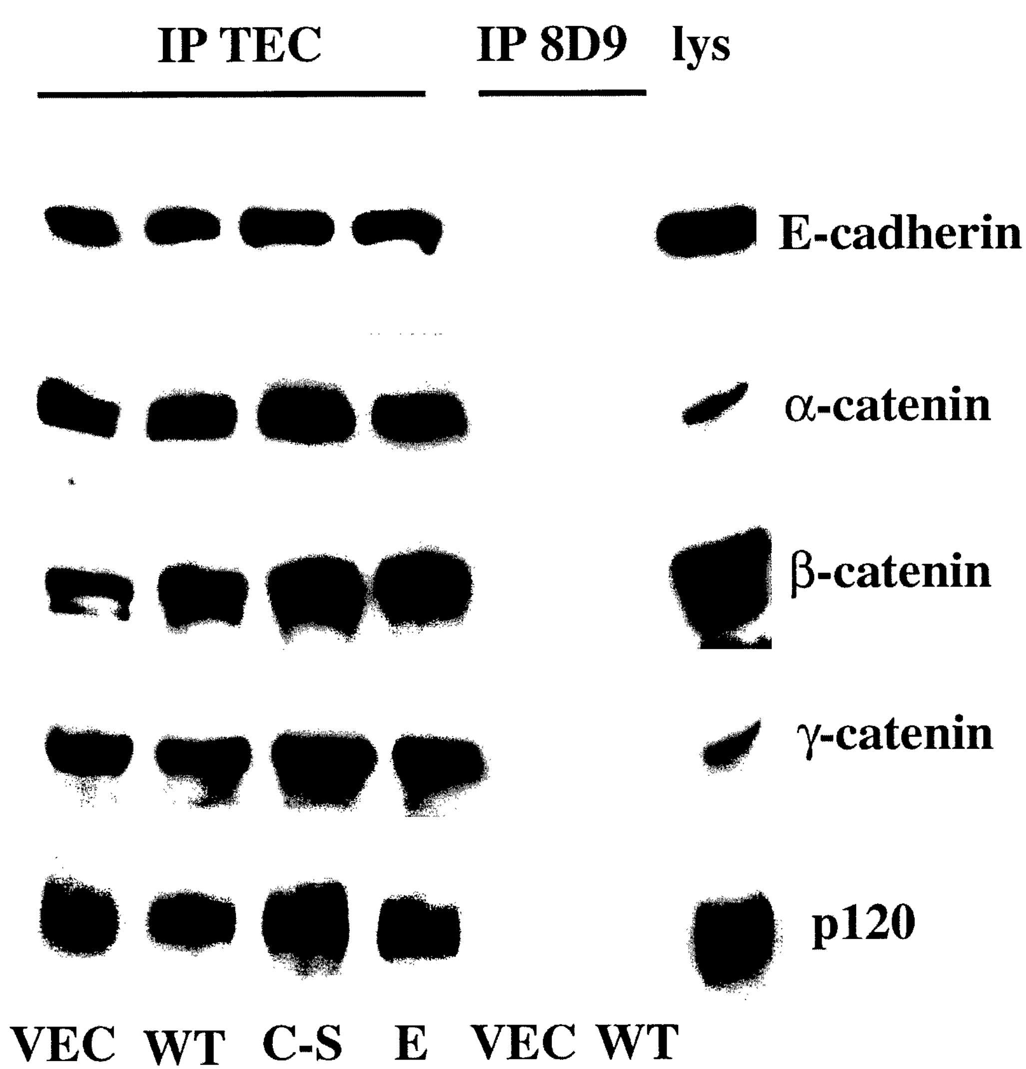
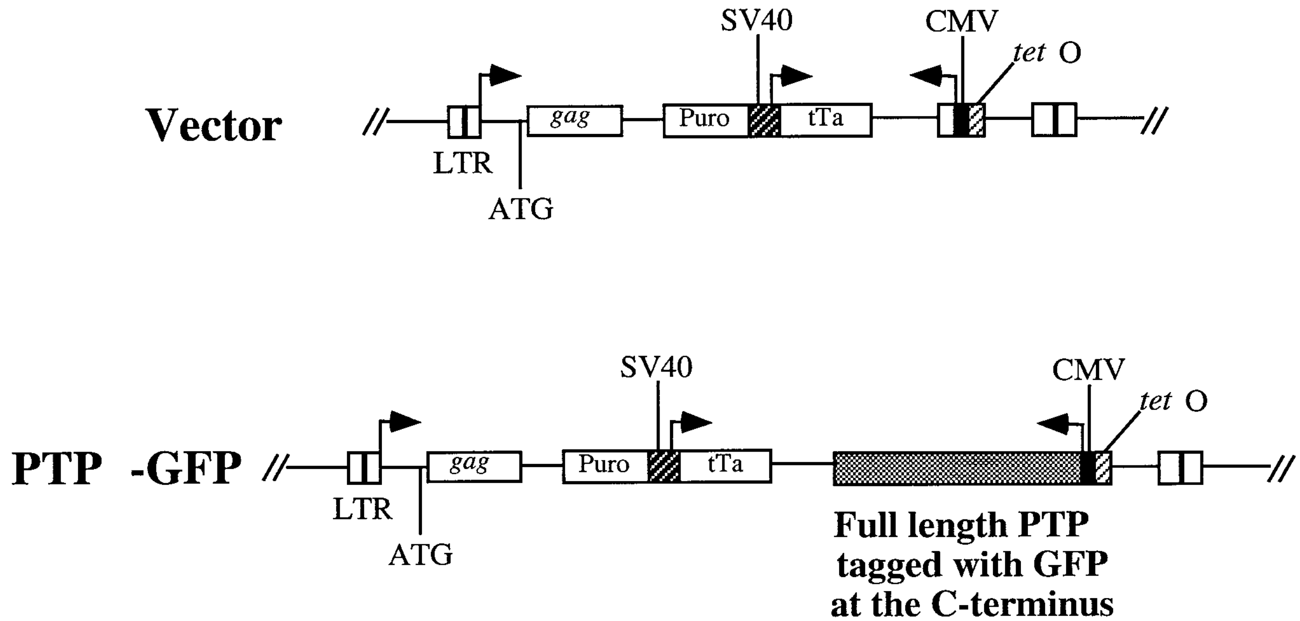


Figure 5. The E-cadherin complex is intact in LNCaP cells regardless of PTPμ expression. LNCaP cells were infected with vector, wild-type PTPμ, the C-S or extra (E) mutant forms of PTPμ. Immunoprecipitations were done with E-cadherin (IP TEC) or control antibodies (IP 8D9). Lysates (lys) were also run on SDS-PAGE gels and immunoblotted with antibodies to the proteins indicated on the right.

Retroviral constructs were generated using the Tet-Off Plasmid (pBSTR1)



[Either wild type PTP , C1095S, D1063A, or R1101M]

Figure 6. The retroviral expression system that we use to re-express wild type and mutant forms of PTP in LNCap cells is shown diagramatically.

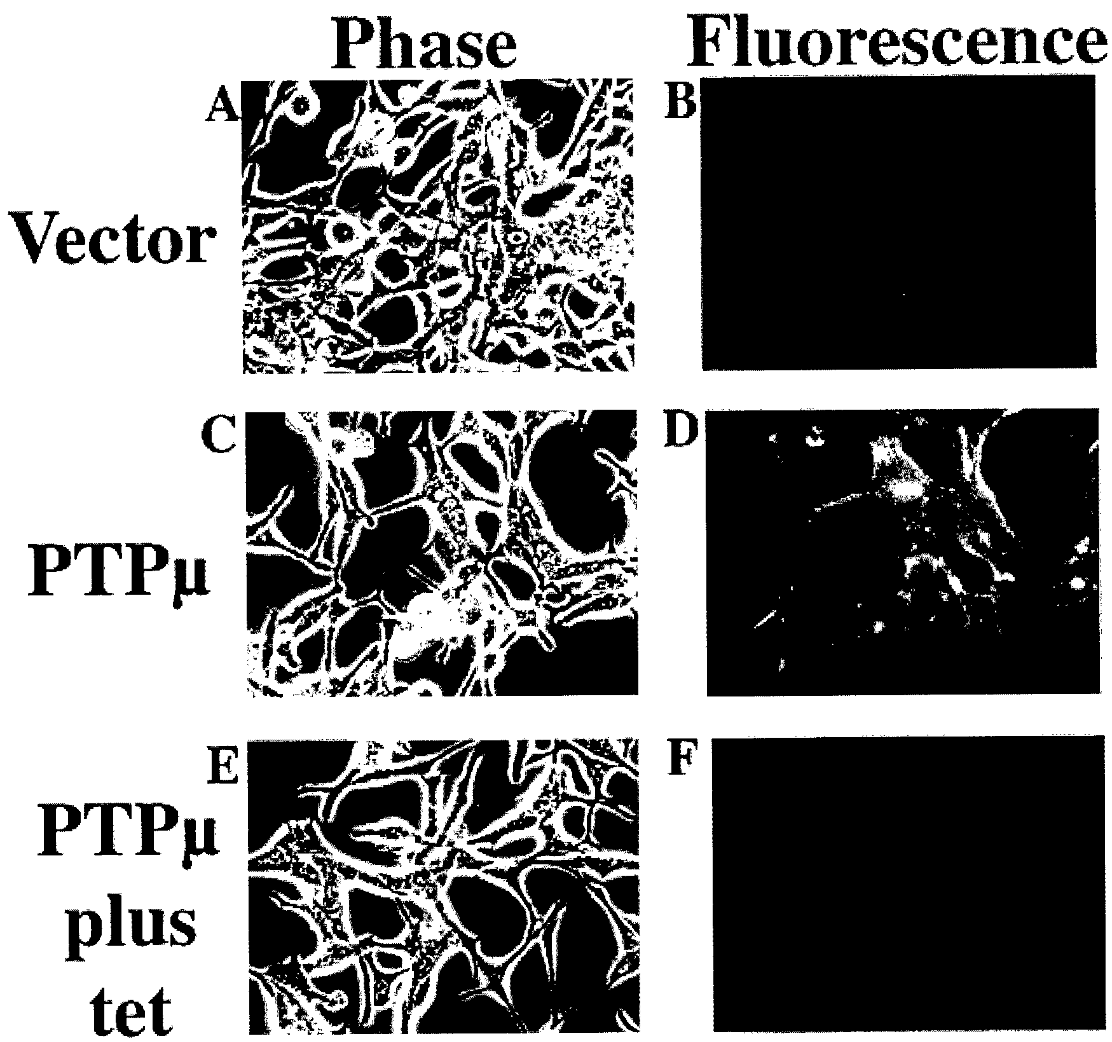


Figure 7. PTPμ-GFP expression is controlled by tetracycline. LNCaP cells were infected with wild-type PTPμ in the presence or absence of tetracycline. Phase and fluorescent micrographs are shown.

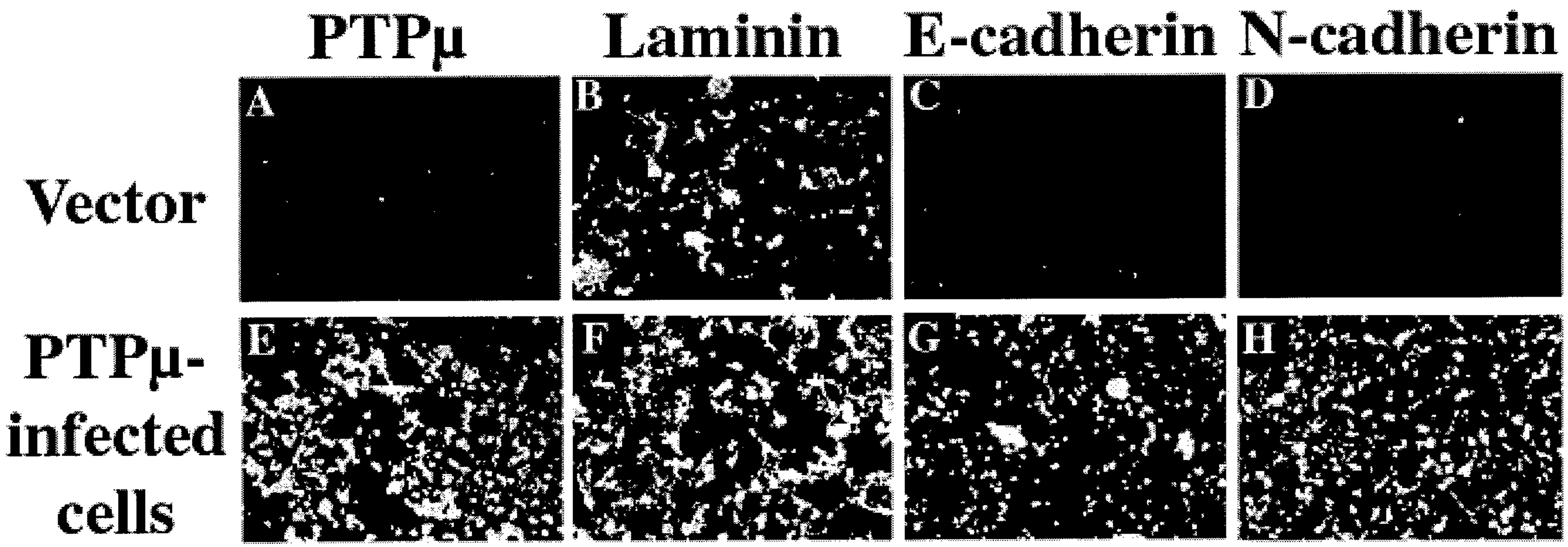
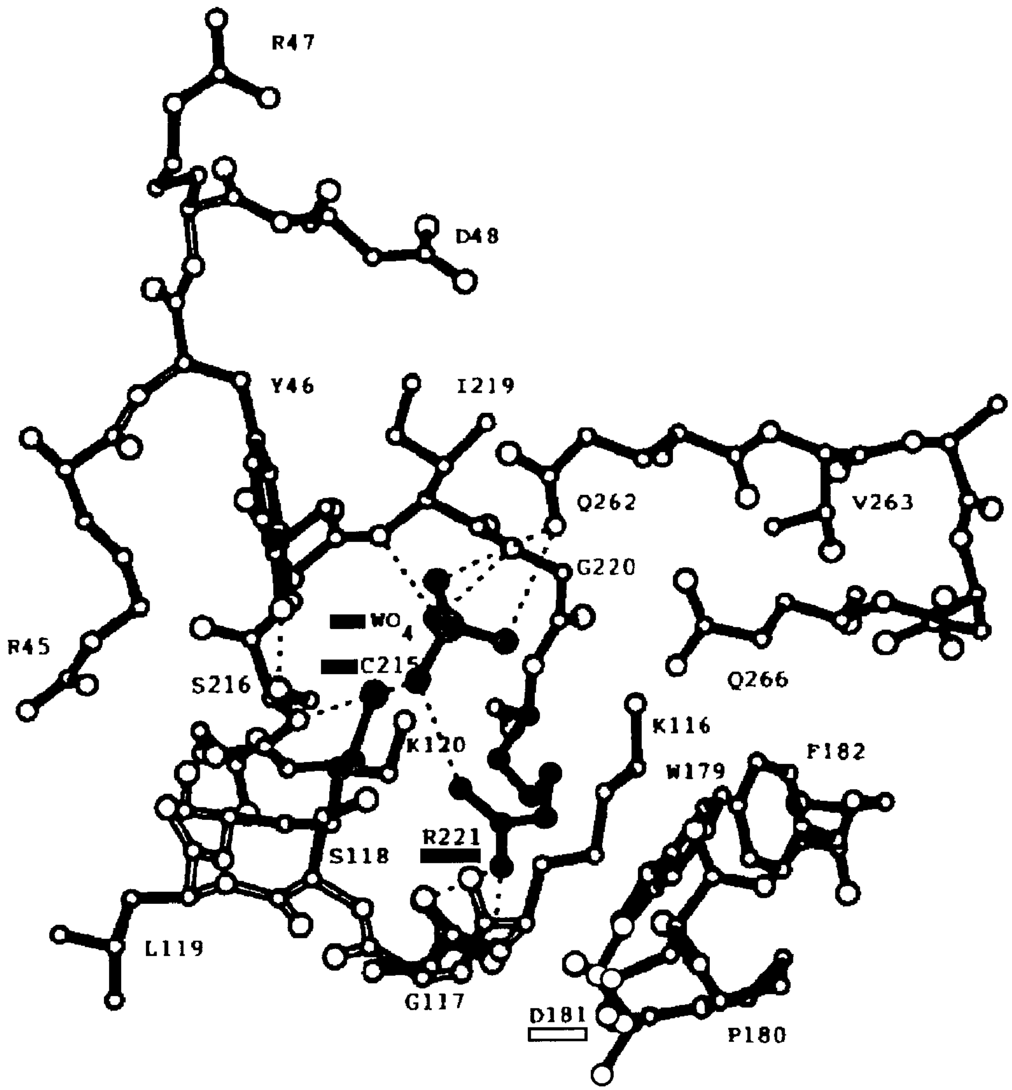


Figure 8. PTPμ restores cadherin-dependent adhesion. LNCaP cells were Infected with vector or wild-type PTPμ and an adhesion assay was performed. Adhesion of the LNCaP cells to PTPμ, laminin, E- and N-cadherin is shown. Dark field micrographs of the adherent cells are shown.



- cys>ser: catalytically inactive
- arg>met: "dominant negative"
- asp>ala: "substrate trapping"

Figure 9. Catalytic site of PTPs with the mutations that alter catalytic activity indicated in color.

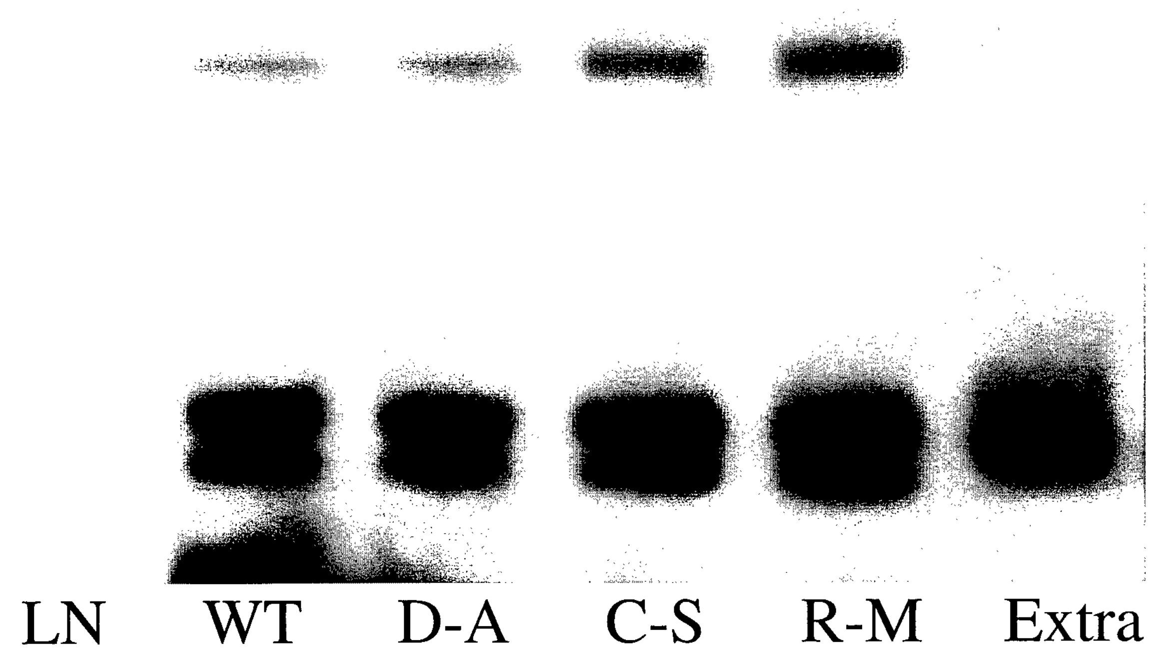


Figure 10. Re-expression of PTPμ mutants in LNCaP cells. LNCaP cells Were infected with wild type, D-A, C-S, R-M or extra mutants of PTPμ. Normal prostate was run as a control.

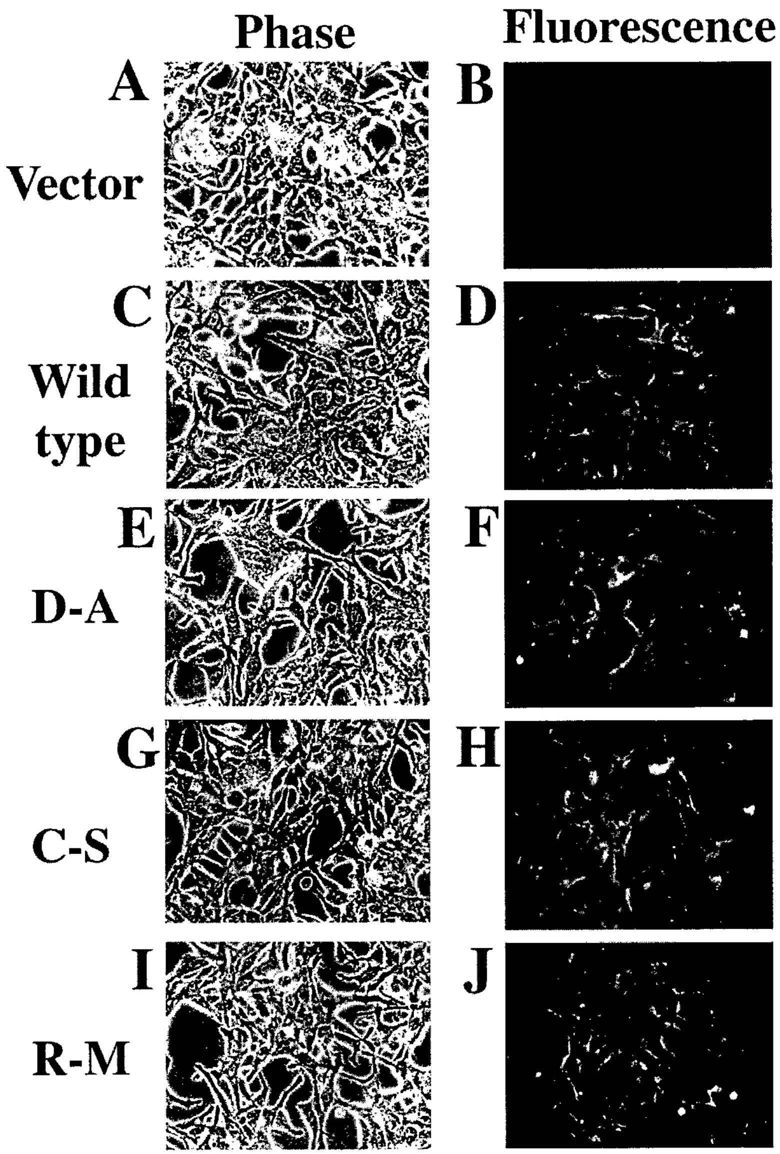


Figure 11. All of the PTPμ-GFP mutants are expressed in LNCaP. LNCaP cells were infected with wild-type or mutant forms of PTPμ. Phase and fluorescent micrographs are shown.

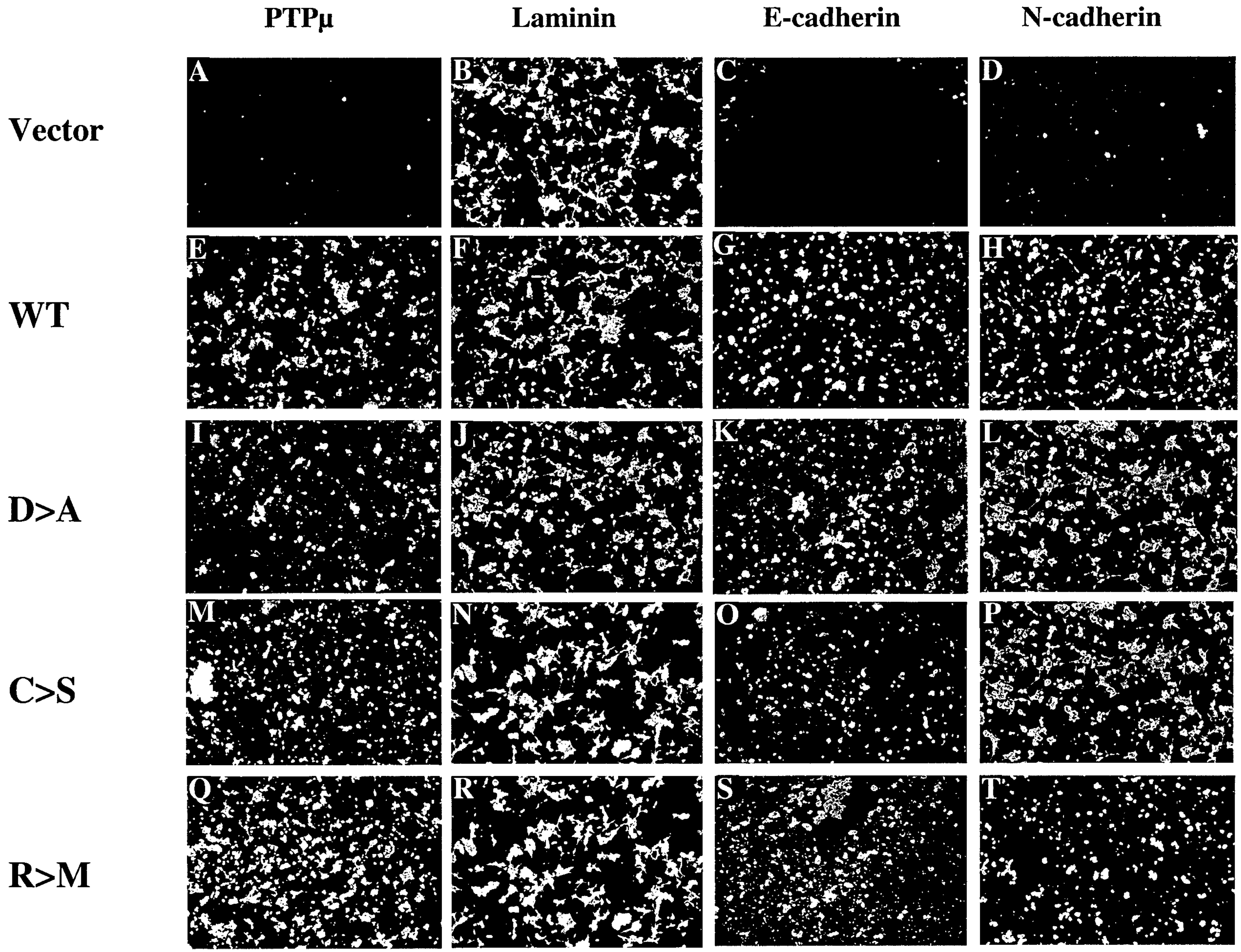


Figure 12. PTPμ re-expression restores both PTPμ and cadherin-dependent adhesion to LNCap cells regardless of catalytic activity of the PTPμ phosphatase.

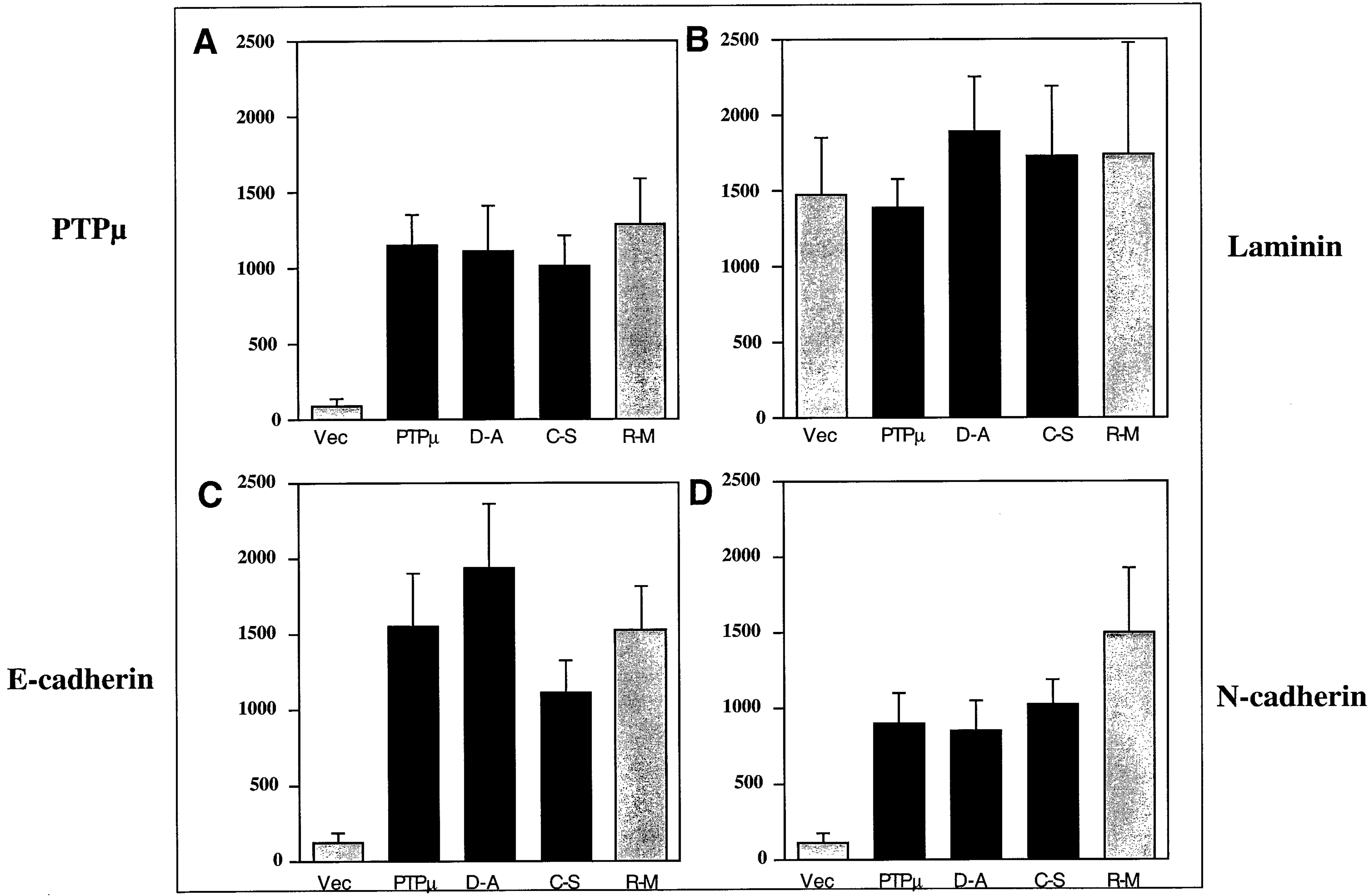


Figure 13. Adhesion of cells expressing wild type and mutant forms of PTPμ to PTPμ, laminin, E-cadherin or N-cadherin substrates.

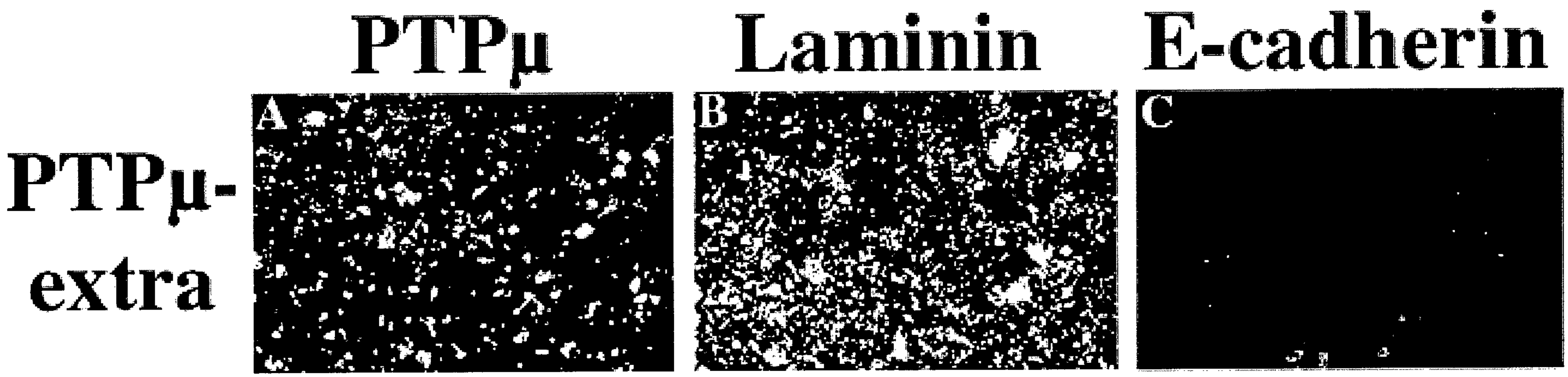


Figure 14. PTPµ-extra does not restore adhesion to E-cadherin but does induce adhesion to PTPµ as expected.

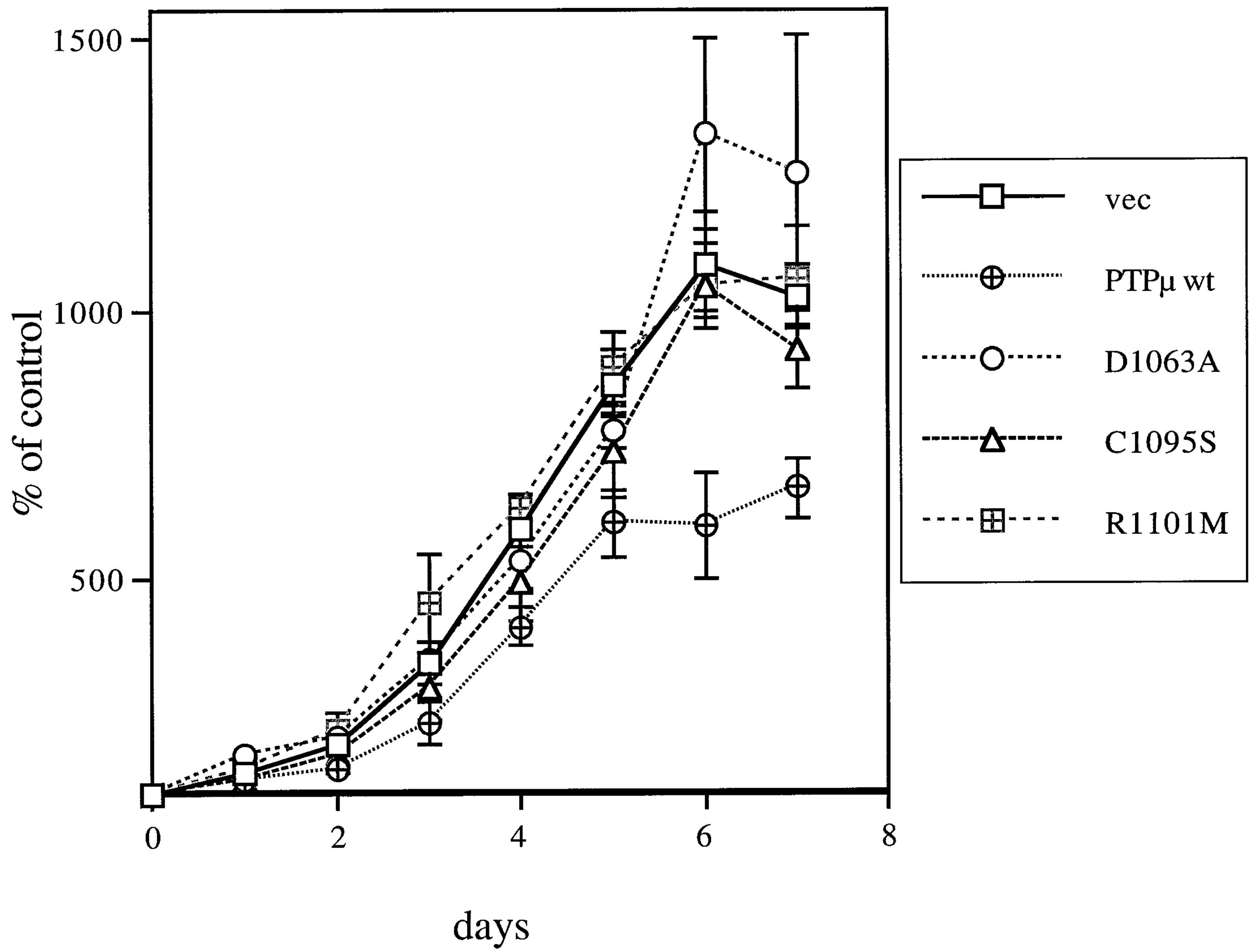


Figure 15. Growth Curve analysis of cells expressing wild-type and mutant forms of PTPμ

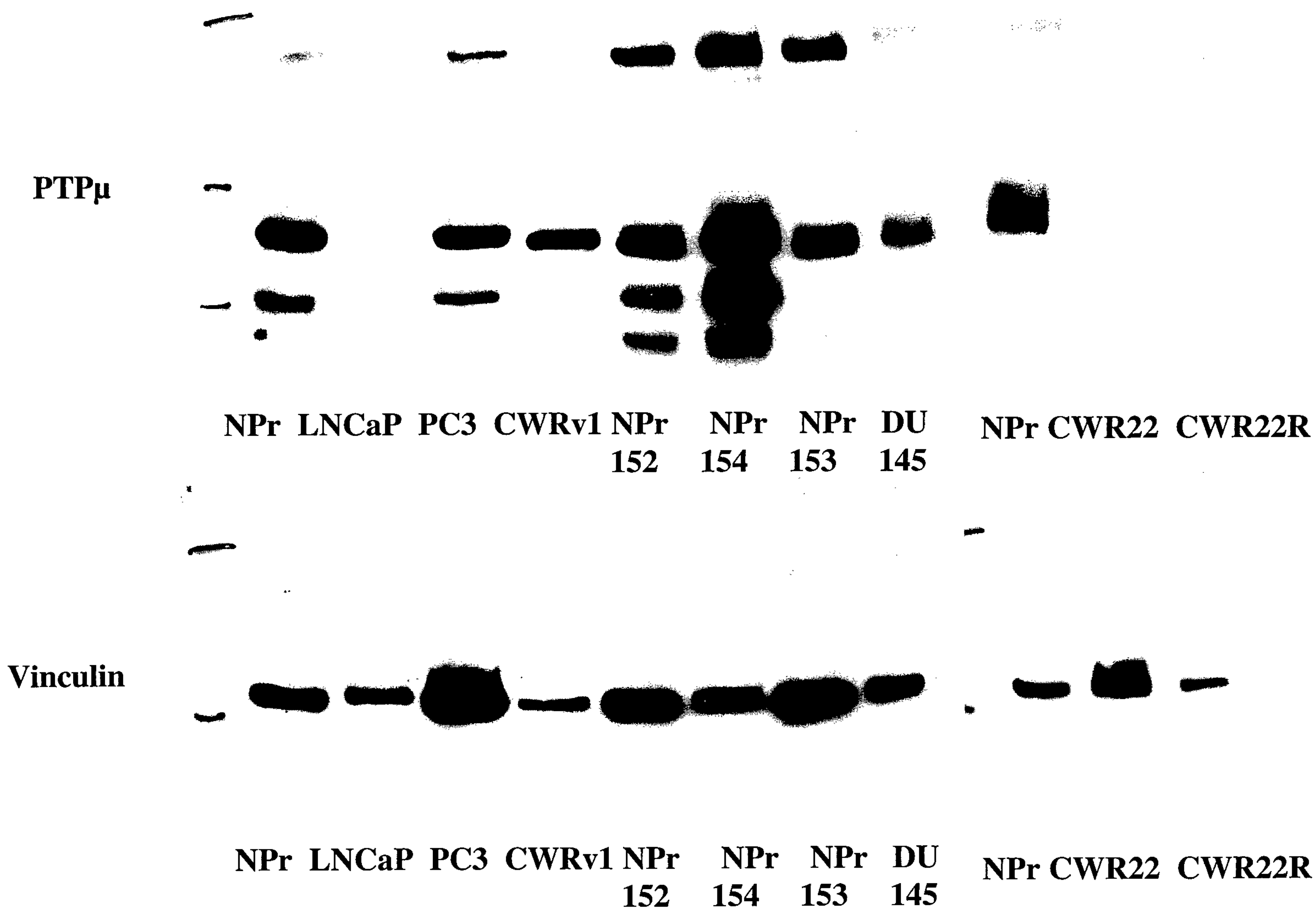


Figure 16. Expression of PTPμ in various prostate cell lines.These cell lines include some that were generated at CWRU (CWR22, 22R, NPr 152-154). The others are available from ATCC.

The protein tyrosine phosphatase PTPμ binds to RACK1, a receptor for activated PKC

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Abstract

Previously, we have shown that PTPμ, an Ig-superfamily receptor protein tyrosine phosphatase (RPTP), promotes cell-cell adhesion and interacts with the cadherin/catenin complex. The signaling pathway downstream of PTPμ is unknown; therefore, we used a yeast two-hybrid screen to identify additional PTPμ interacting proteins. The first phosphatase domain of PTPμ was used as bait. Sequencing of two positive clones identified RACK1 (receptor for activated protein C kinase) as a PTPμ interacting protein. RACK1 interacts with PTPμ when co-expressed in a recombinant baculovirus expression system. RACK1 is known to bind to the *src* protein tyrosine kinase (PTK). Our studies demonstrate that PTPμ and *src* associations with RACK1 are mutually exclusive. RACK1 is thought to be a scaffolding protein that recruits proteins to the plasma membrane via an unknown mechanism. The association of endogenous PTPμ and RACK1 is increased at high cell density. We also demonstrate that RACK1 is recruited to both the plasma membrane and cell-cell contact sites at high cell density. In addition, the RACK1/PTPμ interaction was not affected by phorbol ester stimulation of protein kinase C in MvLu cells. Therefore, PTPμ may be one of the proteins that recruits RACK1 to points of cell-cell contact which may be important for PTPμ-dependent signaling in response to cell adhesion.

Introduction

Protein phosphorylation events within a cell mediate many cellular processes including differentiation and proliferation. Control of these events is regulated by the opposing actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). The PTP superfamily is a diverse group of proteins, which include transmembrane receptors (Brady-Kalnay, 2000). Many of these receptor protein tyrosine phosphatases (RPTPs) are members of the immunoglobulin (Ig) superfamily, a group of proteins responsible for cell recognition or adhesion. RPTPs have cell adhesion molecule-like extracellular segments as well as intracellular domains possessing tyrosine phosphatase activity, suggesting they may play a regulatory role in cell adhesion-induced signaling (Brady-Kalnay, 1998; Brady-Kalnay, 2000; Neel and Tonks, 1997). However, the precise signaling pathways utilized by RPTPs are unknown.

The RPTP, PTPμ, has an extracellular segment featuring a MAM domain, an Ig domain, and four fibronectin type-III repeats. PTPμ promotes cell-cell aggregation when expressed in nonadhesive cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993). Both the MAM domain (Zondag et al., 1995) and the Ig domain (Brady-Kalnay and Tonks, 1994) have been shown to play a role in PTPμ-mediated cell-cell adhesion. Together, these studies demonstrated that the binding is homophilic (i.e., the "ligand" for PTPμ is an identical PTPμ molecule on an adjacent cell). Interestingly, endogenous levels of the PTPμ adhesion molecule have also been shown to promote neurite outgrowth from retinal neurons (Burden-Gulley and Brady-Kalnay, 1999).

The juxtamembrane domain of PTPμ contains a region of homology to the conserved intracellular domain of the cadherins (Tonks et al., 1992). Cadherins are calcium-dependent cell-cell adhesion molecules that are important for many physiological processes such as cell polarity, adherens junction formation and motility (Vleminckx and Kemler, 1999; Gumbiner, 2000). The cytoplasmic domain of cadherins interacts with molecules termed catenins that associate with actin (Gumbiner, 2000). The catenins include α, β, γ (also called plakoglobin) and p120. We previously demonstrated that PTPμ associates with a complex containing classical cadherins, α catenin and ß catenin (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998). We demonstrated that the intracellular segment of PTPμ binds directly to the intracellular domain of E-cadherin, as confirmed later by another group (Hiscox and Jiang, 1998). In addition, we have recently demonstrated that PTPμ is required for an N-cadherin-dependent function (regulation of neurite outgrowth) (Burden-Gulley and Brady-Kalnay, 1999). The signal transduction pathways downstream of the RPTPs and cadherins are not well understood. In this manuscript, we demonstrate that PTPμ interacts with RACK1 and that this protein may be a component of a PTPμ signaling pathway.

A recently identified group of cytosolic proteins called RACKs (receptors for activated protein C kinase) have been shown to bind to PKC only when it is in the activated state (Mochly-Rosen and Kauvar, 1998). A specific RACK, RACK1, has been cloned and is a homolog of the β subunit of heterotrimeric G proteins as determined by the existence of WD repeats (Ron et al., 1994). WD repeats are 40 amino acid motifs proposed to mediate protein-protein interactions (Neer et al., 1994). RACK1 is composed of seven WD repeats that are thought to form propeller-like structures (Garcia-Higuera et al., 1996).

RACK1 was originally identified as a receptor for activated protein kinase C. Activation of some isozymes of PKC is normally regulated *in vivo* by diacylglycerol. Treatment with phorbol esters, known tumor promoters, can stimulate PKC because of their close chemical similarity to diacylglycerol (Mochly-Rosen and Kauvar, 1998). Active PKC is then responsible for stimulating other intracellular targets. It has been suggested that the binding of activated PKC to RACK(s) is necessary for the translocation of PKC to the plasma membrane; a process thought to be required in order for PKC to perform its physiological function (Mochly-Rosen and Kauvar, 1998).

More recent data suggests that RACK1 is a scaffolding protein that recruits a number of signaling molecules into a complex. Theoretically, the seven propellers of the RACK1 structure (Garcia-Higuera et al., 1996) could bind seven different proteins. RACK1 has been shown to bind PKC, PLCγ, the *src* cytoplasmic protein tyrosine kinase (PTK), cAMP phosphodiesterase-4, the β subunit of integrins, and the β chain of IL-5R (an interleukin receptor) (Chang et al., 1998; Disatnik et al., 1994; Geijsen et al., 1999; Liliental and Chang, 1998; Yarwood et al., 1999). RACK1 has also been demonstrated to bind select pleckstrin homology (PH) domains *in vitro* including dynamin, β– spectrin, Ras GRF and oxysterol binding protein (OSBP) (Rodriguez et al., 1999). Some of the interactions between RACK1 and the proteins listed above have been shown to be mutually exclusive (Chang et al., 1998). In addition, only a subset of these interactions depend upon PKC stimulation (Liliental and Chang, 1998). These studies suggest that RACK1 may form distinct signaling complexes in response to unique cellular stimuli.

In this study, we utilized the yeast two-hybrid genetic screen to isolate PTPμ interacting proteins and identified RACK1 as a protein that binds directly to the first phosphatase domain in the cytoplasmic segment of PTPμ (PTPμD1). We characterized this interaction using a recombinant baculovirus expression system and showed that RACK1 and PTPμ interact only when co-expressed. Phorbol ester treatment of cells to stimulate endogenous PKC activity had no affect on the ability of PTPμ and RACK1 to interact. We also demonstrated that PTPμ and *src* compete for RACK1 binding. In addition using MvLu cells, which endogenously express PTPμ and RACK1, we demonstrated that PTPμ and RACK1 associate and that the association is not dependent upon the activation of PKC. PTPμ is up-regulated at high cell density in MvLu cells (Gebbink et al., 1995) and is primarily found at cell-cell contact sites (Brady-Kalnay et al., 1995). At high cell density, RACK1 is recruited to the plasma membrane and points of cell-cell contact. Therefore, the recruitment of RACK1 to both the plasma membrane and cell-cell contact sites may be dependent upon PTPμ. Localization of RACK1 to points of cell-cell contact may be an important part of the PTPμ-dependent signal transduction process in response to adhesion.

Materials and Methods

Yeast two-hybrid screen: We used the LexA version of the yeast two-hybrid system to perform an interaction trap assay (Golemis, 1997). This approach detects protein-protein interactions between a protein from a HeLa cell cDNA library and a construct containing the first phosphatase domain of PTPμ (PTPμD1) as the bait. Amino acids 915-1178 encoding the first phosphatase domain from full length PTPμ were cloned in frame with the LexA coding sequence of pEG202 (*HIS3*) to generate a "bait" plasmid. The resulting construct (pEG202-D1) was sequenced for insertion and correct orientation. The pEG202-D1 plasmid and the betagalactosidase reporter plasmid (pSH18-34) were co-transformed into the yeast strain YPH499. The pSH18-34 (*URA3*) reporter plasmid contains a LexA-operator-*lacZ* fusion gene. The pEG202-D1 plasmid did not activate the betagalactosidase reporter plasmid on its own. A HeLa cell human cDNA library (Golemis, 1997) in the pJG4-5 (*TRP1*) yeast expression vector was introduced into a yeast strain containing a chromosomal copy of the *LEU2* gene (EGY48) where the activating sequences of the *LEU2* gene are replaced with LexA operator sequences. The two strains (EGY48 and YPH499) were mated and the resulting colonies containing the three plasmids were processed according to published methods (Golemis, 1997). Potential interactions were detected by growing the mated yeast strain on minimal media containing 2% galactose and 1% raffinose and lacking the appropriate amino acids to ensure selective pressure of the auxotrophic markers (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). The interaction was confirmed by changing which yeast strain carried the bait and library plasmid and mating the strains to retest the interaction. Two independent positive "prey" clones were identified as full length RACK1 by DNA sequencing of the library plasmid (pJG4-5). The positive control used in Figure 1 is a yeast strain containing a bait and library plasmid that are known to interact. The negative control is a yeast strain containing the bait (pEG 202-D1), reporter plasmid (pSH 18-34) and an empty prey vector (pJG4-5).

A constituitively-activated form of the *src* protein tyrosine kinase ($Y^{527}F$, $Y^{416}F$ double mutant) was obtained from Dr. Jonathan Cooper (Keegan and Cooper, 1996). The BTM116 plasmid containing the *src* gene was restriction digested with BamH1. A partial digest of pSH18-34 was performed with BamH1 and the *src* insert was ligated with this vector. The pSH18-34/*src* and the pEG202-D1 plasmids were used to transform the YPH499 yeast strain. Then this YPH499 strain was mated to the EGY48 strain containing the RACK1 gene. This allowed us to test whether *src* interaction with RACK1 could block the RACK1/PTPμ interaction that drove β– galactosidase transcription.

Antibodies: Monoclonal antibodies against the intracellular (SK7, SK15, SK18) and extracellular (BK2) domains of PTPμ or polyclonal antibody against the intracellular domain (471) of PTPμ have been described (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994). A control monoclonal antibody directed against L1 (8D9), generated from hybridoma cells, was generously provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). A monoclonal antibody to the HA tag (Covance, Denver, PA) was used to detect recombinant RACK1. The HA antibody was purchased in a biotinylated form and strepavidin-HRP was used for visualization (Covance). In addition, a specific antibody to RACK1 was purchased from Transduction Labs (Lexington, KY). The monoclonal antibody to src was purchased from CalBiochem (San Diego, CA).

Baculoviruses: The baculovirus encoding the intracellular domain of PTPμ (intra-PTPμ) has been described (Brady-Kalnay et al., 1993). The *src* baculovirus, which encodes a constituitively active kinase (Y^{527} F mutant), was kindly provided by Dr. Michael Weber and originally constructed by Dr. David Morgan (Dent et al., 1995). The RACK1-pJG4-5 vector was restriction digested so that the resulting 1800 bp fragment contained an HA tag (from pJG4-5) in frame with the full length RACK1 clone. The 1800 bp fragment was cloned into the pAcHLT-C

(BD Pharmingen, San Diego, CA) baculovirus expression vector. The construct was sequenced to confirm orientation and correct insertion of the 1800 bp fragment. This created a form of RACK1 that contained a poly-histidine tag, a PKA site, a thrombin cleavage site and an HA-tag, at the N-terminus followed by the RACK1 cDNA sequence (amino acids 1-317). Baculoviruses were generated using the BaculoGold ™ system (BD PharMingen).

Expression in Insect cells: Sf9 cells (CRL 1711; American Type Culture Collection, Rockville, MD), derived from the ovary of the Fall armyworm *Spodoptera frugiperda*, were maintained at 27°C in Grace's Insect Medium Supplemented (GIBCO-BRL) containing 10% fetal bovine serum and 10 μg/mL gentamicin. The viral stocks were then used to infect Sf9 cells and express the proteins of interest (PTPμ, RACK1 and *src*). Forty-eight hours post-infection, cells were either lysed or treated with 160nM PMA (Calbiochem) (Liliental and Chang, 1998) for 30 min. at 27°C. Cells were lysed in ice-cold buffer (1% Triton X-100, 20 mM Tris pH 7.6, 1 mM benzamidine, 2μl/ml protease inhibitor cocktail (Sigma), 150 mM NaCl, 0.2μM okadaic acid, 200 μM phenylarsine oxide, 1mM vanadate, 0.1mM molybdate) for 30 minutes on ice. The lysates were centrifuged at 14,000g for 3 min. to remove Triton insoluble components.

MvLu cell experiments: MvLu cells (ATCC number CCL 64) were grown at 37°C, 5% CO_2 in DMEM containing 10 μg/mL gentamicin plus 10% fetal bovine serum (GIBCO-BRL). The MvLu cells were grown to 50% or 90% confluence prior to lysis. PKC activation of MvLu cells was performed by adding 10nM PMA (Calbiochem) for 15 min. at 37°C before lysing the cells. MvLu cells were lysed in buffer (20mM Tris pH 7.6, 1% Triton X-100, 2μl/ml protease inhibitor cocktail, 1mM benzamidine, 200 μM phenyl arsine oxide, 1 mM vanadate and 0.1mM molybdate), incubated on ice for 30 min. and centrifuged at 14,000 x g for 3 min.

A replication-defective amphotrophic retrovirus expressing an antisense PTPμ construct and control retrovirus have been previously described (Burden-Gulley and Brady-Kalnay, 1999). MvLu cells were incubated in virus-containing media supplemented with serum (10% final) plus 5μg/ml polybrene for 2-4 days. Reduction in endogenous PTPμ expression was verified by immunoblotting lysates from infected cells with antibodies to PTPμ.

Immunoprecipitations and electrophoresis: For immunoprecipitation, antibodies to PTPμ or RACK1 were incubated with Protein A sepharose (Pharmacia Biotech, Piscataway, NJ) or Goat anti-mouse IgG (or IgM) that had been conjugated to sepharose (Zymed, South San Francisco, CA) for 2 hours at room temperature then washed 3 times with PBS (phosphate buffered saline: 9.5mM phosphate, 137mM NaCl, pH 7.5) before addition to cell lysates. Purified monoclonal antibodies were used at 0.6mg of IgG/ml beads, ascites fluid was used at 1mg of IgG/ml beads and polyclonal serum was used at 3mg of IgG/ml beads. Immunoprecipitates were prepared from 40μg (Sf9 cells) or 300μg (MvLu cells) of a Triton-soluble lysate of cells as measured by the Bradford method. The immunoprecipitates were incubated overnight at 4°C on a rocker, centrifuged at 3,000 x *g* for 1 min. The supernatant was removed and the beads were separated, then washed four times in lysis buffer and the bound material eluted by addition of 100μl of 2X sample buffer and heated for 5 min. at 95°C. One-fifth of the immunoprecipitate (20 μL) was loaded per lane of the gel, the proteins were separated by 10% (for analysis of RACK1 and src), 7.5% (for analysis of the intracellular PTPμ) and 6% (for analysis of endogenous PTPμ) SDS-

PAGE and transferred to nitrocellulose for immunoblotting as previously described (Brady-Kalnay et al., 1993).

Immunocytochemistry: All chemicals were diluted in PBS. Cells were fixed with 2% paraformaldehyde for 30 min. at room temperature (Electron Microscopy Sciences, Fort Washington, PA). The cells were permeabilized with 0.5% saponin, blocked with 20% normal goat serum plus 1% BSA and incubated with primary antibody for 18 hours at 4°C. The cells were washed with TNT buffer (0.1M Tris, 0.15M NaCl, 0.05% Tween-20) containing 0.5% saponin and incubated with Texas-Red or Rhodamine conjugated secondary antibody (Molecular Probes, Eugene, OR or Cappel Research Products, Durham, NC) for 1.5 hours at room temperature. Samples were mounted with Slow fade Light (Molecular Probes, Eugene, OR). The fluorescent labeling was examined using a 40X objective on a Zeiss Axioplan 2 microscope equipped for epifluorescence. Images were captured using a Hamamatsu cooled CCD camera.

Results

Yeast two-hybrid analysis

The yeast two-hybrid interaction trap assay (Golemis, 1997) was used to identify proteins that were capable of binding directly to the first tyrosine phosphatase domain of PTPμ. Potential interactions were detected by growing the mated yeast strain on minimal media containing 2% galactose and 1% raffinose and lacking the appropriate amino acids (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). PTPμ interactors were selected by three criteria. First, they were screened for viability on medium lacking leucine. Only interacting clones will be able to grow on media without leucine. Second, they were screened for formation of blue colonies when grown on medium containing Xgal/galactose compared to Xgal/glucose containing media. Galactose specifically induces expression of the "prey" protein whereas glucose does not. Third, they were discriminated for the level of transcriptional activation of the *lacZ* gene based on the blue color of the colonies when grown on medium containing Xgal. The positive control used in Figure 1 is a yeast strain containing a bait and library plasmid that are known to interact. The negative control is a yeast strain containing the bait and reporter plasmid and an empty prey vector. As shown in Figure 1, two independent clones fulfilled these criteria. The two clones did not grow on glucose but did grow on galactose (Fig. 1 a). Multiple independent colonies are shown for each clone. These two clones also expressed high levels of β– galactosidase (Fig. 1 b). Sequence analysis of two independent positive clones demonstrated that they both encoded RACK1, a member of the heterotrimeric G_β superfamily of proteins.

RACK1 and PTPμ interact in Sf9 cells

To characterize the interaction of PTPμ and RACK1, Sf9 cells were infected with baculoviruses encoding full-length RACK1 or the intracellular domain of PTPμ, singly or in combination. Lysates from Sf9 cells were immunoprecipitated with RACK1 or PTPμ (471) antibodies and resolved by SDS-PAGE. Immunoblots of immunoprecipitates probed with an antibody to RACK1 are shown (Fig. 2 a and b). Immunoblots of PTPμ immunoprecipitates probed with an anti-PTPμ (SK15) antibody are shown (Fig. 2 c). Equal amounts of RACK1 were available in the Triton soluble lysate used for immunoprecipitation based on the ability of RACK1 antibodies to immunoprecipitate RACK1 (Fig. 2 a, lanes 1, 3-6). Figure 2 also illustrates that equal amounts of PTPμ were immunoprecipitated from all of the PTPμ-infected cell samples (Fig. 2 c, lanes 2-6). Anti-PTPμ immunoprecipitates (471 antibody) were immunoblotted with antibody to RACK1 (Fig. 2 b). RACK1 interaction with PTPμ was only detected in cells where both proteins were co-expressed (Fig. 2 b, lane 3-4). The PTPμ antibody did not immunoprecipitate RACK1 from cell lysates in the absence of PTPμ expression (Fig. 2 b, lane 1), thus ruling out the possibility that the antibody recognized RACK1 nonspecifically. An endogenous PKC is expressed in Sf9 cells and can be stimulated by phorbol ester treatment (Geiges et al., 1997). The interaction between RACK1 and PTPμ was unaffected by endogenous PKC stimulation with phorbol esters (Fig. 2 b, lane 4). These data indicate that RACK1 only bound to PTPμ when both proteins were co-expressed in insect cells and the interaction was not affected by PKC activation.

Src blocks the interaction between RACK1 and PTPμ

Since RACK1 is known to bind to *src* (Chang et al., 1998), we tested whether addition of a constituitively active *src* to the yeast cells affected binding between RACK1 and PTPμ. β–galactosidase staining of yeast expressing RACK1 and PTPμ was positive while RACK1/PTPμ/*src* containing yeast did not turn blue on media containing Xgal (Fig. 1 b). Therefore, the *src* PTK appears to block the interaction between RACK1 and PTPμ in yeast.

We then analyzed whether *src* could block the interaction between RACK1 and PTPμ in the Sf9 cell system. We did single, double or triple infections with RACK1, PTPμ and *src*. Figure 2 d shows that the *src* PTK was expressed in the appropriate samples. RACK1 antibody immunoprecipitated RACK1 from all the appropriate samples (Fig. 2 a, lanes 1, 3-6). While RACK1 and PTPμ interact when co-infected (Fig. 2 b, lane 3-4), there was no interaction detected when *src* was added in the triple infection (Fig. 2 b, lane 5-6). Inhibition of src tyrosine kinase activity by PP1 had no effect on the ability of src to block the PTPμ/RACK1 interaction (data not shown). The ability of *src* to block the PTPμ/RACK1 interaction was also unaltered by phorbol ester stimulation of endogenous PKC (Fig. 2 b, lane 6). Therefore, the *src* PTK was able to block the interaction between RACK1 and PTPμ in yeast as well as in the Sf9 cell system. Together, these results suggest that PTPμ and *src* form mutually exclusive complexes with RACK1. One could speculate that PTPμ and *src* may be using the same binding site on RACK1 or *src* may sterically hinder the interaction between PTPμ and RACK1.

Endogenous RACK1 interacts with endogenous PTPμ

To examine whether endogenous PTPμ associates with endogenous RACK1, immunoprecipitation experiments were done using MvLu cells. Endogenous PTPμ in MvLu cells is proteolytically processed. The full length form is cleaved into two noncovalently associated fragments, one (P-subunit) comprising the entire intracellular and transmembrane segments and a short stretch of extracellular sequence, the other (E-subunit) containing the remainder of the extracellular segment (Brady-Kalnay and Tonks, 1994; Gebbink et al., 1995). Both the full-length (200 kDa) and cleaved form (100 kDa) of PTPμ were expressed (Fig. 3 a) as expected (Brady-Kalnay et al., 1995). PTPμ expression in MvLu cells is known to be regulated by cell density (Gebbink et al., 1995). In our studies the expression of PTPμ also increases with cell density (data not shown). However, at 50% and 90% confluence, PTPμ expression appears to be approximately the same (Fig. 3 a). MvLu cell cultures were grown at these two densities to control the amount of cell contact. When MvLu cells are grown to 50% confluence there is little cell contact while at 90% confluence the majority of cells adhere to one another.

We then used a retrovirus encoding antisense PTPμ (Burden-Gulley and Brady-Kalnay, 1999) to infect MvLu cells. Immunoblot analysis demonstrated that the full-length protein (200 kDa band) was substantially reduced in cells infected with PTPμ antisense virus when compared to cells infected with control virus (Fig. 3 a). The 100 kDa immunoreactive band was also reduced (Fig. 3 a). These results confirm that PTPμ antisense expression inhibits the new synthesis of PTPμ.

RACK1 was immunoprecipitated by antibodies to RACK1 (Fig. 3 c), but was not detected when a control mouse antibody was used for immunoprecipitation (Fig. 3 b). PTPμ was immunoprecipitated with a monoclonal antibody to the extracellular domain (BK2) or with a polyclonal antibody to the C-terminus (471). When immunoprecipitates of PTPμ were probed on immunoblots with anti-RACK1 antibody, an association was detected (Fig. 3 d-e). The association increased at high cell density (Fig. 3 d-e-lane 3). The interaction was not substantially altered when PKC was stimulated by phorbol ester (PMA) treatment (Fig. 3 d-e-lane 4). However, when PTPμ expression was reduced in antisense-infected cells, it no longer interacted with RACK1 (Fig. 3 d-e-lane 5). These data suggest that endogenous RACK1 interacts directly with PTPμ when cell-cell contact occurs.

RACK1 localizes to points of cell-cell contact at high cell density

PTPμ localizes to points of cell-cell contact in MvLu cells (Brady-Kalnay et al., 1995) and as shown in Fig. 4 (a-d). Immunocytochemical analysis of subconfluent cultures of MvLu cells is shown in Figure 4 (a-b). PTPμ localized to filopodial extensions that contacted between adjacent MvLu cells (Fig. 4 a and b). When cells were plated at higher density, PTPμ was restricted to points of cell-cell contact (Fig. 4 c and d). To determine the localization of RACK1, we performed immunocytochemistry on MvLu cells. The RACK1 protein is predominately cytoplasmic in subconfluent MvLu cells (Fig. 4 e and f). However, at high cell density, RACK1 also decorated points of cell-cell contact in MvLu cells (Fig. 4 g and h). The translocation of RACK1 to the membrane and points of cell-cell contact at high cell density is likely to be related to its increased association with PTPμ.

Discussion

In this study we used the yeast two-hybrid screen to isolate PTPμ interacting proteins. We identified an interaction between the cytoplasmic phosphatase domain 1 of PTPμ (PTPμD1) and RACK1 (receptors for activated protein C kinase). Since yeast do not have traditional tyrosine kinases, the interaction of PTPμD1 and RACK1 was likely to be mediated by protein-protein interactions and not dependent upon phosphotyrosine. We characterized the association between RACK1 and PTPμD1 using the recombinant baculovirus expression system, and have shown that the intracellular segment of PTPμ binds to RACK1 in insect cells. The RACK1/PTPμ interaction was blocked by the *src* PTK. These data suggest that RACK1/*src* and RACK1/PTPμ form mutually exclusive signaling complexes. In addition, we showed an association between PTPμ and RACK1 using MvLu cells, which express both proteins endogenously. The interaction of PTPμ and RACK1 was not affected by phorbol ester stimulation of PKC, suggesting that when RACK1 is bound to PTPμ it is still likely to be able to bind PKC. PTPμ expression increases with increasing cell density in MvLu cells (Gebbink et al., 1995). At high cell density, we observed an increased association of RACK1 with PTPμ as well as increased translocation of RACK1 to the plasma membrane and points of cell-cell contact. Together these data suggest that PTPμ and RACK1 form a signaling complex at high cell density.

RACK1 is a homolog of the β subunit of heterotrimeric G proteins and is composed of WD repeats (Ron et al., 1994). Both G_{β} and RACK1 form seven propeller structures (seven independently folding loops) proposed to mediate protein-protein interactions (Neer et al., 1994). RACK1 was originally identified as a protein that binds to activated protein kinase C (PKC)

(Ron et al., 1994). It has been suggested that activated PKC binding to RACK1 is required for the translocation of the enzyme to the plasma membrane, its physiologically relevant site of action (Mochly-Rosen and Kauvar, 1998). In addition, RACK1 seems to serve as a general scaffolding protein for a number of signaling enzymes including *src* (Chang et al., 1998).

Receptor protein tyrosine phosphatases are involved in cell adhesion (Brady-Kalnay, 2000). PTPμ has been shown to induce cell adhesion by homophilic binding (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994; Gebbink et al., 1993). In addition, it also appears to regulate cadherin-mediated cell adhesion by binding to the cadherin/catenin complex (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998; Burden-Gulley and Brady-Kalnay, 1999). The PTPμ/RACK1 interaction appears to predominately occur in cells at high cell density. These data indicate that the PTPμ/RACK1 interaction may be induced by cell-cell contact. It clear that the interaction requires the presence of the PTPμ protein based on our antisense experiments. Our hypothesis is that the PTPμ/RACK1 interaction is likely to be induced by cell adhesion, which may recruit other signaling proteins that are important for PTPμ-dependent signal transduction. One could speculate that PKC or other signaling molecules associated with RACK1 might be downstream of PTPμ-dependent signals induced by cell-cell adhesion.

If the association of RACK1 and PTPμ brings activated PKC close to its site of action at the membrane, how might other cell-cell adhesion molecules, like cadherins, be involved? There have been suggestions in the literature that PKC may regulate E-cadherin-dependent adhesion. Adherens junctions serve to anchor the actin cytoskeleton at regions of cell-cell contact. Investigators have postulated that the cellular reorganization occurring during the formation of adherens junctions is induced by PKC activation and that PKC, in turn, may regulate cadherin-dependent adhesion (Lewis et al., 1995). Clearly our data suggest an interesting relationship exists between PKC signal transduction mechanisms and the PTPμ cell-cell adhesion molecule. We hypothesize that the interaction of PTPμ and RACK1 at high cell density recruits PKC or other RACK1 binding partners to sites of cell-cell contact to transduce adhesion-dependent signals.

In a companion paper, we have shown that normal prostate expresses the receptor protein tyrosine phosphatase PTPμ whereas LNCaP prostate carcinoma cells do not. LNCaP cells express normal levels of E-cadherin and catenins, but are defective in both PTPμ- and E-cadherin-dependent adhesion. Re-expression of PTPμ restored cell adhesion to PTPμ and to E-cadherin. A mutant form of PTPμ that is catalytically inactive was re-expressed and it restored adhesion to PTPμ and to E-cadherin. Expression of PTPμ-extra (which lacks most of the cytoplasmic domain) induced adhesion to PTPμ but not to E-cadherin, demonstrating a requirement for the presence of the intracellular domains of PTPμ to restore E-cadherin-mediated adhesion. In addition, we demonstrate that both catalytically active and inactive PTPμ interact with RACK1. Activation of PKC reversed the adhesion of PTPμWT-expressing cells to E-cadherin, whereas treatment of parental LNCaP cells with PKC∂ inhibitors induced adhesion to E-cadherin. Together, these studies suggest that PTPμ regulates the PKC pathway to restore E-cadherin-dependent adhesion via its interaction with RACK1.

Tyrosine phosphorylation by the *src* PTK negatively regulates cadherin-dependent adhesion (Brady-Kalnay, 1998; Brady-Kalnay and Tonks, 1995; Daniel and Reynolds, 1997);

however, the mechanism is unknown. Previously, we tested the ability of *src* tyrosine kinase to regulate PTPμ/cadherin interactions. We used a series of WC5 cell lines, which express PTPμ endogenously, and ectopically expressed E-cadherin. We analyzed the effect of tyrosine phosphorylation on the composition of the PTPμ/cadherin complex and our data suggested that increased tyrosine phosphorylation of E-cadherin results in decreased association with PTPμ (Brady-Kalnay et al., 1998). Interestingly, RACK1 binds to the *src* PTK. In this study we found that *src* and PTPμ compete for binding to RACK1. Since *src* is known to negatively regulate cadherin-dependent adhesion, the ability of PTPμ and *src* to compete for RACK1 may directly affect tyrosine phosphorylation of the cadherin complex via PTPμ or indirectly by regulating the presence of the *src* PTK in the complex. Together, these data suggest that PTPμ may be altering *src* signaling pathways via its interaction with RACK1.

Since RACK1 binds to the conserved PTP catalytic domain of PTPμ, a number of other PTPs may also interact with RACK1. It is interesting to note that many PTPs are known to regulate the *src* cytoplasmic PTK (Brady-Kalnay, 2000). Importantly, RACK1 is known to bind to *src*. Our data suggest that one of the links between PTPs and *src* PTK signaling may be the RACK1 protein. This manuscript demonstrates that PTPμ association with RACK1 is altered in the presence of *src* suggesting that there are mutually exclusive interactions of *src* and PTPμ with RACK1. One caveat of these studies is that they were done using a constituitively active *src* PTK. However, we believe that this the PTP versus PTK competition for binding to RACK1 may be a common form of regulation for signaling complexes. For example, protein-protein interactions with scaffolding molecules such as RACK1 may control tyrosine phosphorylation of substrate proteins via their mutually exclusive interactions with a tyrosine kinase or phosphatase.

More importantly, the ability of *src* and PTPμ to compete may be an important mechanism for regulation of cell-cell adhesion and signal transduction.

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Figure Legends

Figure 1. PTPμ and RACK1 interact in yeast. Panel a illustrates the growth of two yeast clones containing both RACK1 (R) and PTPμ (μ) grown on galactose or glucose. Panel b shows the same PTPμ/RACK1 (R+μ) clones grown on media containing Xgal. + or - equals positive or negative control respectively. The yeast strains containing PTPμ/RACK1/*src* (R, μ, *src*) are also shown.

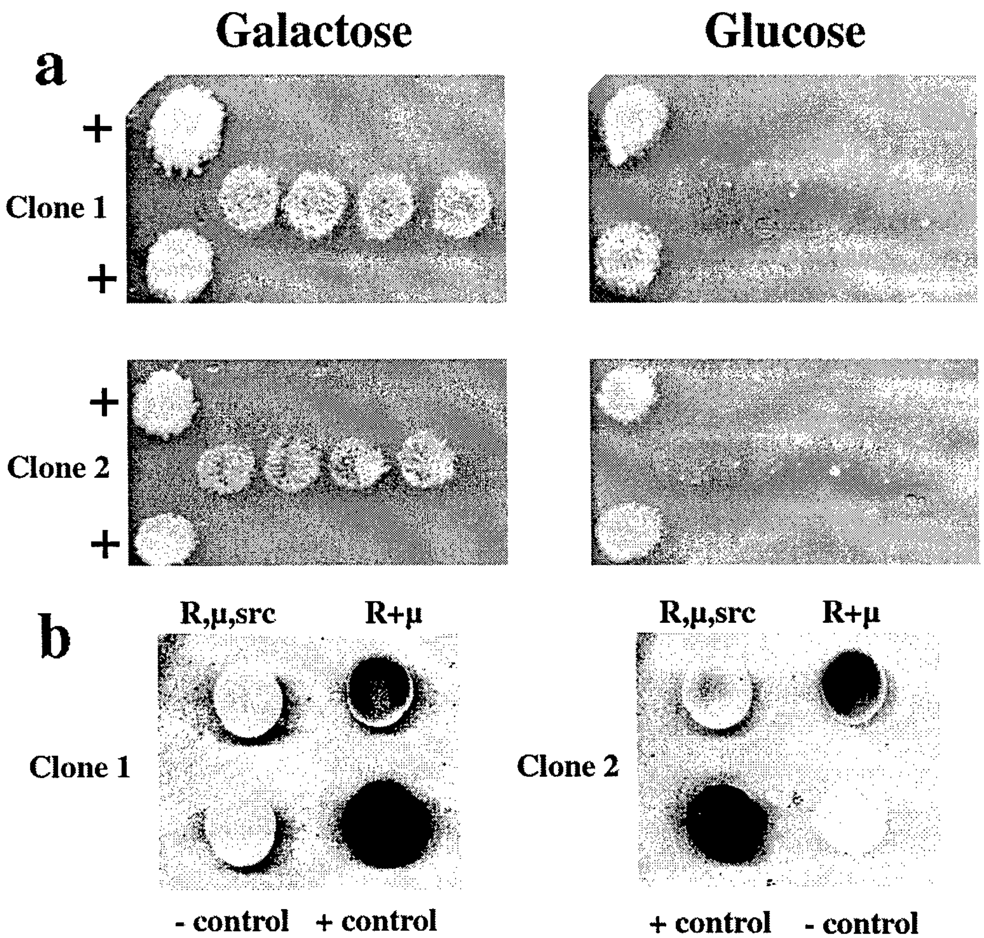
Figure 2. PTPμ and RACK1 interact in Sf9 cells. Panels a-d are immunoblots that were separated by 7.5% or 10% SDS-PAGE and probed with a monoclonal antibodies to either the HA tag of RACK1 (a and b), intracellular domain of PTPμ (c) or *src* (d). Panel A demonstrates that equal amounts of RACK1 are present in RACK1 immunoprecipitates from Sf9 cells infected with either RACK1 (lane 1), RACK1 and PTPμ (lane 3) or RACK1/PTPμ plus PMA (lane 4), and RACK1, PTPμ and *src* (lane 5) or RACK1, PTPμ and *src* plus PMA (lane 6). Cells infected with PTPμ only (a, lane 2), display no detectable RACK1. Panel b illustrates immunoblots of PTPμ immunoprecipitates probed with HA antibody to detect the RACK1 protein. PTPμ and RACK1 interact only when they are co-expressed regardless of the presence or absence of PMA (b, lanes 3-4). The interaction between RACK1 and PTPμ is disrupted by the triple infection with *src* (b, lanes 5-6). The bar to the left of panels a and b represent the 52kDa molecular weight marker. Recombinant RACK1 migrates at this molecular weight due to the addition of various tags. Panel c shows an immunoblot of PTPμ immunoprecipitates that were separated by 7.5% SDS-PAGE and probed with a monoclonal antibody to PTPμ (SK15). In all Sf9 cells infected with PTPμ, equal amounts of PTPμ are present. Cells infected with RACK1 only (lane 1), display no

detectable PTPμ. An immunoblot of lysates using a *src* antibody demonstrates that *src* is expressed only in cells infected with the *src* virus (panel d). The bar in panels c and d represents the 91kDa molecular weight marker.

Figure 3. Endogenous RACK1 interacts with endogenous PTPμ and the interaction is not altered by PMA treatment. MvLu cells were grown to 50% (lanes 1-2) or 90% confluence (lanes 3-5). Then MvLu cells were treated with phorbol esters (PMA). Lysates from MvLu cells were immunoprecipitated with control, RACK1 or PTPμ antibodies as indicated and were separated by 10% or 6% SDS-PAGE. Panel a is an immunoblot using an antibody to PTPμ. Panels b-e are immunoblots of the immunoprecipitates probed with antibody to RACK1. The control antibody (8D9) did not immunoprecipitate RACK1 (panel b). RACK1 was readily detectable in all RACK1 immunoprecipitates (panel c). PTPμ interacted with RACK1 predominately in 90% confluent MvLu cells (panel d and e). Importantly, the interaction was not substantially altered by PMA treatment (panel d and e). Finally, the interaction between PTPμ and RACK1 was abolished by down-regulating PTPμ expression using a virus encoding antisense PTPμ (panel d and e-lane 5). The arrows in each panel indicate the RACK1 band.

Figure 4. Immunocytochemical localization of PTPμ and RACK1 in MvLu cells. Phase contrast (Panels a, c, e and g) or fluorescence (Panels b, d, f and h) micrographs of MvLu cells are shown. Cells labeled with antibodies to PTPμ (SK15-panels a and b) show that it is localized to filopodial extensions of the cells and points of cell-cell contact in subconfluent MvLu cells. When the cells were plated at higher density, PTPμ was localized to points of cell-cell contact (SK15-panels c and d). RACK1 (panels e and f) was localized in the cytoplasm at low cell

density. At high cell density, RACK1 was localized to points of cell-cell contact (panels g and h).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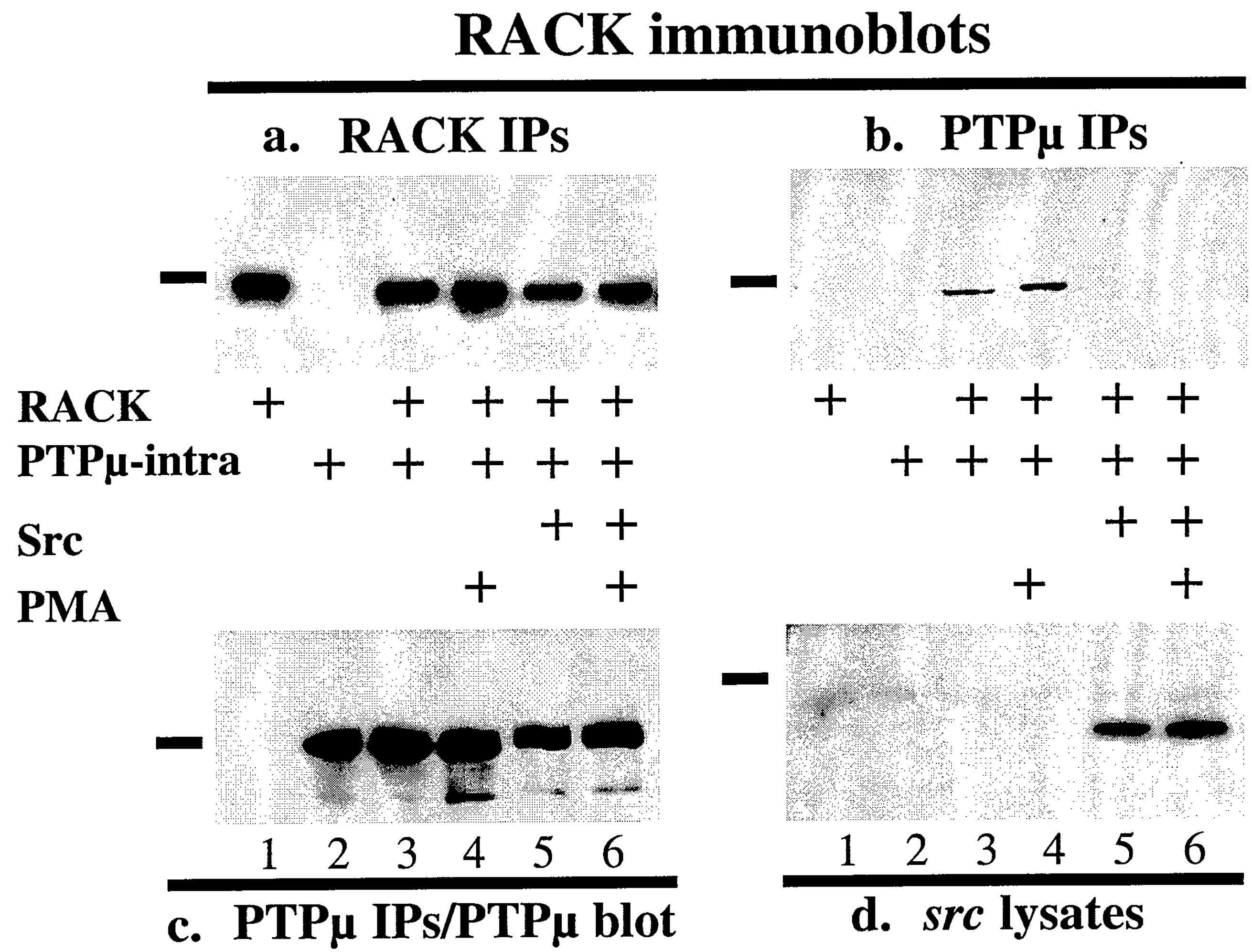
RACK immunoblots
a. RACK IPs
b. PTPμ IPs
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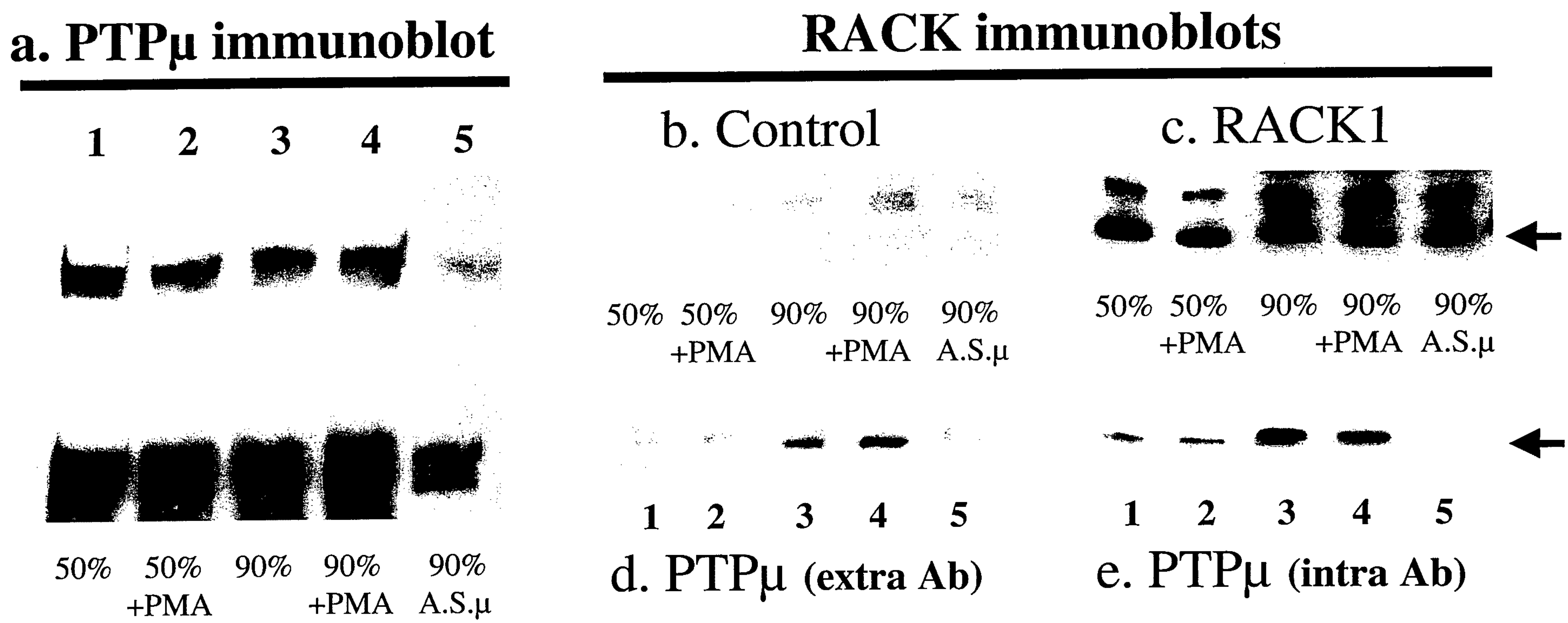
d. src lysates


a. PTPμ immunoblot
1 2 3 4 5
50% 50% +PMA 90% 90% +PMA 90% A.S.μ
RACK immunoblots
b. Control
50% 50% +PMA 90% 90% +PMA 90% A.S.μ
c. RACK1
50% 50% +PMA 90% 90% +PMA 90% A.S.μ
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d. PTPμ (extra Ab)
1 2 3 4 5
e. PTPμ (intra Ab)

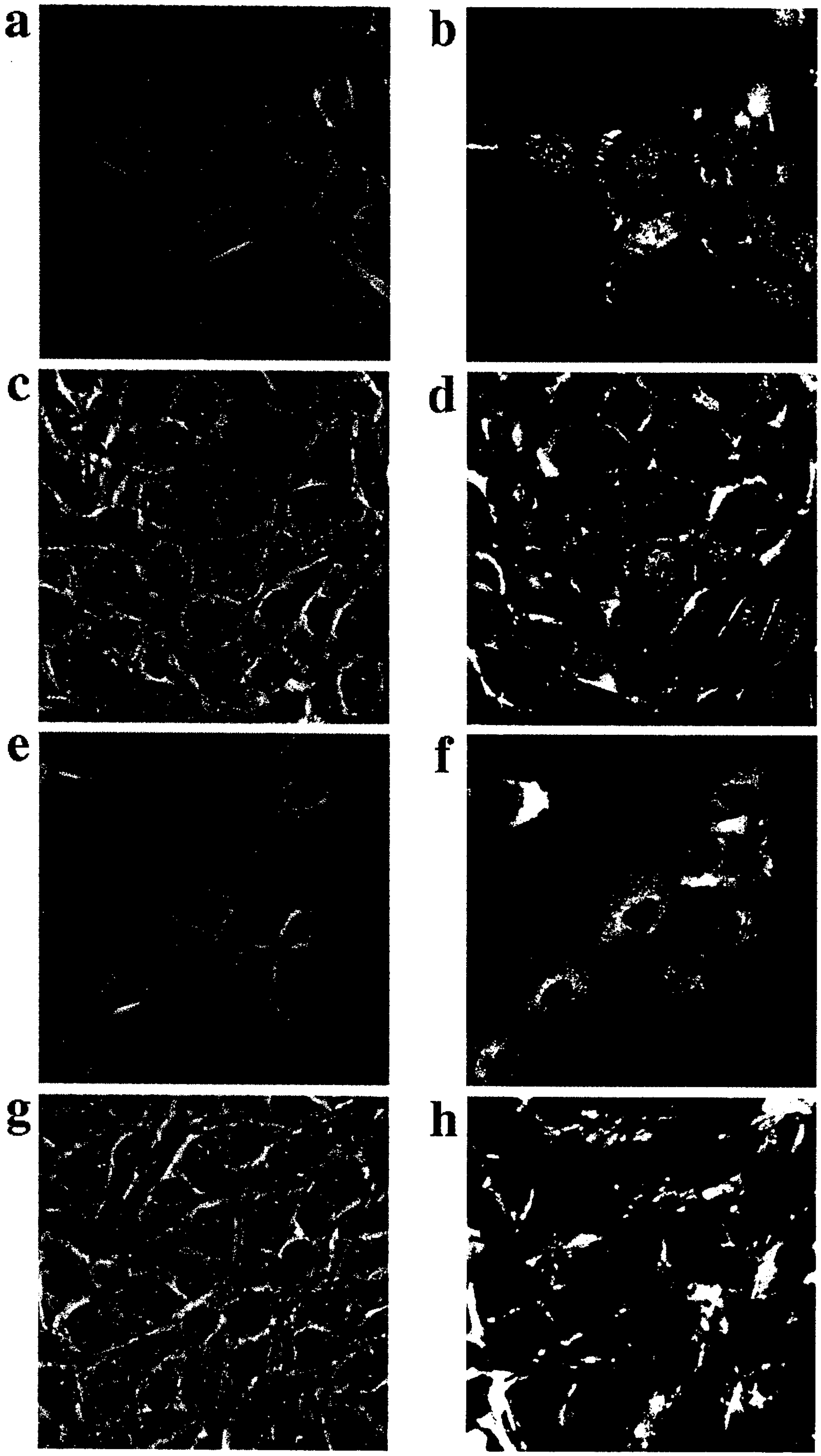

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Figure 4

Expression of the receptor protein tyrosine phosphatase, PTPμ, restores E-cadherin-dependent adhesion in human prostate carcinoma cells

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Number of characters: 54,626 (without spaces)

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Abbreviations: CHE, chelerythrine chloride; GF, GF109203X; GFP, green fluorescence protein; Go, Gö6776; LY, LY294002; PKC, protein kinase C; R, Rotlerrin; PTP, protein tyrosine phosphatase; RPTP, receptor protein tyrosine phosphatase; C-S, PTPμC1095S mutant; PTPμ-extra, construct containing the extracellular-, transmembrane- and 55 amino acids of the intracellular domains of PTPμ

ABSTRACT

Normal prostate expresses the receptor protein tyrosine phosphatase PTPμ whereas LNCaP prostate carcinoma cells do not. PTPμ has been shown to interact with the E-cadherin complex. LNCaP cells express normal levels of E-cadherin and catenins, but are defective in both PTPμ- and E-cadherin-dependent adhesion. Re-expression of PTPμ restored cell adhesion to PTPμ and to E-cadherin. A mutant form of PTPμ that is catalytically inactive was re-expressed and it restored adhesion to PTPμ and to E-cadherin. Expression of PTPμ-extra (which lacks most of the cytoplasmic domain) induced adhesion to PTPμ but not to E-cadherin, demonstrating a requirement for the presence of the intracellular domains of PTPμ to restore E-cadherin-mediated adhesion. In a companion paper, we demonstrate a direct interaction between the intracellular domain of PTPμ and RACK1, a receptor for activated protein kinase C (PKC). Activation of PKC reversed the adhesion of PTPμWT-expressing cells to E-cadherin, whereas treatment of parental LNCaP cells with PKCδ inhibitors induced adhesion to E-cadherin. Together, these studies suggest that PTPμ regulates the PKC pathway to restore E-cadherin-dependent adhesion via its interaction with RACK1.

INTRODUCTION

A diverse set of cellular behaviors including growth, differentiation, adhesion and migration are regulated by protein tyrosine phosphorylation. Protein tyrosine kinases and protein tyrosine phosphatases (PTPs) regulate intracellular phosphotyrosine levels. A family of receptor-like (RPTPs) and nontransmembrane PTPs have been identified and characterized (Neel and Tonks, 1997; Brady-Kalnay, 1998). Some of the RPTPs have extracellular segments containing adhesion molecule-like domains and intracellular segments that possess tyrosine phosphatase activity. This structural arrangement suggests that RPTPs directly send signals in response to cell adhesion.

The receptor protein tyrosine phosphatase PTPμ is a member of the immunoglobulin (Ig) superfamily of adhesion molecules. The extracellular segment of PTPμ contains a MAM (Meprin/A5/PTPμ) domain, an Ig domain and four fibronectin type III repeats (Gebbink et al., 1991). Expression of PTPμ induces aggregation of nonadherent cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993b) through a homophilic binding site that resides within the Ig domain (Brady-Kalnay and Tonks, 1994). The MAM domain plays a role in cell-cell aggregation by determining the specificity of adhesive interactions (Zondag et al., 1995). PTPμ contains a single membrane-spanning region with two cytoplasmic PTP domains. Only the membrane proximal PTP domain has catalytic activity (Gebbink et al., 1993a). The role of the membrane distal PTP domain is not known, but this domain has been implicated in directing protein-protein interactions in other RPTPs (reviewed in Brady-Kalnay, 1998). The intracellular juxtamembrane domain of PTPμ contains a region that is homologous to the conserved intracellular domain of the cadherins (Tonks et al., 1992).

Cadherins are a family of calcium-dependent adhesion molecules that play an essential role in the formation of the cell-cell contacts termed adherens junctions (Gumbiner, 2000). Cadherin-dependent adhesion is important for many physiological processes including establishment of cell polarity, morphogenetic movements such as epithelial/mesenchymal transitions, and cell-type sorting during development (Vleminckx and Kemler, 1999; Gumbiner, 2000). Cadherins interact with the actin cytoskeleton through the bindings of the cytoplasmic domain to catenins (reviewed in Provost and Rimm, 1999). The catenins include α, ß, γ/plakoglobin and p120. α-catenin is an actin binding protein that is homologous to vinculin. ß-catenin (also called *amadillo*) is an "arm repeat" protein and is homologous to γ-catenin and p120. ß- and γ-catenin bind directly to the cytoplasmic segment of cadherin, whereas α-catenin binds to ß- or γ catenin thereby linking the cadherin-catenin complex to the cytoskeleton. Deletions in the catenin binding region of cadherins disrupt cadherin-mediated adhesion despite the presence of an intact extracellular segment (Provost and Rimm, 1999).

Despite the importance of cadherin-mediated cell-cell adhesion, the underlying mechanisms that regulate adhesion are still poorly understood. PTPμ has been shown to associate with the cadherin/catenin complex (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998; Hiscox and Jiang, 1998). Specifically PTPμ interacts with a number of classical cadherins including E-cadherin, N-cadherin and cadherin 4 (also called R-cadherin) (Brady-Kalnay et al., 1998). The classical cadherins have a highly conserved cytoplasmic domain, PTPμ has been shown to bind directly to the C-terminal 38 amino acids of the intracellular domain of E-cadherin, which is the likely binding site in the other classical cadherins as well (Brady-Kalnay et al., 1998). We have recently shown that PTPμ regulates N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999). In fact, expression of a catalytically inactive form of PTPμ perturbed N-cadherin-mediated neurite outgrowth. This demonstrates that the phosphatase activity of PTPμ is required for N-cadherin-mediated signal transduction and/or regulation of the cytoskeleton in neurons.

In this study, we employed the LNCaP prostate carcinoma cell line (Horoszewicz et al., 1983) to investigate the role of PTPμ in cadherin-mediated adhesion. Unlike normal prostate epithelial cells, LNCaP cells do not express endogenous PTPμ. Although these cells express the proteins in the cadherin-catenin complex, we found that they were deficient in E-cadherin -mediated adhesion in aggregation assays and in an *in vitro* adhesion assay. Using a retroviral/tetracycline-repressible system, we re-expressed wild type and mutant forms of PTPμ in LNCaP cells. To study cadherin-dependent cell-cell adhesion, LNCaP cells were trypsinized in the presence of Ca^{2+}, and were subsequently allowed to aggregate. LNCaP cells infected with an empty vector weakly aggregated, whereas re–expression of PTPμ wild type (WT), or a catalytically inactive mutant form of PTPμ, increased the aggregation 3 fold. This increase in aggregation was only partly dependent on E-cadherin function, since it could only partly be blocked by pre-incubating the cells with function-blocking antibodies to E-cadherin. However, the aggregation was dependent on Ca^{2+} since it was blocked by EDTA. In this regard, the LNCaP cells also express N-cadherin (unpublished data). Therefore, to specifically study E-cadherin-dependent adhesion, we employed an *in vitro* adhesion assay that measured binding to a surface coated with purified adhesion molecules. Expression of PTPμ wild type (WT) restored E-cadherin-mediated adhesion, demonstrating that the expression of PTPμ is required for E-cadherin-mediated adhesion to occur. Re-expression of a catalytically inactive mutant form of PTPμ also restored E-cadherin-mediated adhesion in LNCaP cells, demonstrating that PTPμ exerts an effect on the cadherin adhesion complex which is independent of its phosphatase activity. To investigate whether the presence of the intracellular PTP domains of PTPμ were required to affect E-cadherin-dependent adhesion, cells were infected with a construct encoding the extracellular domain, the transmembrane domain and 55 amino acids of the intracellular domain of PTPμ (PTPμ-extra) (Brady-Kalnay et al., 1993). As expected, expression of PTPμ-extra induced LNCaP adhesion to recombinant PTPμ. However, PTPμ-extra did not induce adhesion to E-cadherin, demonstrating a requirement of the intracellular domains of PTPμ to restore E-cadherin-mediated adhesion. These data suggest that PTPμ may be recruiting signaling molecules to the cadherin complex that may be important for inducing E-cadherin-dependent adhesion. In a companion paper, we have shown that PTPμ interacts with RACK1 (Mourton et al., 2000). In this study, we show that both PTPμWT and the C-S mutant, but not PTPμ-extra, interact with RACK1 in LNCaP cells. RACK1 was originally identified as a receptor for activated protein kinase C (Ron et al., 1994), indicating a role for PKC in the regulation of E-cadherin-mediated adhesion. In cells where E-cadherin-dependent adhesion was restored by re-expressing PTPμ, activation of PKC by PMA resulted in detachment of the cells from E-cadherin within 15 min. To investigate whether PTPμ may be regulating the PKC pathway to restore E-cadherin-mediated adhesion, we treated uninfected LNCaP cells with kinase inhibitors. Inhibition of PKCδ, but not PKCα, PKCß or the PI3-kinase, induced LNCaP adhesion to E-cadherin. Taken together, these data indicate that the cytoplasmic domain of PTPμ regulates E-cadherin-mediated adhesion through regulation of the PKC pathway via its interaction with RACK1.

MATERIALS AND METHODS

Antibodies and reagents: Monoclonal antibodies against the intracellular (SK7) and extracellular (BK2) domains of PTPμ have been described (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994). A monoclonal antibody against γ-catenin (5172) was kindly provided by Dr. Pamela Cowin (NYU, NY). Monoclonal antibodies against chick L1 (8D9) were generated in our lab using hybridoma cells generously provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). Monoclonal antibodies against E-cadherin, p120, α- and β catenin, and RACK1 were purchased from Transduction Labs (Lexington, KY). A monoclonal antibody against vinculin, and a monoclonal antibody against the extracellular domain of E-cadherin (DECMA) were purchased from Sigma (St. Louis, MO). Goat anti-mouse IgG and IgM immunobeads were obtained from Zymed Laboratories (San Fransisco, CA), or goat anti-mouse IgG immunobeads were alternatively obtained from Cappel (Costa Mesa, CA). Normal prostate epithelial cells were purchased from Clonetics (San Diego, CA). LY294002 was obtained from New England Biolabs (Beverly, MA). PMA was purchased from Calbiochem (San Diego, CA). RPMI 1640 medium, SMEM medium and laminin were obtained from GIBCO-BRL (Grand Island, NY). Fetal bovine serum was obtained from

Hyclone (Logan, UT). Tween-20 was obtained from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Construction and expression of the PTPμ retroviruses: The retroviral system used is a tetracycline repressible ("tet-off") promoter-based system (Paulus et al., 1996). Using the pBPSTR1 vector generously provided by Dr. Steven Reeves (Harvard Medical School, Charlestown, MA), the following constructs were generated: wild type PTPμ, the C-S mutant form of PTPμ and PTPμ-extra. The wild type PTPμ plasmid (PTPμWT) and the PTPμC1095S (C-S) catalytically inactive mutant have been previously described (Burden-Gulley and Brady-Kalnay, 1999). Briefly, the wild type PTPμ plasmid contained almost the entire coding sequence of PTPμ (base pairs 1-4350, i.e. it only lacked the last two amino acids and the stop codon). This was done to create an in frame fusion with the green fluorescence protein at the C-terminus (PTPμ-GFP). The mutant form of PTPμ is also GFP tagged and contains a cysteine to serine mutation at residue 1095. A construct containing the extracellular, the transmembrane and 55 amino acids of the intracellular domains has been previously described (Brady-Kalnay et al., 1993) (PTPμ-extra is not GFP tagged). This construct was subcloned into the tetracycline-regulatable retroviral vector, pBPSTR1. Replication-defective amphotrophic retroviruses were made by transfecting the PA317 helper cell line (ATCC CRL-9078) with the respective PTPμ-containing plasmid. Control virus was generated by transfecting PA317 helper cells with the pBPSTR1 plasmid.

Expression and purification of GST fusion proteins: An E-cadherin GST fusion protein construct containing amino acids 9-139 of mouse E-cadherin was obtained from Dr Robert Brackenbury (University of Cincinnati, Cincinnati, OH). The E-cadherin GST fusion protein was constructed by restriction digest of pBATEM2 with PvuII and HincII. The fragment was ligated into the Sma I site of pGEX-KG, which results in a fusion protein containing amino acids 9-139 of E-cadherin with GST at the N-terminus. The GST fusion protein construct for expression of the entire extracellular domain of PTPμ has previously been described (Brady-Kalnay et al., 1993). Expression of GST-tagged proteins in *E.coli* was induced by IPTG. The bacteria were collected by centrifugation at 3000 x g for 10 min and lysed in PBS containing 1% Triton X-100, 5μg/ml leupeptin, 5μg/ml aprotinin and 1mM benzamidine, sonicated and centrifuged again at 3000 x g for 10 min to remove debris. The supernatant was passed over glutathione-sepharose beads (Pharmacia Biotech, Piscataway, NJ) washed and the bound protein eluted with 10mM glutathione as described previously (Brady-Kalnay et al., 1993).

Tissue culture and retroviral infection of LNCaP cells: LNCaP cells (Horoszewicz et al., 1983) were grown in RPMI 1640 supplemented with 10% FBS and 1 μg/ml gentamicin at 37°C and 5% CO_2. Cells were infected with retrovirus by the addition of polybrene (5 μg/ml) and virus-containing medium. The cells were incubated overnight at 37°C, and the medium was exchanged with normal culture medium. Five days after infection, the cells were checked for expression of the PTPμ proteins which were tagged with the green fluorescent protein (GFP) by fluorescence microscopy.

Protein Extraction and Immunoblotting: LNCaP cells were rinsed once with PBS and the cells were lysed in Triton-containing buffer (20mM Tris pH 7.6, 1% Triton X-100, 2mM $CaCl_2$, 1mM benzamidine, 200 μM phenyl arsine oxide, 1mM vanadate, 0.1mM ammonium molybdate and 2μl/ml protease inhibitor cocktail) and scraped off the dish. In all experiments where RACK1 was co-immunoprecipitated, the cells were lysed in a buffer containing 20mM Tris pH 7.6, 1% Triton X-100, 50mM NaCl , 1mM benzamidine, 1mM vanadate, and 2μl/ml protease inhibitor cocktail. After incubation on ice for 30 min, the lysate was centrifuged at 14, 000 rpm for 3 min and the Triton-soluble material was recovered in the supernatant. The amount of protein was determined by the Bradford method using BSA as a standard. Lysates were boiled in equal volume of 2X sample buffer and the proteins were separated by 6% or 10% SDS-PAGE and transferred to nitrocellulose for immunoblotting as described previously (Brady-Kalnay et al., 1993).

Immunoprecipitations: Antibodies (5 μg IgG/IP or 1.25μg IgM/IP) were incubated with goat anti-mouse IgG or IgM immunobeads respectively for 2 hours at room temperature then washed 3 times with PBS (phosphate buffered saline: 9.5mM phosphate, 137mM NaCl, pH 7.5). Immunoprecipitates were prepared by incubating lysates containing either 250 μg protein (Figure 7) or 400μg protein (Figure 8) with antibody-coupled beads overnight at 4°C. The beads were washed extensively with lysis buffer, then boiled in sample buffer and separated by 6% or 10% SDS-PAGE. The immunoprecipitate was loaded per lane. Proteins were transferred to nitrocellulose membrane and immunoblotted as described (Brady-Kalnay et al., 1993).

Aggregation Assay: Aggregation assays were performed as previously described (Brackenbury et al., 1977). Briefly, uninfected LNCaP cells or cells infected with either PTPμWT or C-S mutant were rinsed twice in HCMF buffer (10mM HEPES, pH7.4, 137mM NaCl, 5.4mM KCl, 0.3mM Na_2HPO_4-$7H_2O$, 5.5mM glucose) containing 2mM $CaCl_2$, and trypsinized into single cells by incubation with 0.04% trypsin in HMCF buffer supplemented with 2mM $CaCl_2$. The trypsin was inactivated by the addition of RPMI containing 10% serum. The cells were pelleted and resuspended in 2ml SMEM, followed by a 20 min incubation with 20 U/ml DNase on ice. Some of the cells re-expressing PTPμWT were treated with 200μg/ml of an E-cadherin function blocking antibody (DECMA) for an additional 20 min on ice. $2x10^6$ cells were added to scintilation vials containing HMCF buffer with a final concentration of 2mM $CaCl_2$. Where indicated, the $CaCl_2$ was substituted with 5mM EDTA (final concentration). Aggregation was initiated by placing the vials at 37°C at 90 rpm in a gyratory shaker. Aliquotes of the samples were diluted 50 fold in PBS and the number of particles were determined using a Coulter Counter. The Coulter Counter was set at: lower threshold-10%, 1/aperture current-16, 1/amplification-2, The percent aggregation was calculated by subtracting the number of particles after 1h (N_t) from the initial particle number and dividing by the initial number $\{((N_0 - N_t)/N_0)x100\}$.

LNCaP Adhesion to Purified Proteins: Sterile coverslips were coated overnight with 100μg/ml Poly-L-lysine (Sigma), washed twice in sterile water and were allowed to dry. Subsequently, the coverslips were coated with nitrocellulose in methanol (Lagenaur and Lemmon, 1987) and allowed to dry. Purified recombinant proteins were diluted in PBS containing 2mM $CaCl_2$ to a concentration of 75 μg/ml for PTPμ and E-cadherin respectively, and 40 μg/ml for laminin. To identify the individual protein spots on the coverslips, the protein solutions were supplemented with 20μg/ml Texas Red BSA (Sigma). Three distinct spots, each containing a single adhesion molecule, were generated by spotting 20 μl of each protein solution on one coverslip. After a 20 min. incubation at room temperature, the protein solutions were aspirated, and this procedure was repeated once. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in PBS, and the dishes were rinsed with RPMI-1640 medium. LNCaP cells infected with the indicated retrovirus were fully trypsinized with 0.05% trypsin/0.53mM EDTA (GIBCO) and 3 x 10^5 cells were added to coverslips, and the cells were allowed to adhere overnight to regenerate cell surface proteins. In the control experiments, function-blocking antibodies to either PTPμ (BK2, 10μl/ml of ascites) or E-cadherin (DECMA, 1:1dilution of culture supe), or 5mM EDTA (final concentration) was added to the dishes just prior to the addition of cells. In some experiments, the overnight incubation was followed by a 15 min. incubation with 20nM PMA or an equal volume of DMSO. Alternatively, uninfected LNCaP cells were added to coverslips and incubated over night followed by a 45 min. incubation with either 5μM Rotlerrin, 10μM chelerythrine chloride, 0.5μM GF109203X, 15 nM Gö6976, 10μM LY294002 or DMSO alone. At the concentrations used, chelerythrine chloride (IC_{50}=0.66μM) and GF109203X (IC_{50} ranges between 8nM-5.8μM for different isoforms of PKC) are specific for PKC, whereas Rotlerrin is specific for PKCδ (IC_{50}=6μM), and Gö6976 is specific for PKCα and -ß (IC_{50}=2.3nM and 6nM, respectively). LY294002 inhibits the PI 3-kinase (IC_{50}=1.4μM). The medium was then removed and the coverslips were rinsed once in PBS to remove unattached cells. The cells were subsequently fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80mM Pipes, 5mM EGTA, 1mM $MgCl_2$, 3% sucrose), pH 7.4 for 30 min. at room temperature. The coverslips were washed twice in PBS and mounted in IFF mounting medium (0.5M Tris-HCl pH 8.0 containing 20% glycerol and 0.1% p-phenylenediamine). Adherent cells were detected by dark field microscopy, using a 5x objective, and photographed. To quantify the number of adherent cells, the 35 mm negatives were scanned and the digitized images were analyzed using the Metamorph image analysis program (Universal Imaging Corp., West Chester, PA). The number of adherent cells per image was approximated by highlighting the cells using the threshold function, and the total number of highlighted cells per image was calculated. The data obtained in 4-6 separate experiments were analyzed by Student's t test (Statview 4.51, Abacus Concepts, Inc.).

RESULTS

Re-expression of PTPμ

The receptor tyrosine phosphatase PTPμ has previously been shown to interact with E-cadherin in a variety of tissues by immunoprecipitation (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998; Hiscox and Jiang, 1998). To investigate whether PTPμ plays a functional role in E-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line (Horoszewicz et al., 1983). Unlike normal prostate epithelial cells (NPr), these cells do not express PTPμ (Fig. 1A, VEC). To re-express PTPμ in LNCaP cells, we generated a tetracycline-regulatable retrovirus encoding the PTPμ cDNA sequence tagged with the green fluorescence protein (PTPμ-GFP) (Burden-Gulley and Brady-Kalnay, 1999). Using this retrovirus, we re-expressed wild type PTPμ (PTPμWT) in the LNCaP cells. Five days after retroviral infection, the cells were analyzed for expression of PTPμWT-GFP by immunoblot and by fluorescence microscopy. Immunoblot analysis showed that LNCaP cells infected with retrovirus containing an empty vector do not express PTPμ (Fig. 1A, VEC), whereas cells infected with retrovirus containing PTPμWT (Fig 1A, WT) expressed both the full length protein (200 kDa) as well as the proteolytically processed forms (~100 kDa) (Brady-Kalnay and Tonks, 1994).

Due to the GFP-tag, both the full length- and the proteolytically processed forms of the re-expressed PTPμWT migrated at a higher molecular weight than the PTPμ expressed in normal prostate cells (Fig.1A, NPr). The retroviral system we used is a tet-off system, in the presence of tetracycline the gene is not expressed. The re-expression of PTPμWT was inhibited by treating the cells with 4μg/ml of tetracycline (Fig 1A, WT+T). Fluorescence microscopy revealed that between 70-90 % of the LNCaP cells expressed PTPμWT, and that PTPμ was primarily localized to the plasma membrane as expected (Fig. 2C and D). This expression was repressed when the cells were grown in the presence of 4 μg/ml of tetracycline (Fig. 2E and F). Control cells infected with a virus containing an empty vector did not show any fluorescence (Fig. 2A and B).

To assess the functional role of PTPμ catalytic activity in the regulation of E-cadherin-mediated adhesion, we have generated tetracycline regulatable retrovirus encoding a mutant form of PTPμ-GFP containing a single amino acid mutation in the catalytic site (Burden-Gulley and Brady-Kalnay, 1999). Mutation of the conserved cysteine residue PTPμC1095S (C-S) results in a catalytically inactive enzyme. Immunoblot analysis showed that the C-S mutant was expressed at a similar level to PTPμWT in LNCaP cells (Fig. 1A, C-S). Fluorescence microscopy confirmed that infection with the C-S mutant (Fig. 2G and H) resulted in expression at the plasma membrane at a similar level as

PTPμWT, demonstrating that the expression and subcellular localization are not affected by the change in catalytic activity.

Expression of cadherins and catenins

Cadherin-mediated cell-cell adhesion is dependent on the expression of both cadherins and catenins. Immunoblot analysis demonstrated that LNCaP cells expressed E-cadherin as well as α, ß and γ-catenin and p120 (Fig 3, LNCaP). This is in accordance with normal prostate epithelial cells, which were found to express similar amounts of E-cadherin as well as α, ß and γ-catenin, and vinculin (Fig. 3, NPr). However, LNCaP cells express significantly higher levels of p120 than normal prostate epithelial cells. Infection of LNCaP cells with an empty vector (Fig. 3, VEC), PTPμWT (Fig. 3,WT) or the C-S mutant form of PTPμ (Fig 3, C-S) did not alter the expression of any of the proteins in the cadherin/catenin complex. It is possible that re-expression of wild type or mutant forms of PTPμ may alter the subcellular localization of the proteins in the cadherin/catenin complex, thereby altering the function of the complex. To address this question, we performed immunocytochemical analysis on LNCaP cells using antibodies to E-cadherin as well as α, ß and γ-catenin and p120. However, re-expression of either

PTPμWT or the C-S mutant did not significantly alter the subcellular localization of any of the proteins examined (data not shown).

Re-expression of PTPμ enhanced LNCaP cell aggregation

To investigate whether PTPμ plays a role in E-cadherin-mediated adhesion in LNCaP cells, we trypsinized the cells in the presence of $CaCl_2$ to preserve the cadherins (Takeichi, 1977). The cells were then allowed to aggregate for one hour. LNCaP cells infected with an empty vector weakly aggregated (Fig. 4, VEC). Re-expression of PTPμWT increased the aggregation 3 fold (Fig. 4, WT), as did expression of the C-S mutant (Fig. 4, C-S). The increased aggregation was only partly dependent on E-cadherin function, since the presence of an E-cadherin function-blocking antibody did not completely reduce the aggregation induced by re-expression of PTPμ (Fig. 4, WT+E-cad. Ab). However, the increased aggregation was Ca^{2+}-dependent (Fig. 4, WT+EDTA), since the presence of EDTA reduced the aggregation to a level below that seen in cells infected with an empty vector (Fig. 4, VEC). The residual Ca^{2+}-dependent adhesion is at least due in part to the fact that LNCaP cells express N-cadherin (data not shown). Taken together, these findings demonstrate that re-expression of PTPμ in LNCaP cells induced Ca^{2+}-dependent aggregation that is partly due to E-cadherin-dependent cell-cell adhesion.

Thus, aggregation assays were not ideal to specifically study E-cadherin-dependent adhesion in LNCaP cells. Therefore, we utilized an *in vitro* adhesion assay that measures specific binding to a given adhesion molecule which is similar to a previously published assay (Brady-Kalnay et al., 1993).

Re-expression of PTPμ induced adhesion to purified PTPμ

To study the specific interactions between cell-cell adhesion molecules in LNCaP cells, we developed an *in vitro* adhesion assay where purified, recombinant proteins were immobilized on nitrocellulose-coated coverslips. PTPμ has been shown to mediate cell-cell adhesion via homophilic binding (Brady-Kalnay et al., 1993; Gebbink et al., 1993b). To verify that the re-expressed forms of PTPμ were able to mediate homophilic binding in LNCaP cells, we investigated the adhesion of LNCaP cells to purified recombinant PTPμ that was immobilized on nitrocellulose-coated coverslips. As expected, cells infected with an empty vector did not adhere to PTPμ (Fig. 5A) since these cells do not express PTPμ. Re-expression of PTPμWT induced LNCaP adhesion to purified PTPμ (Fig. 5D), as did re-expression of the C-S mutant (Fig. 5G). Quantitation of the adhesion assays (N=6) showed that the number of cells that adhered to purified PTPμ was significantly higher for cells infected with both the WT and the C-S mutant form of PTPμ as compared to cells infected with vector only (Table I). However, there was no difference between cells expressing PTPμWT compared to the C-S mutant in their ability to adhere to purified PTPμ (Table I). Taken together, these data confirm that the re-expressed PTPμ is capable of mediating homophilic binding, and that the phosphatase activity is not necessary for this adhesion to occur, as previously demonstrated (Brady-Kalnay et al., 1993). To ensure the specificity of the adhesion assay, we repeated the experiments in the presence of function-blocking antibodies to either PTPμ or E-cadherin. The presence of an antibody to the extracellular domains of PTPμ specifically inhibited the adhesion to recombinant PTPμ of LNCaP cells re-expressing PTPμWT (6A, WT+ PTPμ Ab), or cells expressing the C-S mutant (Fig. 6A, C-S+PTPμ Ab). As expected, the presence of the E-cadherin antibody had no effect on adhesion to PTPμ (Fig. 6A, WT+E-cad. Ab, C-S+E-cad. Ab).

As an internal control in each experiment, cells were allowed to adhere to laminin. Adhesion to extracellular matrix proteins such as laminin is mediated through integrin receptors. Since there is no evidence indicating that PTPμ regulates integrin function, LNCaP adhesion to laminin should not be affected by the re-expression of PTPμ. As expected, LNCaP cells infected with an empty vector adhered to laminin (Fig. 5B), and this adhesion was not significantly affected by re-expression of either WT (Fig. 5E) or C-S mutant forms of PTPμ (Fig. 5H, Table I). None of the retroviral infected cells adhered to nitrocellulose coated with BSA only (data not shown).

Re-expression of PTPμ restores E-cadherin-mediated adhesion

To study the role of PTPμ in the regulation of E-cadherin-mediated adhesion in LNCaP cells, we immobilized purified recombinant E-cadherin on the nitrocellulose-coated coverslips. Despite the fact that these cells express E-cadherin as well as α-, ß- and γ-catenin and p120 (Fig. 3), LNCaP cells infected with an empty vector did not adhere to E-cadherin (Fig. 5C). Re-expression of PTPμWT restored the ability of LNCaP cells to adhere to E-cadherin (Fig. 5F). Quantitation of the adhesion assays show that the number of cells infected with PTPμWT that adhered to E-cadherin was significantly higher than the number of cells infected with vector only (Table I). These data show that expression of PTPμ is necessary for E-cadherin-mediated adhesion in LNCaP cells.

Since tyrosine phosphorylation has been reported to negatively regulate cadherin-mediated adhesion, we investigated whether PTPμ restored E-cadherin-mediated adhesion by dephosphorylating key components of the cadherin-catenin complex. To do this, we repeated the adhesion assays with cells expressing the C-S mutant form of PTPμ. Expression of the C-S mutant restored E-cadherin-mediated adhesion (Fig. 5I). As shown in Table I, re-expression of WT or the C-S mutant form of PTPμ induced a significant increase in adhesion to E-cadherin as compared to LNCaP cells infected with an empty vector. In contrast, there was no difference in adhesion between cells infected with

PTPμWT compared to cells infected with the C-S mutant. The statistical analysis for LNCaP cell adhesion to E-cadherin is summarized in Table I. In control experiments, the adhesion to E-cadherin was totally blocked by a function-blocking antibody to E-cadherin (Fig. 6B, WT+E-cad. Ab, and C-S+E-cad. Ab, respectively). In contrast, the PTPμ antibody did not affect the adhesion to E-cadherin induced by the re-expression of PTPμWT (Fig. 6B, WT+PTPμ Ab) or by the expression of the C-S mutant (Fig. 6B, C-S+PTPμ Ab). E-cadherin-mediated adhesion in this assay is Ca^{2+}-dependent, addition of 5 mM EDTA abolished adhesion to E-cadherin (Fig. 6B, WT+EDTA, and C-S+EDTA, respectively). However, the presence of EDTA did not affect the adhesion to PTPμ of cells either re-expressing PTPμ-WT (Fig. 6A, WT+EDTA) or expressing the C-S mutant (Fig. 6A, C-S+EDTA), Taken together, these data indicate that although the presence of the PTPμ protein is required for E-cadherin-mediated adhesion in LNCaP cells, it does not require PTPμ catalytic activity.

It is possible that the PTPμ intracellular domain may recruit other proteins that aid in restoring E-cadherin-mediated adhesion. To determine whether the intracellular PTP domains of PTPμ were required to affect E-cadherin-dependent adhesion, we constructed a retrovirus encoding for the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (PTPμ-extra) (Brady-Kalnay et al., 1993). Western blot analysis confirmed that this construct was expressed in LNCaP cells (Fig. 1B, Extra).

Expression of PTPμ-extra induced LNCaP adhesion to purified recombinant PTPμ (Fig. 5J; Table I), confirming that the intracellular domains are not required for PTPμ to mediate homophilic binding (Brady-Kalnay et al., 1993). In addition, this adhesion was blocked by an antibody to PTPμ (Fig. 6A, Extra+PTPμ Ab). The antibody to E-cadherin and 5mM EDTA had no major effect on the adhesion to PTPμ (Fig. 6A, Extra+E-cad. Ab and Extra+EDTA, respectively). However, PTPμ-extra did not restore LNCaP adhesion to recombinant E-cadherin (Fig. 5L), demonstrating that the intracellular domains of PTPμ are necessary for E-cadherin-mediated adhesion. Since LNCaP cells expressing PTPμ-extra did not adhere to E-cadherin (Fig. 5L; Fig. 6B, Extra), the presence of either the PTPμ antibody, the E-cadherin antibody or 5 mM EDTA had no effect on adhesion to E-cadherin (Fig. 6B, Extra + PTPμ Ab, Extra + E-cad. Ab, and Extra + EDTA, respectively). As expected, the expression of PTPμ extra did not affect LNCaP adhesion to laminin (Fig. 5K).

As shown in Fig. 5, LNCaP cells expressing an empty vector did not adhere to either PTPμ or E-cadherin (Fig. 6A, VEC, and Fig. 6B, VEC, respectively), and this was not altered by the presence of either the PTPμ antibody, the E-cadherin antibody or 5 mM EDTA (data not shown). Taken together, these experiments demonstrate that this *in vitro* adhesion assay can be used to study specific binding to cell-cell adhesion molecules.

PTPμ does not alter the association of α-, ß-, γ-catenin or p120 to E-cadherin

To examine the possibility that the presence of a full length PTPμ affects the binding of α-, ß-, γ-catenin or p120 to E-cadherin, we immunoprecipitated E-cadherin from cells infected with an empty vector (VEC), PTPμWT (WT), C-S or PTPμ-extra (Extra). As shown in Fig. 7, the immunoprecipitates from cells infected with an empty vector, PTPμWT as well as the C-S and PTPμ-extra contained equal amounts of E-cadherin. The western blot was stripped and reprobed with antibodies to the catenins. Immunoprecipitates from cells infected with an empty vector contained α-, β-, and γ-catenin as well as p120. Infection of cells with various forms of PTPμ did not significantly alter the amounts of the catenins that co-immunoprecipitated with E-cadherin. As a control, a monoclonal antibody to chick L1 (8D9) was used. This antibody did not immunoprecipitate either E-cadherin or any of the catenins.

The PTPμ cytoplasmic domain is required for the interaction with RACK1

Even though the presence of PTPμ does not alter the composition of the E-cadherin/catenin complex, it is possible that full length PTPμ regulates E-cadherin-dependent adhesion by recruiting other signaling molecules to the cadherin/catenin complex. In a companion paper (Mourton et al., 2000), we describe an interaction between the membrane-proximal phosphatase domain of PTPμ and RACK1, a receptor for activated PKC (Ron et al., 1994). Immunoprecipitation of RACK1 showed that LNCaP cells infected with an empty vector expressed RACK1(Fig. 8A, VEC), and that infection of cells with various forms of PTPμ did not alter the expression of RACK1 (Fig. 8A, WT, C-S and E, respectively). To investigate whether PTPμWT and the C-S mutant associate with RACK1 in LNCaP cells, we immunoprecipitated PTPμ using an antibody to the extracellular domain of PTPμ (BK2). RACK1 was found to associate with both PTPμWT (Fig. 8B, WT) and the C-S mutant (Fig. 8B, C-S), but not with PTPμ-extra (Fig. 8B, E). As expected, PTPμ antibody did not immunoprecipitate RACK1 from cells infected with an empty vector (Fig. 8B, VEC). This experiment was repeated with an antibody to the intracellular domain of PTPμ (SK7). As seen in Figure 8C, this antibody also co-immunoprecipitated RACK1 from cells infected with PTPμWT or C-S, but not from cells infected with PTPμ-extra or an empty vector. Taken together, these data demonstrate that full length PTPμ regardless of its catalytic activity associates with RACK1.

The association between PTPμ and RACK1 suggests that the presence of PTPμ may alter signaling through the PKC pathway to regulate E-cadherin-mediated adhesion. Several studies have shown that activation of the PKC pathway can either upregulate or downregulate E-cadherin-mediated adhesion depending on cell type (Lewis et al., 1994; Skoudy and Garcia de Herreros, 1995; Llosas et al., 1996). Therefore, we investigated whether activation of PKC by PMA affects the ability of PTPμ to restore E-cadherin-mediated adhesion. LNCaP cells infected with retrovirus containing PTPμWT were allowed to adhere to PTPμ, laminin or E-cadherin as described above. The cells were then treated with PMA for 15 min. to activate PKC, which did not affect adhesion to either PTPμ or laminin (Fig. 9A). However, activation of PKC detached the PTPμ-expressing cells from E-cadherin (Fig.9A). The statistical analyses for the effects of PMA on LNCaP adhesion are shown in Table I.

Inhibition of PKCδ induced LNCaP adhesion to E-cadherin

To further examine the signal transduction pathways involved in restoring E-cadherin-mediated adhesion, we studied the effect of various kinase inhibitors on the ability of LNCaP cells to adhere to E-cadherin. Uninfected LNCaP cells were added to coverslips with immobilized E-cadherin, and the cells were subsequently treated with the inhibitors. We found that treatment of the cells with three different PKC inhibitors, chelerythrine chloride, GF109203X, and a PKCδ-specific inhibitor (Rotlerrin) induced LNCaP adhesion to E-cadherin (Fig. 9B, CHE, GF and R, respectively). This effect was specific for inhibition of PKCδ , since treatment of LNCaP cells with neither DMSO, the PKCα- and PKCß-specific inhibitor Gö6976, nor the PI 3-kinase inhibitor LY294002 induced LNCaP adhesion to E-cadherin (Fig. 9B, DMSO, Go and LY, respectively). Also, the effect of the PKCδ inhibition was specific in that it only affected the ability of LNCaP cells to adhere to E-cadherin, but did not alter adhesion to PTPμ or laminin (data not shown). Furthermore, PKCδ was found to associate with RACK1 in LNCaP cells, although this association was not affected by the presence of either PTPμWT or the C-S mutant (Fig. 8D). Our data suggests that PTPμ negatively regulates PKCδ activity to restore E-cadherin-dependent adhesion. However, the precise mechanism of PKCδ regulation by PTPμ is not clear but is likely to involve RACK1. Taken together, these data indicate that PTPμ may restore E-cadherin-mediated adhesion in LNCaP cells by regulating the PKC pathway through the recruitment of RACK1 to the PTPμ complex.

DISCUSSION

Alterations in the function of the E-cadherin/catenin adhesion system occur frequently in a wide variety of human carcinomas (Behrens, 1999). The molecular mechanisms underlying the loss of expression or functionality of individual components of the cadherin/catenin complex is still only partly understood. Previous studies have shown that PTPμ associates with classical cadherins (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998; Hiscox and Jiang, 1998). The functional importance of this interaction was illustrated in a recent study from our lab where we demonstrated that PTPμ regulates N-cadherin-mediated neurite outgrowth of retinal ganglion cells (Burden-Gulley and Brady-Kalnay, 1999). Therefore, it is possible that a loss of expression or function of PTPμ may result in defect in cadherin-mediated adhesion. To investigate the role of PTPμ in E-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line (Horoszewicz et al., 1983). These cells provide a good model system in that they, unlike normal prostate epithelial cells, do not express endogenous PTPμ. This allowed us to re-express PTPμ and study the effects of wild type (WT) as well as mutant forms of PTPμ without the interference of endogenous PTPμ. Retroviral re-expression of both WT and the catalytically inactive mutant form of PTPμ induced LNCaP cell adhesion to purified recombinant PTPμ, demonstrating that PTPμ was indeed expressed at the cell surface at a level that could mediate homophilic binding. These results also show that perturbation of the phosphatase activity did not alter the subcellular localization or the ability of PTPμ to mediate homophilic binding as expected (Brady-Kalnay et al., 1993).

Although LNCaP cells express E-cadherin, as well as α-, ß- and γ-catenin and p120, they were found to be deficient in E-cadherin-mediated adhesion. Re-expression of PTPμWT restored this adhesion, demonstrating a functional role for PTPμ in E-cadherin-mediated adhesion. The fact that the re-expression of the catalytically inactive mutant also restored E-cadherin-mediated adhesion indicates that PTPμ exert an effect on E-cadherin-dependent adhesion that is independent of its catalytic activity. This process requires the presence of one or both of the phosphatase domains, since expression of PTPμ-extra failed to restore E-cadherin-mediated adhesion. It is possible that PTPμ alters the cadherin function by recruiting some signaling protein(s) to the cadherin complex through protein-protein interactions involving the PTPμ intracellular domain.

Others have reported that the intracellular domain of RPTPs mediate protein-protein interactions that are independent of their tyrosine phosphatase activity. For example, the RPTPs LAR, PTPδ and PTPσ have been shown to interact with a cytoplasmic protein termed LAR-interacting protein 1 (LIP-1) (Pulido et al., 1995) through their second, catalytically inactive phosphatase domain. LAR also interacts with Trio (Debant et al., 1996), a multifunctional protein containing four spectrin-like repeats, a rac-specific and rho-specific guanidine nucleotide exchange factor domain, two pleckstrin –like domains, an Ig-like domain and a serine/threonine kinase domain. Neither LIP-1 nor Trio is tyrosine phosphorylated indicating that they are not likely to be substrates of the LAR family phosphatases. Therefore, the interaction between RPTPs and various proteins may serve to regulate either the subcellular localization of RPTPs, or to recruit other signaling molecules to form a larger signaling complex. The fact that PTPμ, regardless of its catalytic activity, could restore E-cadherin-mediated adhesion suggests that part of its role in the cadherin complex is to recruit other signaling molecules that may be needed for functional E- cadherin-dependent adhesion. The importance of the intracellular domain of PTPμ is clearly demonstrated by the finding that LNCaP adhesion to E-cadherin was not restored by the expression of a construct where the majority of the intracellular domain of PTPμ had been deleted (PTPμ-extra). In this regard, we have demonstrated that the first phosphatase domain of PTPμ binds to RACK1 (Mourton et al., 2000). RACK1 is a homologue of the G_{β} subunit of heterotrimeric G-proteins (Ron et al., 1994) and consists of seven WD repeats that are believed to form a propeller-like structure (Garcia-Higuera et al., 1996). RACK1 is thought to be a scaffolding molecule since each of the seven WD repeats could potentially mediate protein-protein interactions. RACK-1 was originally described as a receptor for activated PKC (Ron et al., 1994), but more recent studies have described its interaction with a variety of signaling proteins such as src (Chang et al., 1998), and with select pleckstrin homology domains *in vitro* (Rodriguez et al., 1999). In this study, we found that full length PTPμ interacts with RACK1, and that this interaction is not dependent upon the catalytic activity of PTPμ. The interaction between PTPμ and RACK1 suggests that PTPμ regulates E-cadherin-mediated adhesion by recruiting RACK1 to the PTPμ adhesion complex.

Despite numerous attempts to clarify the regulation of cadherin function by tyrosine phosphorylation, it is not fully understood. Components of the of the cadherin/catenin complex are phosphorylated by a number of cytoplasmic and receptor protein tyrosine kinases including src, EGF receptor and met, (scatter factor receptor) (Brady-Kalnay, 1998). Other PTPs in addition to PTPμ were also shown to interact with cadherins and catenins (Brady-Kalnay, 1998). The association of the cadherins with both kinases and phosphatases indicates a critical role for dynamic tyrosine phosphorylation in cadherin function. Tyrosine phosphorylation of components of the cadherin/catenin complex has been correlated with loss of cadherin-mediated adhesion and destabilization of adherens junctions (Brady-Kalnay, 1998). Therefore, adhesive function may be controlled by reversible tyrosine phosphorylation. Alterations in the expression of tyrosine kinases or PTPs may result in changes in the association of catenins and/or adhesive function of the cadherins. Treatment of MvLu cells with the broad-specific tyrosine phosphatase inhibitor, pervanadate, resulted in the tyrosine phosphorylation of the PTPμ-associated cadherins (Brady-Kalnay et al., 1995). This data was supported by studies performed on cells transformed with a temperature-sensitive form of the Rous Sarcoma Virus (RSV) (Brady-Kalnay et al., 1998). The mutant RSV is temperature sensitive for pp60*src* tyrosine kinase activity. When grown at the permissive temperature, increased tyrosine phosphorylation induced by src resulted in an increased tyrosine phosphorylation of E-cadherin, which correlated with a decreased association between PTPμ and E-cadherin. The association between PTPμ and the cadherin/catenin complex suggested that cadherins and/or catenins may be substrates for PTPμ. However, in this study we show that PTPμ regulates the cadherin function independently of its phosphatase activity, indicating that the cadherin/catenin complex may not be the primary substrates for PTPμ. We have previously shown that PTPμ catalytic activity is required for N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999). These data indicate that PTPμ catalytic activity may be required for signaling events that regulate the cytoskeleton and thus other cadherin-dependent functions downstream of adhesion. An alternative hypothesis is that the interaction between RACK1 and PTPμ may regulate the presence of the src protein tyrosine kinase in the cadherin/catenin complex. In a companion paper, we found that PTPμ and src compete for binding to RACK1. RACK1 binds to the SH2 domain of src, an interaction that inhibits src kinase activity (Chang et al., 1998). The presence of PTPμ would recruit RACK1 to the plasma membrane where it could bind and inactivate src, which could indirectly regulate the tyrosine phosphorylation of either E-cadherin or the catenins, thereby restoring E-cadherin-mediated adhesion.

Several studies have indicated that PKC is involved in the regulation of E-cadherin-mediated adhesion and the formation of adherens junctions. The molecular mechanisms whereby PKC regulates E-cadherin function are unknown. Additionally, the activation of PKC has been reported to have the opposite effects on E-cadherin function in different cell types. For example, the calcium-induced formation of adherens junctions in keratinocytes is dependent on the activation of PKC (Lewis et al., 1994). On the other hand, activation of PKC has been shown to induce a dissociation of E-cadherin from the cytoskeleton (Skoudy and Garcia de Herreros, 1995), followed by cell scattering in the HT29 intestinal cell line (Llosas et al., 1996). In this study, we show that the inhibition of PKCδ restored E-cadherin function in LNCaP cells. PTPμ restored E-cadherin-dependent adhesion which could be reversed by PMA stimulation of PKC. These data suggest that PTPμ may negatively regulate PKC activity. Although the precise mechanism is unclear, it is likely to involve the PTPμ/RACK1 complex.

Others have shown that serine/threonine phosphorylation of p120 negatively regulates E-cadherin-mediated adhesion (Aono et al., 1999; Ohkubo and Ozawa, 1999). It is therefore possible that the inactivation of PKCδ leads to decreased phosphorylation of p120 and thereby increased E-cadherin-mediated adhesion. However, we could not detect any alteration in the phosphorylation of p120 either after re-expression of PTPμ or after treatment of uninfected LNCaP cells with the PKCδ inhibitor Rotlerrin (data not shown). In addition, activation of PKC caused cells expressing PTPμWT to dissociate from an E-cadherin substrate. The fact that this dissociation occurred within 15 min. after the addition of PMA argues that PKC directly affects the E-cadherin complex, rather than downregulating the expression of either E-cadherin or the catenins. Therefore, the role of PTPμ in regulating E-cadherin-mediated adhesion could be to recruit RACK1 to the plasma membrane, thereby regulating the PKC pathway.

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8586, was supported by the Department of Defense Prostate Cancer Research Program, which is managed by the U.S. Army Medical Research and Materiel Command, and a prostate pilot grant from the Case Western Reserve University Cancer Center. C. Hellberg was supported by The Swedish Society for Medical Research.

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LEGENDS TO THE FIGURES

Figure 1. Re-expression of PTPμ in LNCaP cells. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), a mutant form of PTPμ containing a single point mutation in the catalytic site (C-S), or a construct the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (Extra). To verify that protein expression is under tetracycline control, 4μg/ml of tetracycline was added daily to cells infected with retrovirus containing PTPμWT (WT+T). Five days after infection, the cells were lysed, and 30 μg of lysate from normal prostate epithelial cells (NPr) and LNCaP cells were separated by 6 % SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal antibodies to PTPμ. Note that both PTPμWT and the C-S mutant have a GFP tag and therefore migrate at a higher molecular weight than PTPμ expressed in the normal prostate epithelial cells. **A.** Western blot using the monoclonal antibody SK7 against the intracellular domain of PTPμ. **B.** Western blot using the monoclonal antibody BK2 against the extracellular domain of PTPμ.

Figure 2. GFP-tagged forms of PTPμ are expressed at the cell surface and regulated by tetracycline. LNCaP cells were infected with a retrovirus containing an empty vector

(A and B), with retrovirus containing wild type PTPμ tagged with GFP in the absence (C and D) or presence (E and F) of 4 μg/ml tetracycline, or with a mutant form of PTPμ containing a single C-S point mutation in the catalytic site (G and H). Five days after infection, the expression of GFP-tagged proteins was visualized by fluorescence microscopy (128x magnification). Representative phase contrast (A, C, E and G) and fluorescent (B, D, F and H) images are shown.

Figure 3. Expression of proteins in the cadherin/catenin complex. LNCaP cells were left untreated (LNCaP) or infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), or mutant forms of PTPμ containing a single point mutation in the catalytic site (C-S). Five days after infection the cells were lysed and 15 μg of lysate from normal prostate epithelial cells (NPr) and the LNCaP cells were separated by 6 % SDS-PAGE, transferred to nitrocellulose and blotted with antibodies against the indicated proteins.

Figure 4. Aggregation assays. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), or a mutant form of PTPμ containing a C-S point mutation in the catalytic site (C-S). Five days after infection, the cells were trypsinized in the presence of 2mM $CaCl_2$ to preserve the cadherins. The cells were either left untreated or, where indicated, incubated with 200μg/ml of DECMA, an E-cadherin adhesion

blocking antibody (E-cad. Ab) for 20 min. on ice. The cells were induced to aggregate by incubating in the presence of 2mM $CaCl_2$ at 37°C at 90 rpm in a gyratory shaker. In some experiments, the $CaCl_2$ was substituted with 5mM EDTA (+ EDTA). The number of particles were determined by using a Coulter Counter, and the percent aggregation is shown.

Figure 5. LNCaP adhesion to purified recombinant proteins. LNCaP cells were infected with retrovirus containing an empty vector (A-C), PTPμWT (D-F), or a catalytically inactive mutant form of PTPμ(C-S) (G-I) or PTPμ-extra (J-L). Five days after infection, the cells were incubated with coverslips spotted with purified recombinant PTPμ (A, D, G, and J), laminin (B, E, H, and K) or E-cadherin (C, F, I and L). Adherent cells were visualized by dark-field microscopy (16x magnification) and photographed using a 35 mm camera.

Figure 6. Specificity of the adhesion assay. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), or a catalytically inactive mutant form of PTPμ (C-S) or PTPμ-extra (Extra). Five days after infection, the cells were incubated with coverslips spotted with purified recombinant PTPμ (A) or E-cadherin (B) in the presence of either a PTPμ antibody (BK2), an E-cadherin antibody (DECMA), 5mM EDTA, or were left untreated. Adherent cells were fixed after an overnight

incubation, and were visualized by dark-field microscopy and photographed using a 35 mm camera. The 35 mm negatives from six experiments were scanned and the digitized images were analyzed using the Metamorph image analysis program. To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data is presented as mean ± SEM.

Figure 7. Immunoprecipitation of E-cadherin. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), a catalytically inactive mutant form of PTPμ (C-S), or PTPμ-extra (Extra). Five days after infection, the cells were lysed, and 250 μg of the lysates were subjected to immunoprecipitation using antibodies to E-cadherin or L1(8D9). The immunoprecipitates were separated by 6 % SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies to the indicated proteins. As a positive control, 30 μg of lysate from vector-infected cells (lysate) is shown in each panel.

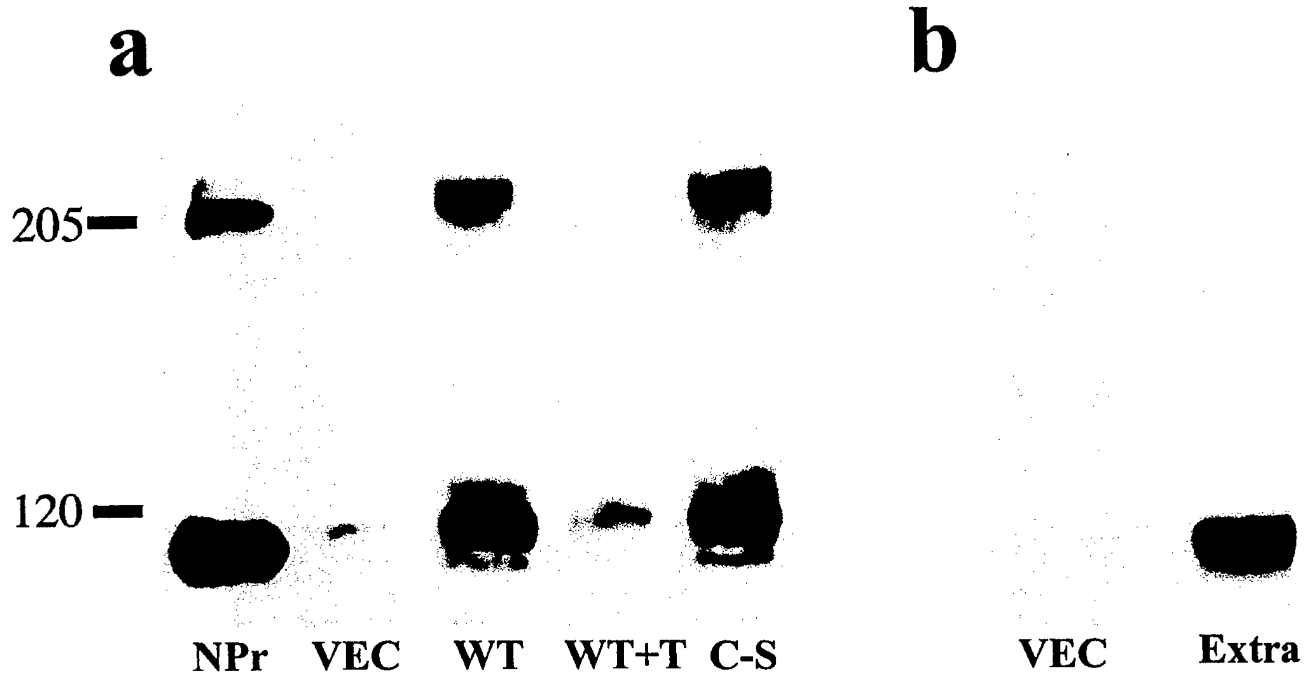
Figure 8. Full length PTPμ interacts with RACK1. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), a catalytically inactive mutant form of PTPμ (C-S), or PTPμ-extra (E). Five days after infection, the cells were lysed, and 400 μg of the lysates were subjected to immunoprecipitation using **A.** a monoclonal antibody to RACK1, **B.** a monoclonal antibody to the extracellular domain of PTPμ (BK2), or **C.** a monoclonal antibody to the intracellular domain of PTPμ (SK7). The immunoprecipitates were separated by 10 % SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies to RACK1 (A-C). **D.** The immunoblot of RACK1 immunoprecipitates shown in panel A was stripped and reprobed with a polyclonal antibody to PKCδ .

Figure 9. Protein kinase C regulates LNCaP adhesion E-cadherin.

A. PMA induced the detachment of cells re-expressing PTPμWT from E-cadherin, but not from PTPμ or laminin. LNCaP cells were infected with retrovirus containing PTPμWT. Five days after adhesion, the cells were added to coverslips spotted with purified recombinant PTPμ, laminin or E-cadherin. After an overnight incubation, the cells were treated with DMSO or 20nM PMA for 15 min. **B.** Inhibition of PKCδ induced adhesion to E-cadherin in uninfected LNCaP cells. Uninfected LNCaP cells were incubated overnight with coverslips spotted with purified recombinant E-cadherin, followed by a 45 min. incubation with 10 μM chelerythrine chloride (CHE), 0.5 μM

GF109203X (GF), 5 μM Rotlerrin (R), 15 nM Gö6976 (Go), 10 μM LY294002 (LY), or with DMSO alone. Adherent cells were fixed and visualized by dark-field microscopy and photographed using a 35 mm camera the 35 mm negatives from six experiments were scanned and the digitized images were analyzed using the Metamorph image analysis program. To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data is presented as mean ± SEM.

Table I. Statistical analysis of LNCaP adhesion to purified recombinant proteins. The data shown in figure 5 and 8A is presented as mean number of adherent cells ± SEM. For each substrate, Student's t test was used to compare the number of cells infected with PTPμ to the number of adherent cells infected with an empty vector.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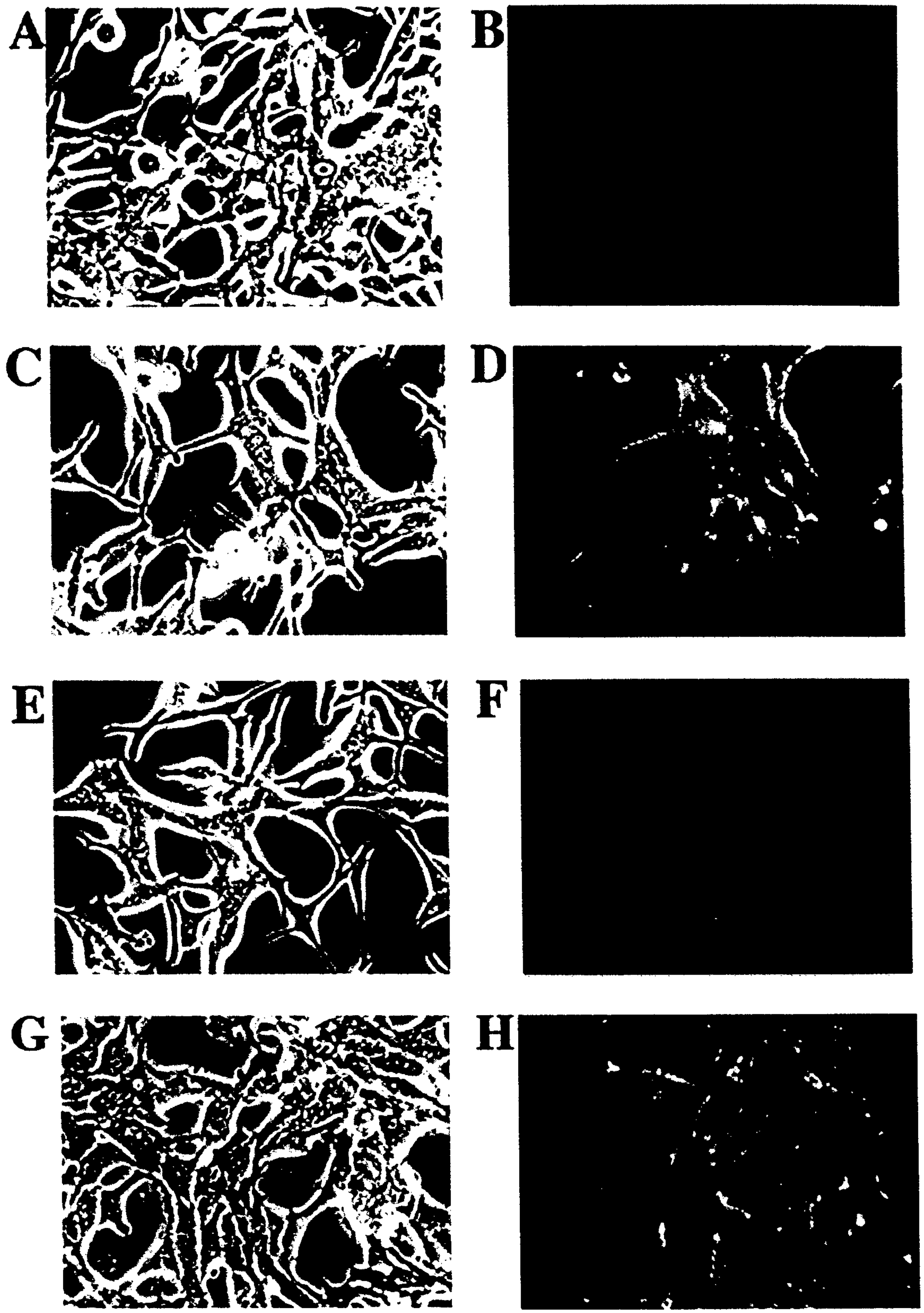
VEC
Extra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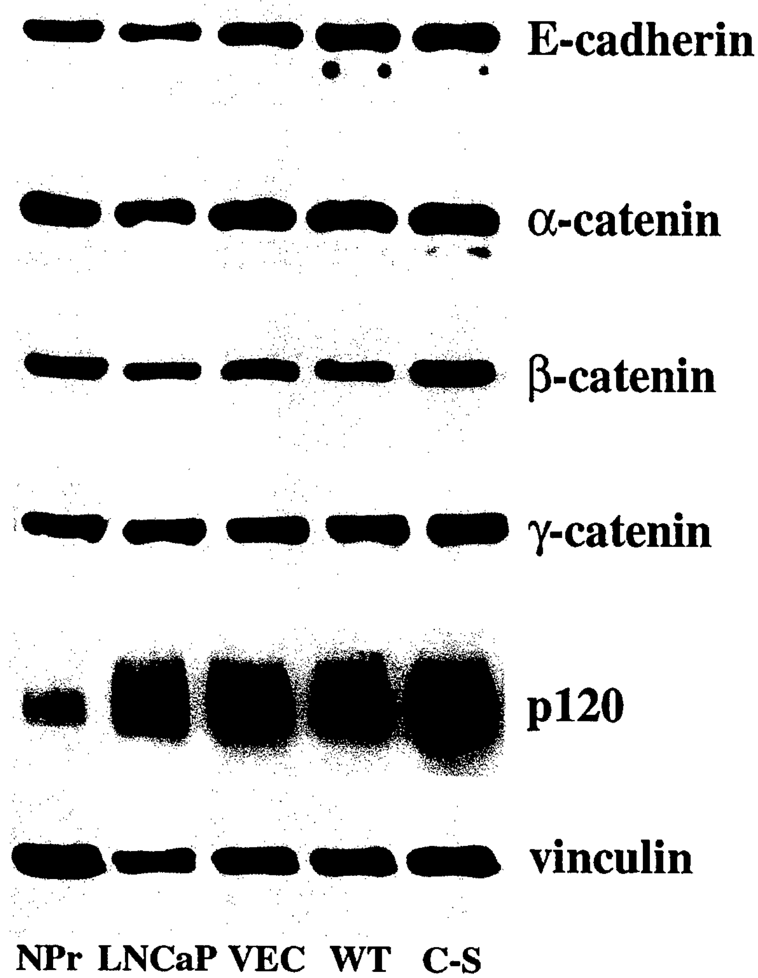
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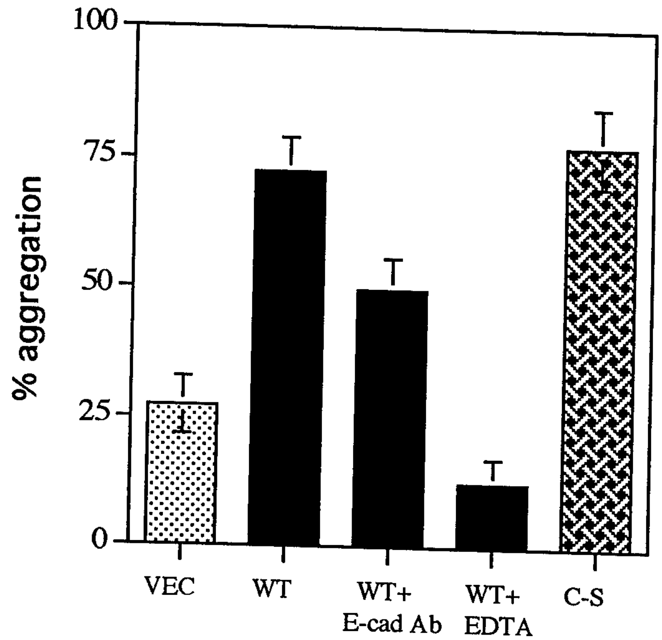
β-catenin
γ-catenin
p120
vinculin
NPr LNCaP VEC WT C-S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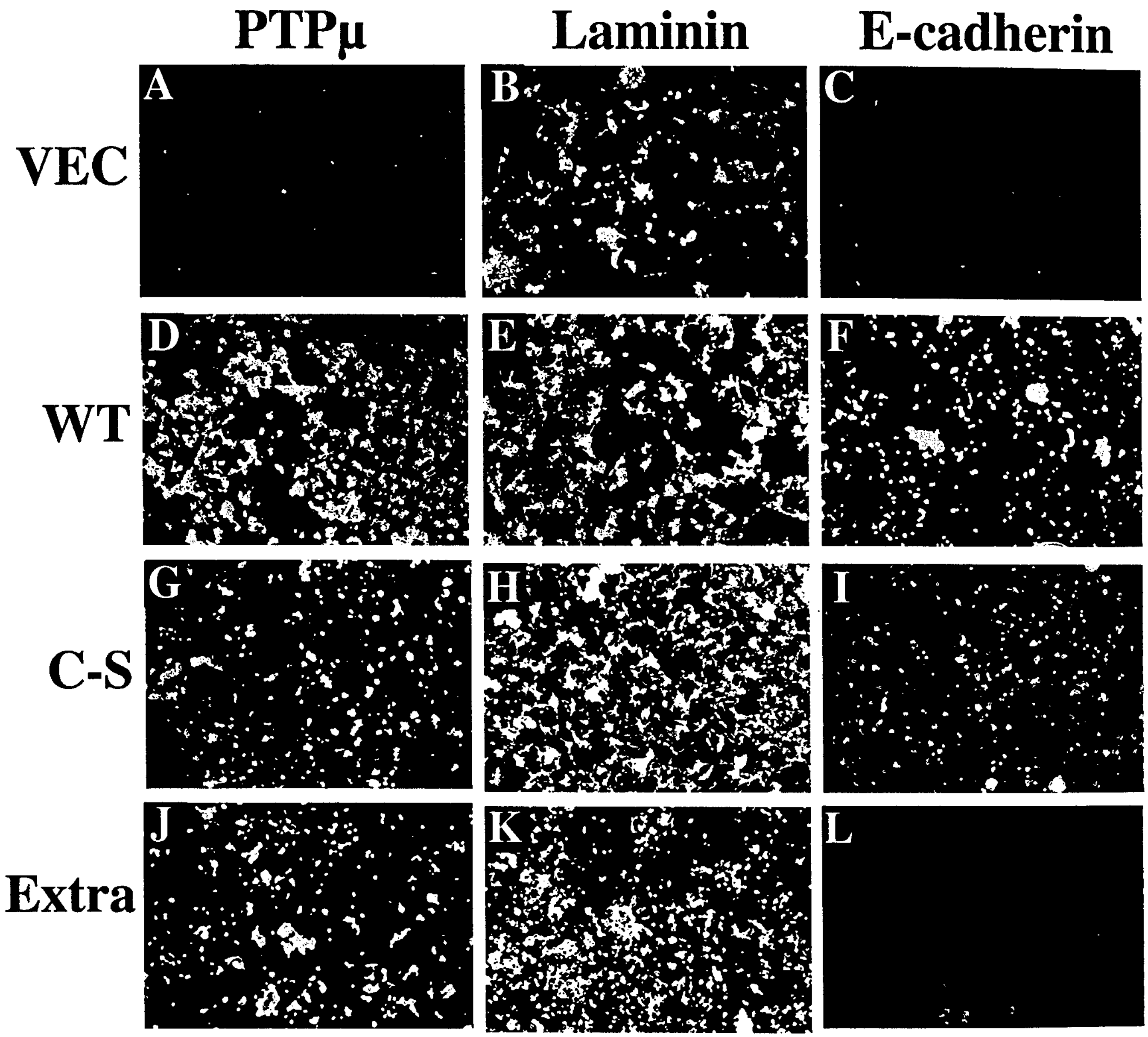
WT+
EDTA
C-S


PTPμ
Laminin
E-cadherin
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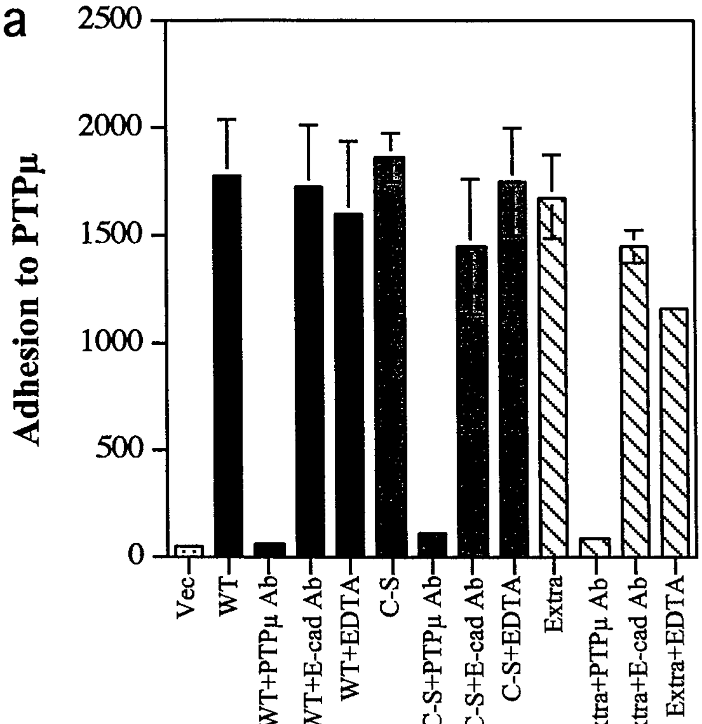
a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2000
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500
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Adhesion to PTPμ
Vec
WT
WT+PTPμ Ab
WT+E-cad Ab
WT+EDTA
C-S
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C-S+E-cad Ab
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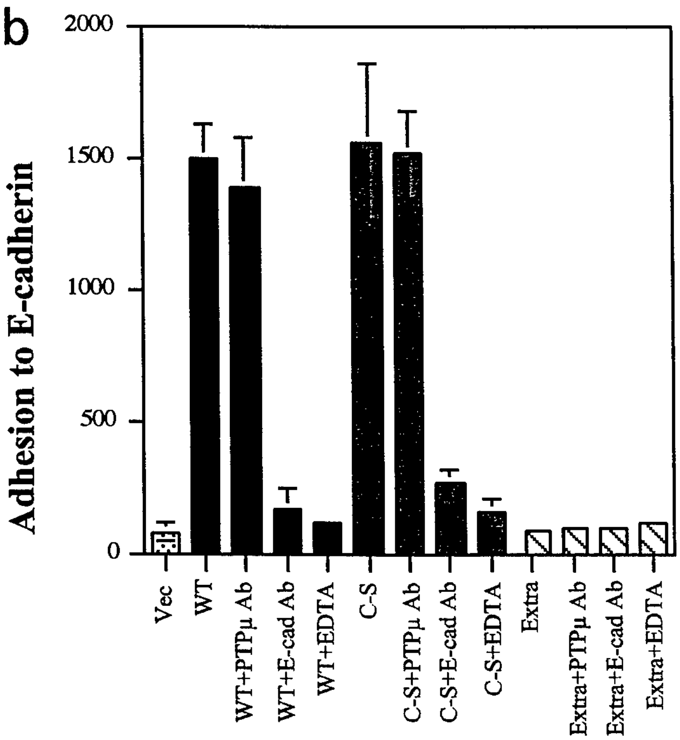
Extra
Extra+PTPμ Ab
Extra+E-cad Ab
Extra+EDTA

b


2000
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Adhesion to E-cadherin
Vec
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WT+PTPμ Ab
WT+E-cad Ab
WT+EDTA
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C-S+EDTA
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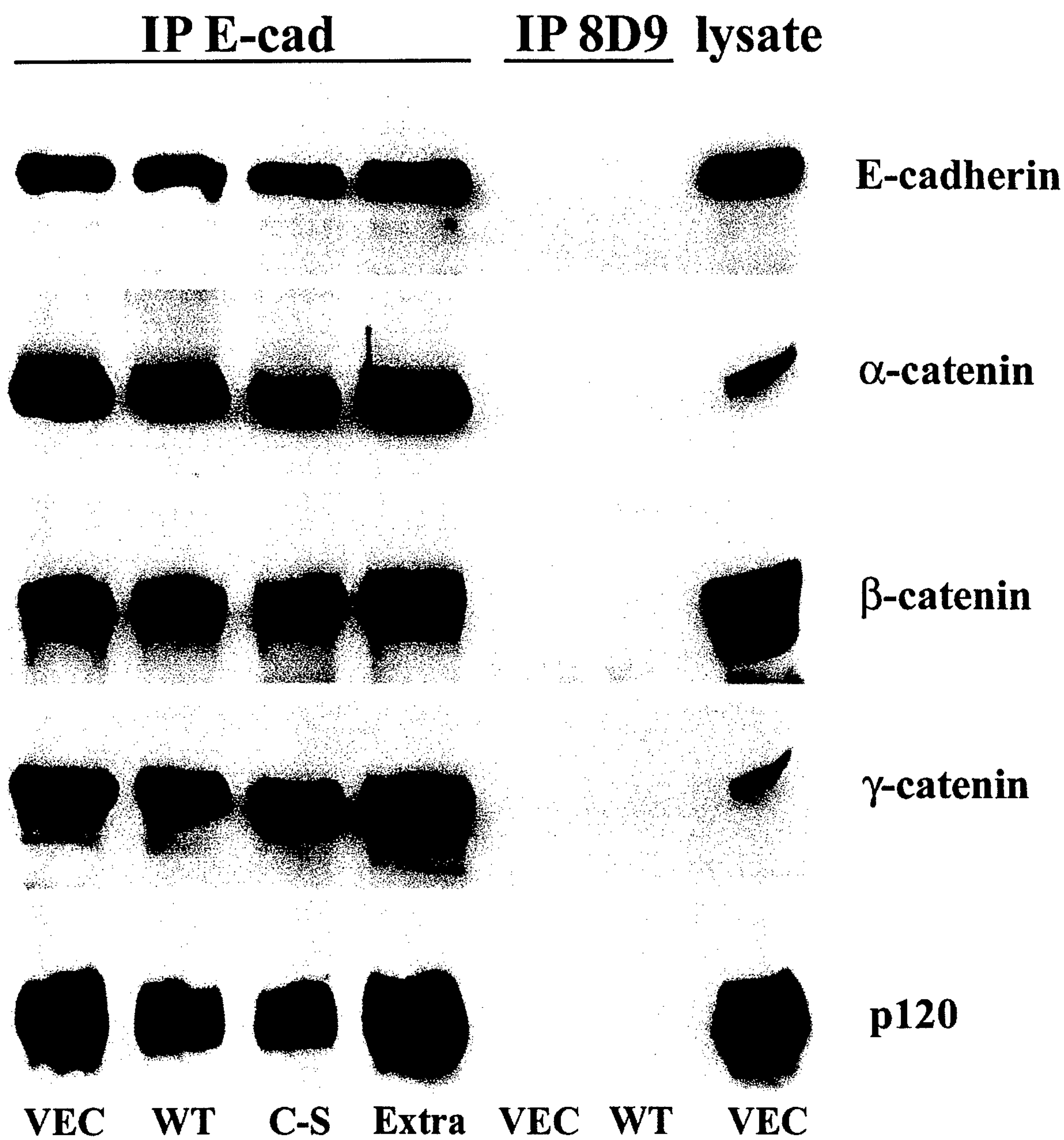
Extra+EDTA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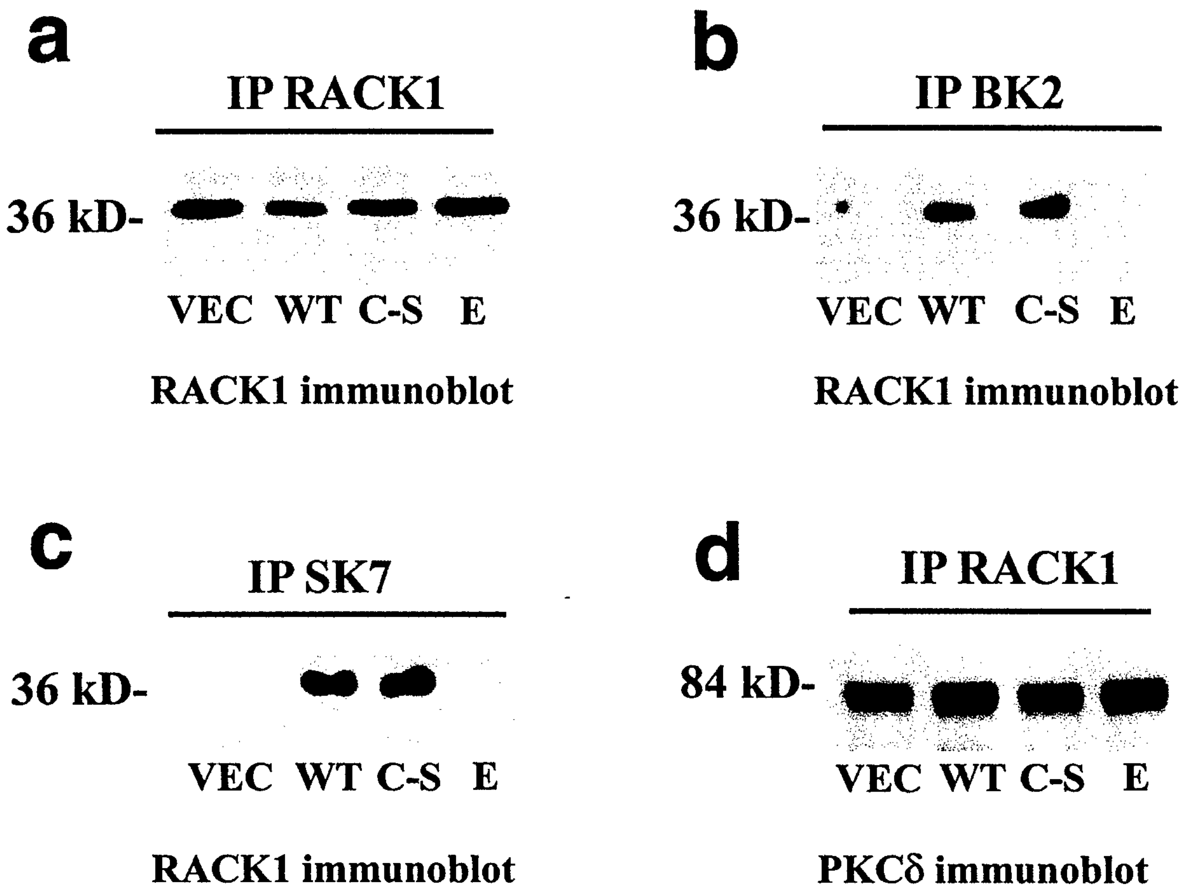
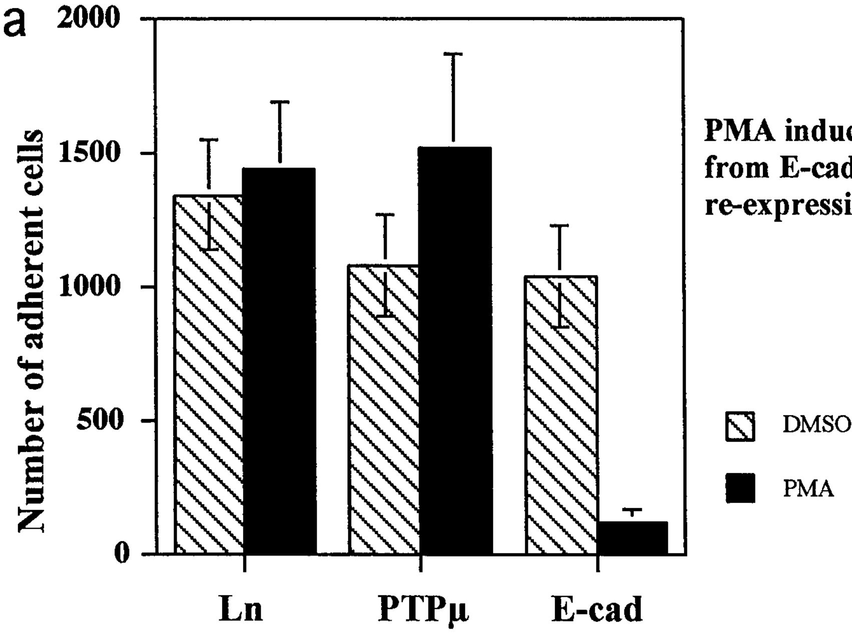
VEC

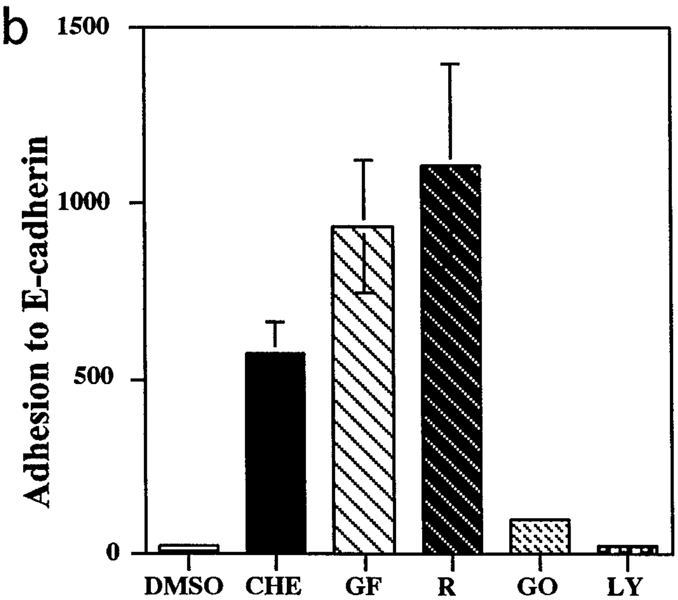

a
IP RACK1
36 kD-
VEC WT C-S E
RACK1 immunoblot
b
IP BK2
36 kD-
VEC WT C-S E
RACK1 immunoblot
c
IP SK7
36 kD-
VEC WT C-S E
RACK1 immunoblot
d
IP RACK1
84 kD-
VEC WT C-S E
PKCδ immunoblot

a



PMA induces detatchment from E-cadherin in LNCaP cells re-expressing PTPμ

b



Inhibition of PKCδ induces adhesion to E-cadherin in uninfected LNCaP cells

Table I. Statistical Analysis of LNCaP Adhesion to Purified Proteins

	Substrate					
	PTPμ		Laminin		E-cadherin	
Virus type	Adherent cells	*P	Adherent cells	*P	Adherent cells	*P
Vector	95.5 ± 45.7	-	1483.4 ± 368.2	-	132.7 ± 62.8	-
PTPμWTGFP	1150.0 ± 210.8	0.0006	1391.8 ± 192.7	0.8311	1561.5 ± 350.0	0.0024
C1095S	1022.5 ± 192.0	0.0008	1736.2 ± 463.0	0.6804	1117.8 ± 210.9	0.0012
PTPμ-extra	1102.0 ± 240.1	0.0021	2432.4 ± 431.3	0.1328	4 8.0 ± 20.0	0.2281
Vector	45.2 ± 6.82	-	1335.2 ± 573.9	-	34.8 ± 14.0	-
Vector +PMA	54.8 ± 11.5	0.5039	1799.2 ± 543.3	0.5733	36.0 ± 6.2	0.8985
PTPμWT	1082.8 ± 189.3	0.0015	1346.6 ± 206.1	0.9855	1047.8 ± 189.5	0.0003
PTPμWT+PMA	1521.3 ± 349.4	0.0055	1442.8 ± 257.1	0.8684	120.2 ± 50.5	0.1312

The data from six adhesion assays were subjected to statistical analysis. The data is given as number of adherent cells ± SEM. The P values were obtained by students t test, 99% confidence interval.

Version of 4-3-00

N-cadherin-dependent adhesion is regulated by the receptor tyrosine phosphatase PTPμ.

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Abbreviations: PTP, protein tyrosine phosphatase; RPTP, receptor tyrosine phosphatase; C-S, PTPμC1095S mutant; PTPμ-extra, construct containing the extracellular-, transmembrane- and 55 amino acids of the intracellular domains of PTPμ; GFP, green fluorescence protein; BSA, bovine serum albumin; PBS, phosphate buffered saline

INTRODUCTION

The receptor protein tyrosine phosphatase PTPμ is a member of the immunoglobulin (Ig) superfamily of adhesion molecules. The extracellular segment of PTPμ contains a MAM (Meprin/A5/PTPμ) domain, an Ig domain and four fibronectin type III repeats [2]. Expression of PTPμ induces aggregation of nonadherent cells [3, 4] through a homophilic binding site that resides within the Ig domain [5]. In addition, the MAM domain plays a role in cell-cell aggregation by determining the specificity of adhesive interactions [6]. The cytoplasmic segment of PTPμ contains two phosphatase domains. Only the membrane-proximal PTP domain has catalytic activity. The membrane distal phosphatase domain in other receptor tyrosine phosphatases has been implicated in directing protein-protein interactions [7-10]. The intracellular juxtamembrane domain contains a region that is homologous to the conserved intracellular domain of the cadherins [5].

N-cadherin belongs to the family of classical cadherins. These cadherins are calcium-dependent cell-cell adhesion molecules [11]. Cadherin-mediated cell adhesion requires interactions between the cytoplasmic domain of cadherins and molecules termed catenins [12-14]. Catenins serve to link cadherins to the actin cytoskeleton. The interaction between cadherins/catenins and the actin cytoskeleton is essential for cell-cell adhesion [15]. Mutations in the catenin binding site on cadherins have been shown to disrupt adhesion even in the presence of an intact extracellular segment [15, 16]. Expression of E-cadherin promotes cell-cell adhesion and formation of adherens junctions [11]. Unlike E-cadherin, N-cadherin has been found to promote dynamic cellular processes such as neurite outgrowth and cell motility [17, 18]. Despite the importance of cadherin-mediated cell-cell adhesion, the underlying mechanisms that regulate adhesion are still poorly understood.

The receptor tyrosine phosphatase PTPμ associates with the cadherin/catenin complex [19, 20] through interactions with the cytoplasmic domain of classical cadherins such as N-cadherin, E-cadherin and cadherin 4 (also called R-cadherin) [21]. The association between N-cadherin and PTPμ suggests that PTPμ could regulate N-cadherin function. Indeed, we recently demonstrated that the interaction between PTPμ and N-cadherin is critical for the ability of N-cadherin to promote neurite outgrowth [22]. Furthermore, overexpression of a catalytically inactive form of PTPμ also perturbed the N-cadherin-mediated neurite outgrowth, demonstrating that the phosphatase activity of PTPμ is required for N-cadherin-mediated signal transduction and/or cytoskeletal association [22].

EXPERIMENTAL PROCEDURES

Antibodies and reagents: Monoclonal antibodies against the intracellular (SK7) and extracellular (BK2) domains of PTPμ have been described [5, 26]. A polyclonal antibody against N-cadherin (7873) was kindly provided by Dr. John Hemperly (Becton Dickinson Labs, Research Triangle Park, NC). Normal prostate epithelial cells were from Clonetics (San Diego, CA). RPMI 1640 medium and laminin were obtained from GIBCO-BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). Tween-20 was obtained from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Construction and expression of the PTPμ retroviruses: The retroviral system used is a tetracycline repressible ("tet-off") promoter-based system [27]. Using the pBPSTR1 vector generously provided by Dr. Steven Reeves (Harvard Medical School, Charleston, MA), the following constructs were generated. The wild type PTPμ plasmid (PTPμWT) and the C-S mutant have been previously described [22]. The plasmids containing either wild type or mutant PTPμ were subcloned into the tetracycline regulatable retroviral vector, pBPSTR1. A construct containing the extracellular, the transmembrane and 55 amino acids of the intracellular domain has been previously described [26]. This construct was subcloned into the tetracycline-regulatable retroviral vector, pBPSTR1. A replication-defective amphotrophic retrovirus was made by transfecting the PA317 helper cell line (ATCC CRL-9078) with the respective PTPμ-containing plasmids. Control virus was generated by transfecting PA317 helper cells with the pBPSTR1 plasmid alone.

Tissue culture and retroviral infection of LNCaP cells: LNCaP cells were grown in RPMI 1640 supplemented with 10% FBS and 1 μg/ml gentamicin at 37°C and 5% CO_2. Cells were infected with retrovirus by the addition of polybrene (5 μg/ml) and 1 ml of virus-containing medium per ml of culture medium. The cells were incubated for 4-16 hours at 37°C, and the medium was exchanged with normal culture medium. Five days after infection, the cells were analyzed for GFP expression by fluorescence microscopy.

Protein Extraction and Immunoblotting: LNCaP cells were rinsed once with PBS and the cells were lysed in Triton-containing buffer (20mM Tris pH 7.6, 1% Triton X-100, 2mM $CaCl_2$, 1mM benzamidine, 200 μM phenyl arsine oxide, 1mM vanadate, 2μl/ml protease inhibitor cocktail (Sigma) and 0.1mM ammonium molybdate) and scraped off the dish. After incubation on ice for 30 min, the lysate was centrifuged at 14,000 rpm for 3 min and the Triton-soluble material was recovered in the supernatant. The amount of protein was determined by the Bradford method using BSA as a standard. Lysates were boiled in equal volume of 2X sample buffer, the proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting as described previously [3].

Expression and purification of GST fusion proteins: A N-cadherin GST fusion protein construct containing amino acids 1-110 of N-cadherin was obtained from Dr Robert Brackenbury (University of Cincinnati). The GST fusion protein expressing the entire extracellular domain of PTPμ has previously been described [26]. Expression of GST-tagged proteins in *E.coli* was induced by IPTG. The bacteria were collected by centrifugation at 3000 x g for 10 min and lysed in PBS containing 1% Triton X-100, 5μg/ml leupeptin, 5μg/ml aprotinin and 1mM benzamidine, sonicated and centrifuged again at 3000 x g for 10 min to remove debris. The supernatant was passed over glutathione-sepharose beads (Pharmacia Biotech, Piscataway, NJ) washed and the bound protein eluted with 10mM glutathione as described previously [26].

LNCaP Adhesion to Purified Proteins: Sterile coverslips were coated overnight with 100μg/ml polylysine. Subsequently, the coverslips were coated with nitrocellulose in methanol [28] and allowed to dry. Purified recombinant proteins were diluted in PBS containing 2mM $CaCl_2$ to a concentration of 75 μg/ml for PTPμ and N-cadherin respectively, and 40 μg/ml for laminin. To identify the individual protein spots on the coverslips, the protein solutions were supplemented with 20μg/ml Texas Red BSA (Sigma). Three distinct spots, each containing a single adhesion molecule, were generated by spotting 20 μl of each protein solution on one coverslip. After a 20 min. incubation, the protein solution was aspirated, and this procedure was repeated once. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in PBS, and the dishes were rinsed with RPMI-1640 medium. 3×10^5 LNCaP cells infected with the indicated retrovirus were added to coverslips, and the cells were allowed to adhere overnight. The medium was removed and the coverslips were rinsed once in PBS to remove unattached cells. The cells were subsequently fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80mM Pipes, 5mM EGTA, 1mM $MgCl_2$, 3% sucrose), pH 7.4 for 30 min. at room temperature. The coverslips were washed twice in PBS and mounted in IFF mounting medium (0.5M Tris-HCl pH 8.0 containing 20% glycerol and 0.1% p-phenylenediamine). Adherent cells were detected by dark field microscopy and photographed. The number of adherent cells was quantified using the Metamorph image analysis program (Universal Imaging Corp., West Chester, PA). The data obtained were analyzed by Student's t test (Statview 4.51, Abacus Concepts, Inc.).

RESULTS

Re-expression of PTPμ in LNCaP prostate carcinoma cells–To investigate the role of PTPμ in N-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line. These cells provide a good model system for studying PTPμ-mediated events since they, unlike normal prostate cells, do not express endogenous PTPμ (Fig. 1; [1]). We have generated tetracycline regulatable retroviruses encoding the cDNA sequence of the wildtype (WT) and a catalytically inactive mutant of PTPμ tagged with the green fluorescence protein (GFP). The catalytically inactive mutant contains a C-S point mutation in the catalytic site and has been previously described [22]. In addition, we have generated a virus encoding the extracellular and transmembrane domains and 55 AA of the intracellular domain of PTPμ (PTPμ-extra).

To investigate the functional role of PTPμ in regulating N-cadherin-mediated adhesion, we re-expressed various constructs of PTPμ in LNCaP cells. Western blot analysis confirmed that LNCaP cells infected with retrovirus containing an empty vector did not express detectable levels of PTPμ (Fig. 1, Vec). Cells infected with retrovirus containing PTPμWT (Fig 1, WT) or C-S (Fig. 1, C-S) expressed both the full length protein (200 kDa) as well as the proteolytically processed forms (100 kDa)[5]. Cells infected with virus containing PTPμ-extra displayed only the 100 kDa form (Fig 1, Extra). As a control, the expression of PTPμ in normal prostate epithelial cells is shown (Fig. 1, NPr). The expression of PTPμGFP was confirmed by fluorescence microscopy. Control cells infected with a virus containing an empty vector did not show any fluorescence (Fig. 2A and B). As previously shown [1], 70-90 % of the LNCaP cells expressed PTPμWT (Fig. 2C and D) and C-S (Fig. 2E and F), respectively. Both the WT and C-S mutant form of PTPμ were primarily localized to the plasma membrane.

Normal prostate cells as well as LNCaP cells expressed N-cadherin (Fig. 3, NPr and LNCaP, respectively). Infection of LNCaP cells with an empty vector (Fig. 3, VEC), PTPμWT (Fig. 3,WT) or the mutant forms of PTPμ (Fig 3, C-S) did not alter the expression of N-cadherin.

Re-expression of PTPμ induced LNCaP adhesion to PTPμ–To verify that the re-expressed forms of PTPμ were able to mediate homophilic binding [2, 26] in LNCaP cells, we employed an *in vitro* adhesion assay in which purified recombinant PTPμ was immobilized on nitrocellulose-coated coverslips [1]. As expected, cells infected with an empty vector did not adhere to PTPμ (Fig. 4A) since these cells do not express PTPμ. Re-expression of PTPμWT induced LNCaP adhesion to purified PTPμ (Fig. 4D), as did re-expression of C-S (Fig.4G). Quantitation of the adhesion assays showed that the number of cells that adhered to purified PTPμ was significantly greater for cells infected with WT and C-S as compared to cells infected with vector only (Fig. 5A, Table I). However, there was no significant difference in adhesion to PTPμ between cells expressing PTPμWT or the C-S mutant of PTPμ (Fig. 5A, Table I). Expression of PTPμ-extra also induced LNCaP adhesion to purified recombinant PTPμ (Fig. 4J). As an internal control in each experiment, cells were allowed to adhere to laminin. As expected, LNCaP cells infected with an empty vector adhered to laminin because they express functional laminin receptors (Fig. 4B). The adhesion to laminin was not significantly affected by re-expressing PTPμWT (Fig. 4E), the C-S mutant (Fig. 4H; Fig. 5B, Table I), or PTPμ extra (Fig. 4L). None of the retroviral infected cells adhered to nitrocellulose coated with BSA only (data not shown).

Re-expression of PTPμ restored N-cadherin-mediated adhesion–To investigate whether PTPμ plays a role in N-cadherin-mediated adhesion in LNCaP cells, we immobilized purified recombinant N-cadherin on the nitrocellulose-coated coverslips. Despite the fact that these cells express N-cadherin (Fig. 3) as well as α-, ß-, γ- catenin and p120 [1], LNCaP cells infected with an empty vector did not adhere to N-cadherin (Fig. 4C). Interestingly, re-expression of PTPμWT restored the ability of LNCaP cells to adhere to N-cadherin (Fig. 4F). To investigate if the phosphatase activity of PTPμ is required for N-cadherin-mediated adhesion, we repeated the adhesion assays with LNCaP cells expressing the C-S mutant form of PTPμ. Expression of the C-S mutant also restored N-cadherin-mediated adhesion (Fig. 4J). As shown in Fig 5C, re-expression of PTPμWT or C-S induced a significant increase in adhesion to N-cadherin as compared to LNCaP cells infected with an empty vector. No significant difference in adhesion to N-cadherin was detected between cells infected with PTPμWT compared to cells infected with the C-S mutant. The statistical analysis for LNCaP cell adhesion to N-cadherin is summarized in Table I. These data indicate that although the presence of PTPμ is required for N-cadherin-mediated adhesion in LNCaP cells, PTPμ catalytic activity is not required.

In a recent study, we showed that the intracellular domain of PTPμ is required for E-cadherin-mediated adhesion in LNCaP cells. To determine whether the intracellular domain of PTPμ was required for N-cadherin-dependent adhesion, we repeated the adhesion assay with cells expressing PTPμ-extra. Unlike the adhesion to E-cadherin, PTPμ-extra was capable of restoring LNCaP adhesion to recombinant N-cadherin (Fig. 4M). These results suggest that the presence of the extracellular and transmembrane domains and 55 AA of the intracellular domain of PTPμ are required for N-cadherin-mediated adhesion in LNCaP cells.

DISCUSSION

The expression of N-cadherin in epithelial tumor cells has been linked to increased cell motility, invasion and metastasis [23, 24]. In this study, we show that the receptor tyrosine phosphatase PTPμ is required for N-cadherin-mediated adhesion in LNCaP prostate carcinoma cells. The LNCaP line provides a good model system for studying the role of PTPμ. Unlike normal prostate epithelial cells, these cells do not express endogenous PTPμ (Fig. 1).

We have re-expressed PTPμ and studied the effects of wild type (WT) and mutant forms of PTPμ in LNCaP cells. Although LNCaP cells express N-cadherin (Fig. 3) as well as α-, ß- and γ-catenin and p120 [1], they were found to be deficient in N-cadherin-mediated adhesion. Re-expression of PTPμWT restored this adhesion, indicating that N-cadherin function can be regulated by PTPμ in the LNCaP prostate carcinoma cell system. Re-expression of a catalytically inactive C-S mutant form of PTPμ also restored N-cadherin-mediated adhesion, demonstrating that the ability of PTPμ to restore adhesion is not dependent on its phosphatase activity. This is in concert with our previous finding that PTPμ restored E-cadherin-mediated adhesion in LNCaP cells independent of its phosphatase activity [1]. Expression of the extracellular and transmembrane domains and 55 AA of the juxtamembrane domain of PTPμ also restored N-cadherin-mediated adhesion. This is intriguing, since this construct was not able to restore E-cadherin-mediated adhesion in LNCaP cells [1]. These data indicate that PTPμ regulates N-cadherin- and E-cadherin-mediated adhesion through separate mechanisms. Further studies are required to determine which domains of PTPμ that are involved in N-cadherin- and E-cadherin-mediated adhesion, respectively.

Previous studies have shown that PTPμ is found in complex with classical cadherins [19, 21, 29]. The importance of the interaction between PTPμ and N-cadherin was illustrated in a recent study from our lab where we demonstrated that downregulation of PTPμ expression perturbed neurite outgrowth on N-cadherin [22]. Expression of the C-S mutant also reduced neurite outgrowth on a N-cadherin substrate, demonstrating a functional role for the phosphatase activity of PTPμ in N-cadherin-mediated signal transduction. However, the expression of the C-S mutant also restored N-cadherin-mediated adhesion in LNCaP cells. These results indicate that the expression of PTPμ is required for N-cadherin-mediated adhesion, whereas the phosphatase activity of PTPμ is required for the signaling events downstream of N-cadherin-mediated adhesion that regulate the cytoskeleton and thus N-cadherin-dependent migration.

Several recent studies have shown that expression of N-cadherin in epithelial tumor cells promotes an invasive phenotype [18, 25]. Overexpression of N-cadherin in breast cancer cells was shown to induce an increased ability to migrate [23, 24]. The ability of cells to migrate correlated with the expression levels of N-cadherin, even though E-cadherin was expressed. Increased levels of N-cadherin also increased the ability of breast cancer cells to adhere to endothelial cells, which may in turn increase the ability of these cells to form metastases [24]. Taken together, these studies clearly demonstrate the importance of N-cadherin expression and function in cancer cells. The fact that PTPμ can regulate N-cadherin-mediated adhesion in prostate carcinoma cells opens up the possibility that loss of expression or catalytic activity of PTPμ may result in alterations of N-cadherin-mediated motility in human carcinomas.

ACKNOWLEDGEMENTS

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LEGENDS TO THE FIGURES

Figure 1. Re-expression of PTPμ in LNCaP cells. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT (WT), a mutant form of PTPμ containing a single C-S point mutation in the catalytic site (C-S), or the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (Extra). Five days after infection, the cells were lysed, and 30 μg of each lysate was separated by 6 % SDS-PAGE, transferred to nitrocellulose and blotted with a monoclonal antibody (SK7) to PTPμ. Lysate from normal prostate epithelial cells (NPr) was used as a positive control.

Figure 2. Re-expression of GFP-tagged WT and C-S mutant form of PTPμ. LNCaP cells were infected with retrovirus containing an empty vector (A and B), PTPμWT (C and D) or a mutant form of PTPμ containing a single C-S point mutation in the catalytic site (E and F). Five days after infection, the expression of GFP-tagged proteins was visualized by fluorescence microscopy. Representative phase contrast (A, C and E) and fluorescent (B, D and F) images are shown.

Figure 3. Re-expression of PTPμ did not alter the expression of N-cadherin. LNCaP cells were left untreated (LNCaP) or infected with retrovirus containing an empty vector (VEC), PTPμWT-GFP (WT), or the C-S mutant for five days. The cells were lysed and 15 μg of each lysate, together with a lysate from normal prostate epithelial cells (NPr), was separated by 6 % SDS-PAGE, transferred to nitrocellulose and blotted with a polyclonal antibody against N-cadherin.

Figure 4. LNCaP adhesion to purified recombinant proteins. LNCaP cells were infected with retrovirus containing an empty vector (A-C), PTPμWT (D-F), the C-S mutant form of PTPμ (G-J), or the PTPμ-extra (K-M). Expression of PTPμ was confirmed by fluorescence microscopy, then the cells were incubated with coverslips spotted with purified recombinant PTPμ (A, D, G and K), laminin (B, E, H and L) or N-cadherin (C, F, J and M). Adherent cells were visualized by dark-field microscopy and photographed using a 35 mm camera.

Figure 5. Quantitation of LNCaP adhesion to purified recombinant proteins. To quantify the number of adherent cells from the experiments shown in Figure 4, the 35 mm negatives from six experiments were scanned and the digitized images were analyzed

using the Metamorph image analysis program. To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data is presented as mean ± SEM. **A.** Adhesion to PTPμ. **B.** Adhesion to laminin. **C.** Adhesion to N-cadherin. For statistical analysis see Table I.

Table I. Statistical analysis of LNCaP adhesion to purified recombinant proteins. The data shown in figure 5 is presented as mean number of adherent cells ± SEM. For each substrate, Student's t test was used to compare the number of cells infected with PTPμ to the number of adherent cells infected with an empty vector.

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Figure 1

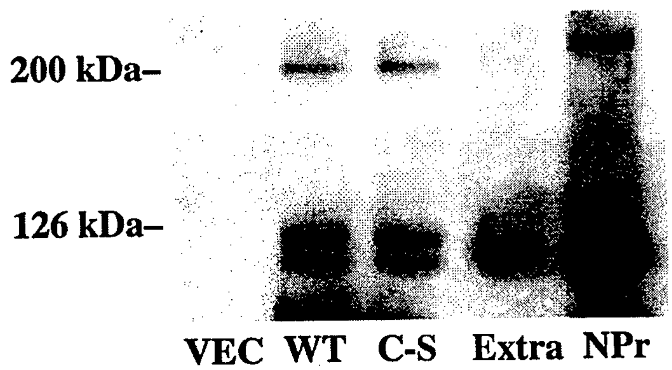


Figure 2

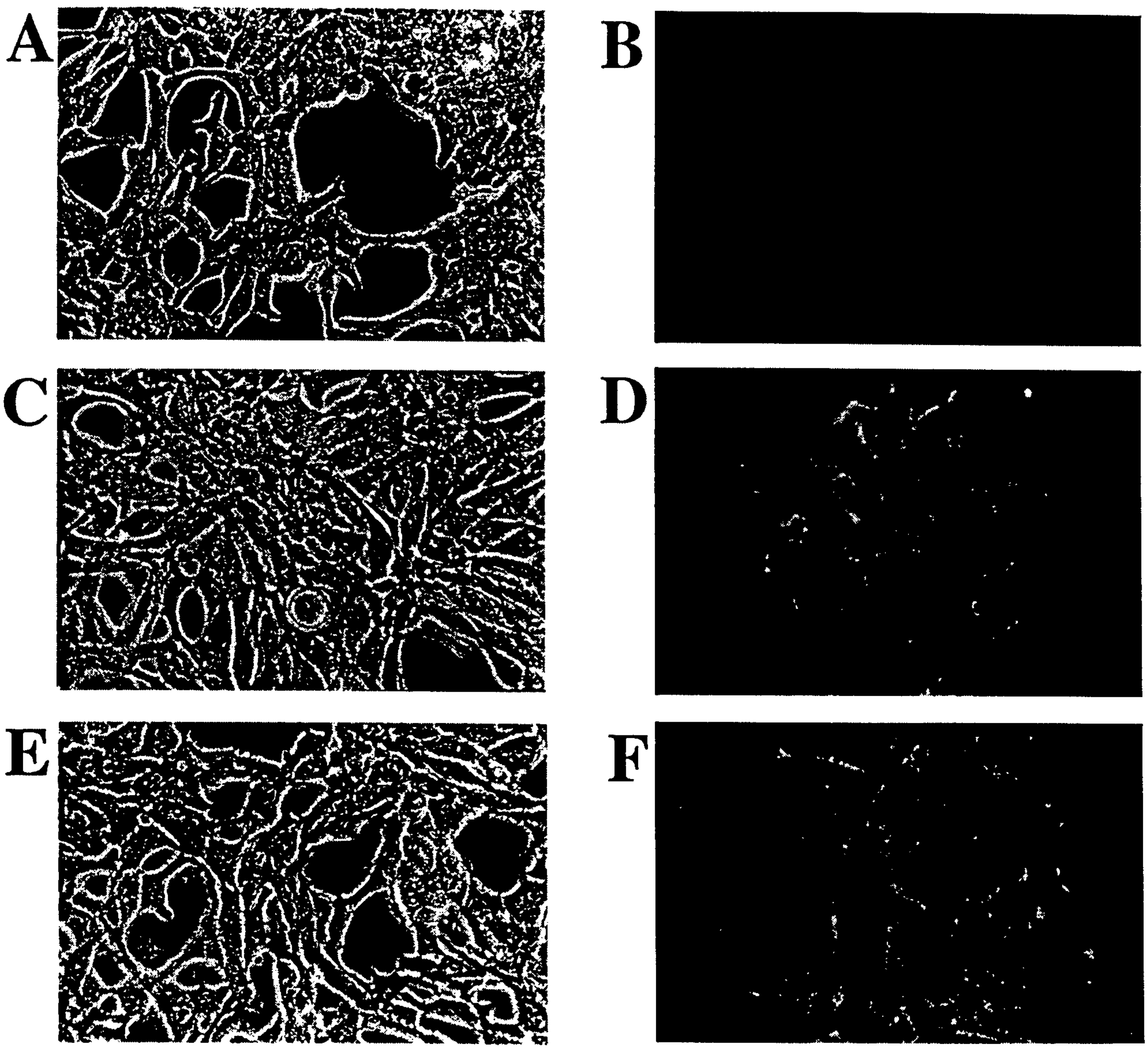
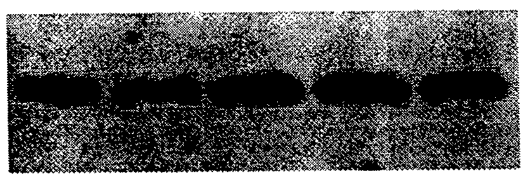
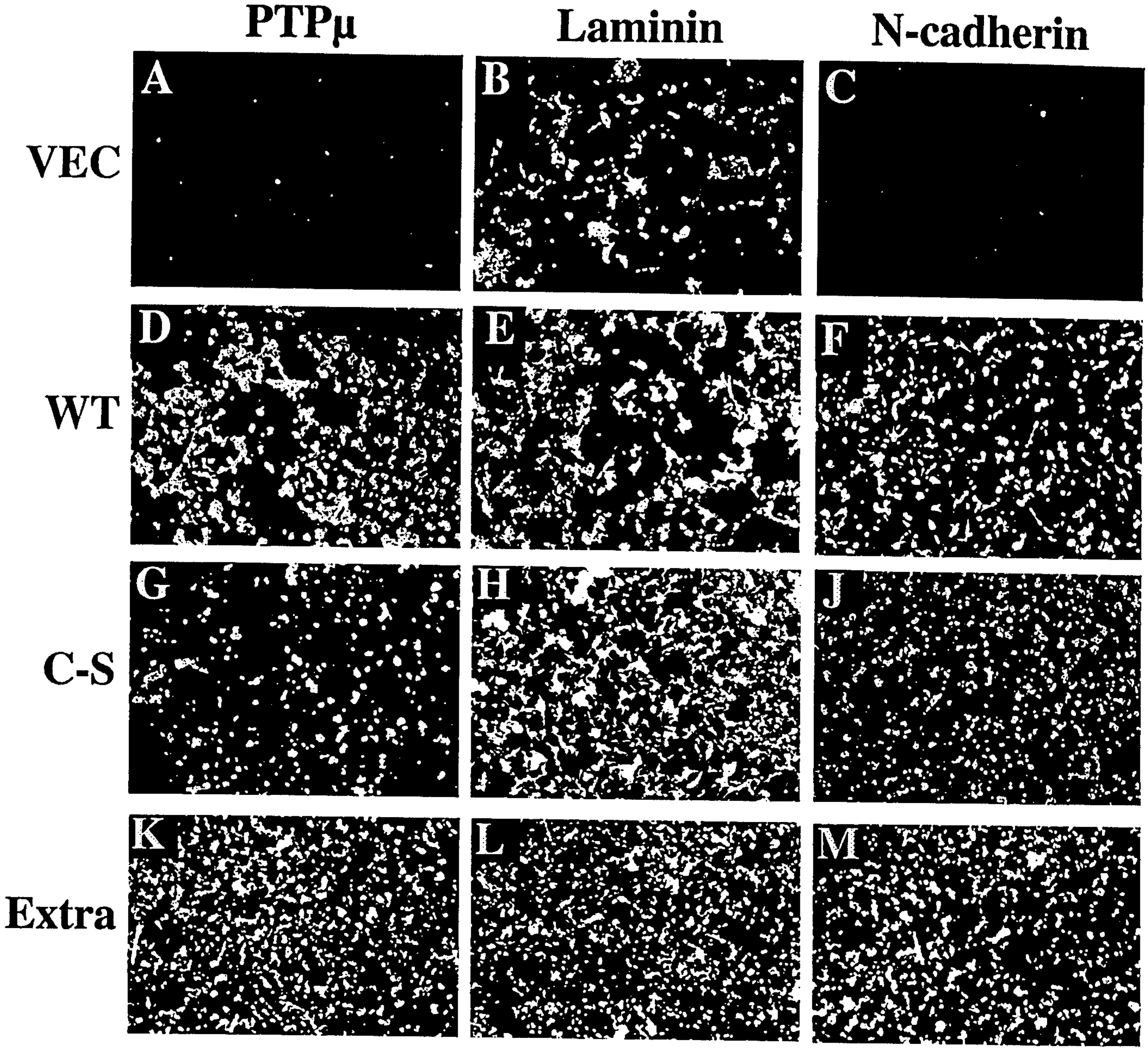


Figure 3



NPr LNCaP VEC WT C-S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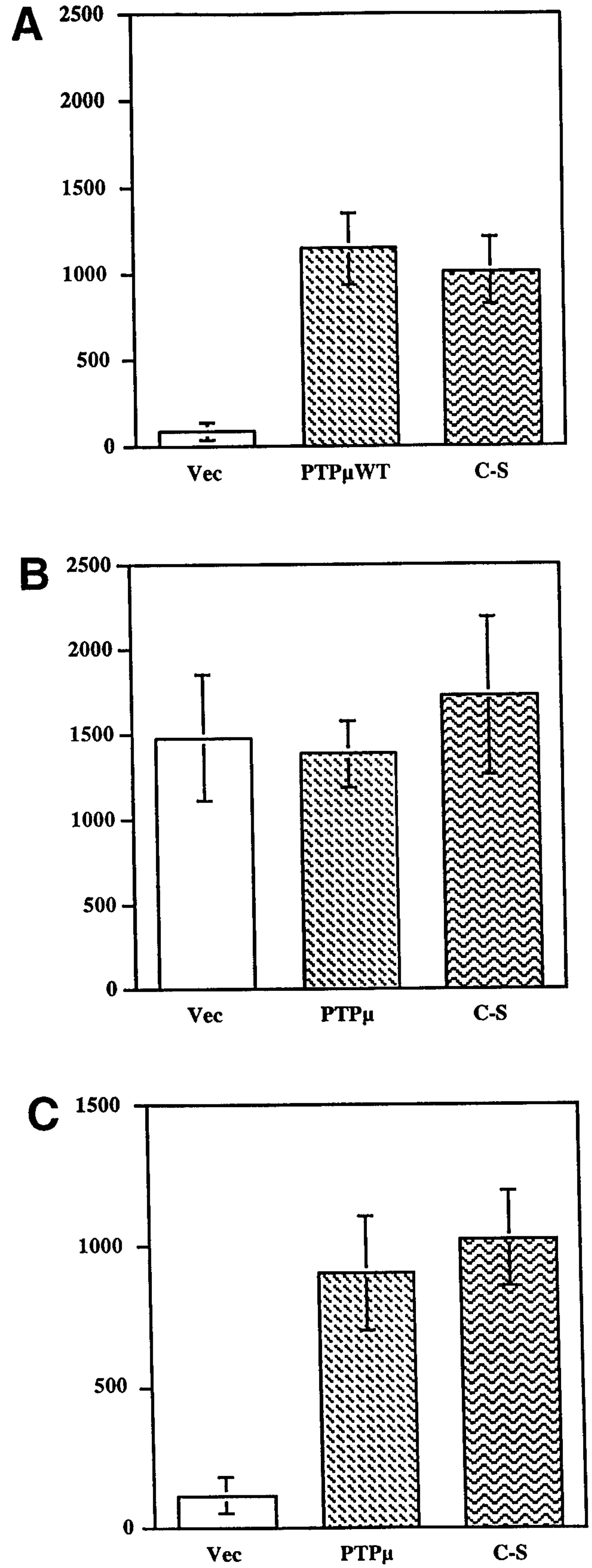
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1000
500
0
Vec
PTPμWT
C-S
B
2500
2000
1500
1000
500
0
Vec
PTPμ
C-S
C
1500
1000
500
0
Vec
PTPμ
C-S

Table I. Statistical Analysis of LNCaP Adhesion to Purified Proteins

	Substrate					
	PTPμ		Laminin		N-cadherin	
Virus type	Adherent cells	*P	Adherent cells	*P	Adherent cells	*P
Vector	95.5 ± 45.7	-	1483.4 ± 368.2	-	118.0 ± 63.7	-
PTPμwtGFP	1150.0 ± 210.8	0.0006	1391.8 ± 192.7	0.8311	905.3 ± 202.0	0.0040
C1095S	1022.5 ± 192.0	0.0008	1736.2 ± 463.0	0.6804	1026.2 ± 169.8	0.0005

The data from six adhesion assays were subjected to statistical analysis. The data is given as number of adherent cells ± SEM. The P values were obtained by students t test, 99% confidence interval.

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The receptor tyrosine phosphatase PTPμ negatively regulates prostate carcinoma cell growth which is dependent upon phosphatase activity.

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Abbreviations: PTP, protein tyrosine phosphatase; RPTP, receptor tyrosine phosphatase; C-S, PTPμC1095S mutant; D-A, PTPμ D1063A mutant; R-M, PTPμR1101M mutant; PTPμ-extra, construct containing the extracellular-, transmembrane- and 55 amino acids of the intracellular domains of PTPμ; GFP, green fluorescence protein; BSA, bovine serum albumin; PBS, phosphate buffered saline

ABSTRACT

Cell-cell adhesion plays a fundamental role in contact inhibition of growth. The receptor tyrosine phosphatase PTPμ has been shown to mediate homophilic cell-cell adhesion (Brady-Kalnay and Tonks, 1994). In addition, it has been implicated in the regulation of cadherin-mediated adhesion and signal transduction (Burden-Gulley and Brady-Kalnay, 1999; Hellberg et al., 2000a; Hellberg et al., 2000b). To investigate whether PTPμ expression and signal transduction play a role in the regulation of cell growth, we employed the LNCaP prostate carcinoma cell system since these cells have downregulated expression of PTPμ. LNCaP cells express normal levels of E- and N-cadherin as well as α-, ß- and γ-catenin, but are defective in cadherin-dependent adhesion (Hellberg et al., 2000a; Hellberg et al., 2000b). Full length PTPμ was re-expressed in the cells using a retroviral/tetracycline-repressible system. To investigate the role of PTPμ phosphatase activity in cadherin-mediated adhesion, we re-expressed mutant forms of PTPμ with altered catalytic activity or altered substrate binding. LNCaP cells expressing the mutant forms of PTPμ adhered to purified recombinant PTPμ as well as to E-cadherin and N-cadherin with the same efficiency as cells expressing wild type PTPμ. This verified that all the mutant forms of PTPμ restored cadherin-mediated adhesion. We then investigated how re-expression of PTPμ affected LNCaP cell growth. Re-expression of PTPμWT, but not the mutant forms of PTPμ, reduced the growth rate of LNCaP cells, indicating that PTPμ negatively regulates cell growth. This was not accompanied by an increased rate of apoptosis. Taken together, these data indicate that PTPμ, through its catalytic activity, is involved in the negative regulation of cell growth.

INTRODUCTION

Cells that are grown at a high density exit the cell cycle in G0/G1 phase even in the presence of growth factors, a phenomenon termed contact inhibition of growth. The ability of cell-cell contact to negatively regulate cell growth is lost in many tumors cells. The molecular events underlying contact inhibition of growth remain largely unknown.

The cell cycle is governed by sequential activation and inactivation of cyclin-dependent kinases (Cdks). Cdks are activated by their binding to molecules termed cyclins. The expression of cyclins is tightly regulated, and the level of each cyclin oscillates throughout the cell cycle. Progression from G1 phase to S-phase is regulated by cyclin D-induced activation of cdk4 or cdk6, and by cyclin E-induced activation of cdk2 (Morgan, 1997). The onset of mitosis is accompanied by increased expression of cyclin D. Cyclin D binds to and activates cdk4 or cdk6, leading to hyperphosphorylation and inactivation of the retinoblastoma protein, Rb (Weinberg, 1995). Phosphorylation of Rb disrupts an inhibitory complex between Rb and members of the E2F family of transcription factors. The release of E2F from Rb induces E2F-mediated transcription of genes necessary for S-phase entry. One of the genes regulated by the E2F transcription factors is cyclin E. Expression of cyclin E leads to the activation of cdk2, which is necessary for the onset of DNA replication.

The activity of the cyclin-Cdk complex can be inhibited by cyklin-dependent kinase inhibitors (CKI) (Morgan, 1997). So far, two classes of CKIs have been identified. The INK4 family contains four members, termed $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$. These inhibitors specifically bind to and inhibit cyklin D-dependent cdk:s (Morgan, 1997). On the other hand, the three members of the KIP family, $p21^{CIP1/WAF1}$, $p27^{KIP}$ and $p57^{KIP2}$, have been found to inhibit both cyclin D-cdk4/6 and cyclin E-cdk2. Several studies have implicated a role for p27 in contact inhibition of growth. In non-transformed fibroblasts and epithelial cells, the formation of cell-cell contacts have been shown to increase the level of p27 (Hengst et al., 1994; Hengst and Reed, 1996; Kato et al., 1997; Polyak et al., 1994). Several studies have implicated that adhesion receptors belonging to the cadherin family mediate contact inhibition of growth through the induction of p27. For example, exogenous expression of E-cadherin in mouse mammary carcinoma cells resulted in growth reduction mediated by increased expression of p27 (St.Croix, 1998). Overexpression of N-cadherin in CHO cells induced G1 arrest through an increased expression of p27 (Levenberg et al., 1999). These data show that cadherin-mediated adhesion is involved in the G0/G1 cell cycle arrest that accompanies contact inhibition of growth.

In several recent studies, we have shown that the receptor tyrosine phosphatase PTPμ is involved in the regulation of cadherin-mediated adhesion and signal transduction. First, PTPμ, through its catalytic activity, regulates N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999). Second, PTPμ restored cadherin-mediated adhesion in the LNCaP prostate carcinoma cell line (Hellberg et al., 2000a; Hellberg et al., 2000b). Although the LNCaP cells expressed all proteins in the cadherin-catenin complex, we found that they were deficient in both E-cadherin- and N-cadherin-mediated adhesion in an *in vitro* adhesion assay. Using a retroviral/tetracycline-repressible system, we re-expressed wild type and mutant forms of PTPμ in LNCaP cells. Expression of PTPμ restored both E-cadherin- and N-cadherin-mediated adhesion *in vitro,* demonstrating that the expression of PTPμ is required for cadherin-mediated adhesion to occur.

The notion that PTPμ regulates cadherin-mediated function prompted us to investigate whether this phosphatase is also involved in growth regulation. To assess the role of PTPμ, we have generated three mutant forms of PTPμ containing single amino acid mutations in the catalytic domain. The PTP enzymes share a catalytic domain with ~30% amino acid identity. The catalytic domain is characterized by a unique sequence motif {(I/V)HCXAGXXR(S/T)G}, which forms the phosphate-binding pocket. The cysteine residue sits at the base of the active site cleft and is essential for catalysis (Denu et al., 1996). The structural data on the PTP catalytic domain suggested mutations that could be made to alter either the affinity for substrate (K_m) or the rate of catalysis (V_{max}) (Flint et al., 1997; Garton et al., 1996). Mutation of the conserved cysteine residue PTPμC1095S (C-S) results in a catalytically inactive enzyme. Mutation of a conserved aspartate residue D1063A (D-A) is likely to create a substrate trap by affecting only V_{max} and not K_m. The "substrate trapping" mutant retain normal affinity for its substrate but catalytic activity is markedly reduced resulting in irreversible binding of the substrate (Neel and Tonks, 1997). While mutation of a conserved arginine R1101M (R-M) residue is likely to decrease both K_m and V_{max} of the enzyme which may act in a "dominant negative" fashion (Neel and Tonks, 1997).

To investigate the role of PTPμ in growth regulation, we employed the LNCaP prostate carcinoma cell line. Unlike normal prostate epithelial cells, LNCaP cells do not express endogenous PTPμ. Re-expression of the D-A, the C-S and the R-M mutant forms of PTPμ were found to restore cadherin-mediated adhesion in LNCaP cells, demonstrating that PTPμ exerts an effect on the cadherin/catenin adhesion complex which is independent of its catalytic activity (Hellberg et al., 2000a; Hellberg et al., 2000b). Re-expression of PTPμWT, but not the mutant forms of PTPμ, reduced the growth rate of LNCaP cells. This indicates that PTPμ, via its catalytic activity, is involved in growth suppression. The reduced growth rate was not accompanied by an increased rate of apoptosis, but FACS analysis indicated that cells expressing PTPμWT spent a longer time in the G0/G1 phase of the cell cycle. Taken together, these data indicate a novel role for PTPμ in the regulation of cell growth.

MATERIALS AND METHODS

Antibodies and reagents: Monoclonal antibodies against the intracellular (SK7) domain of PTPμ have been described (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994). RPMI 1640 medium and laminin were obtained from GIBCO-BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). Tween-20 was obtained from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Construction and expression of the PTPμ retroviruses: The retroviral system used is a tetracycline repressible ("tet-off") promoter-based system (Paulus et al., 1996). Using the pBPSTR1 vector generously provided by Dr. Steven Reeves (Harvard Medical School, Charleston, MA), the following constructs were generated. The wild type PTPμ plasmid (PTPμWT) and the C-S mutant have been previously described (Burden-Gulley and Brady-Kalnay, 1999). The mutant forms of PTPμ containing the D1063A or R1101M mutation were generated by PCR and a BglII/BspE1 fragment was subcloned to replace that same fragment in the wild type form of PTPμ-GFP in bluescript. The resulting plasmids were sequenced to confirm that the single amino acid mutation was present. The plasmids containing either wild type or mutant PTPμ were subcloned into the tetracycline regulatable retroviral vector, pBPSTR1. A construct containing the extracellular, the transmembrane and 55 amino acids of the intracellular domains has been previously described (Brady-Kalnay et al., 1993). This construct was subcloned into the tetracycline-regulatable retroviral vector, pBPSTR1 as described (Hellberg et al., 2000b). A replication-defective amphotrophic retrovirus was made by transfecting the PA317 helper cell line (ATCC CRL-9078) with the respective PTPμ-containing plasmids. Control virus was generated by transfecting PA317 helper cells with the pBPSTR1 plasmid alone.

Tissue culture and retroviral infection of LNCaP cells: LNCaP cells were grown in RPMI 1640 supplemented with 10% FBS and 1 μg/ml gentamicin at 37°C and 5% CO_2. Cells were infected with retrovirus by the addition of polybrene (5 μg/ml) and 1 ml of virus-containing medium per ml of culture medium. The cells were incubated overnight at 37°C, and the medium was exchanged with normal culture medium. Five days after infection, the cells were checked for GFP expression by fluorescence microscopy.

Protein Extraction and Immunoblotting: LNCaP cells were rinsed once with PBS and the cells were lysed in Triton-containing buffer (20mM Tris pH 7.6, 1% Triton X-100, 2mM $CaCl_2$, 1mM benzamidine, 200 μM phenyl arsine oxide, 1mM vanadate and 0.1mM molybdate) and scraped off the dish. After incubation on ice for 30 min, the lysate was centrifuged at 14, 000 rpm for 3 min and the Triton-soluble material was recovered in the supernatant. The amount of protein was determined by the Bradford method using BSA as a standard. Lysates were boiled in equal volume of 2X sample buffer, separated by SDS PAGE and transferred to nitrocellulose for immunoblotting as described previously (Brady-Kalnay and Tonks, 1993).

Expression and purification of GST fusion proteins: Two GST fusion protein constructs were obtained from Dr Robert Brackenbury (University of Cincinnati): a construct encoding amino acids 9-139 of mouse E-cadherin and a construct encoding amino acids 1-110 of N-cadherin. The GST fusion protein expressing the entire extracellular domain of PTPμ has previously been described (Brady-Kalnay et al., 1993). Expression of GST-tagged proteins in *E.coli* was induced by IPTG. The bacteria were collected by centrifugation at 3000 x g for 10 min and lysed in PBS containing 1% Triton X-100, 5μg/ml leupeptin, 5μg/ml aprotinin and 1mM benzamidine, sonicated and centrifuged again at 3000 x g for 10 min to remove debris. The supernatant was passed over glutathione-sepharose beads, (Pharmacia Biotech, Piscataway, NJ) washed and the bound protein eluted with 10mM glutathione as described previously (Brady-Kalnay et al., 1993).

LNCaP Adhesion to Purified Proteins: Sterile coverslips were coated overnight with 100μg/ml polylysine, washed twice in sterile water and were allowed to dry. Subsequently, the coverslips were coated with nitrocellulose in methanol (Lagenaur and Lemmon, 1987) and allowed to dry. Purified recombinant proteins were diluted in PBS containing 2mM $CaCl_2$ to a concentration of 75 μg/ml for PTPμ, E-cadherin and N-cadherin respectively, and 40 μg/ml for laminin. To identify the individual protein spots on the coverslips, the protein solutions were supplemented with 20μg/ml Texas Red BSA (Sigma). Four distinct spots, each containing a single adhesion molecule, were generated by spotting 20 μl of each protein solution on one coverslip. After a 20 min. incubation, the protein solution was aspirated, and this procedure was repeated once. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in PBS, and the dishes were rinsed with RPMI-1640 medium. 3×10^5 LNCaP cells infected with the indicated retrovirus were added to coverslips, and the cells were allowed to adhere overnight. The medium was removed and the coverslips were rinsed once with PBS to remove unattached cells. The cells were subsequently fixed with cold 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80mM Pipes, 5mM EGTA, 1mM $MgCl_2$, 3% sucrose), pH 7.4 for 30 min. at room temperature. The coverslips were washed twice in PBS and mounted in IFF mounting medium (0.5M Tris-HCl pH 8.0 containing 20%

glycerol and 0.1% p-phenylenediamine). Adherent cells were detected by dark field microscopy and photographed. To quantify the number of adherent cells, the 35 mm negatives were scanned and the digitized images were analyzed using the Metamorph image analysis program (Universal Imaging Corp., West Chester, PA). To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data obtained were analyzed by Student's t test (Statview 4.51, Abacus Concepts, Inc.).

LNCaP cell growth analysis: LNCaP cells were plated at 40,000 cells/well in 12 well plates. The following day, one well per plate was trypsinized and the cells were counted in a hemacytometer giving the number of adherent cells at time 0. The cells in the remaining wells were then infected with the indicated virus as described above. The following day, the medium was changed, and the cells in one well/plate were trypsinized and counted each day. The data is given as percent of cells at time 0 ±SEM.

DNA cell cycle analysis: Cells were infected as described above and cultured for six days. The DNA cell cycle analysis was performed as previously described (Schimenti and Jacobberger, 1992). Briefly, the cells were trypsinized and washed twice in ice-cold PBS. The cells were then resuspended in ice-cold PBS to a concentration of 10^6 cells in 50 μl and fixed by the addition of 450 μl ice-cold methanol for at least 30 min. The fixed cells were washed twice in PBS followed by a 30 min. incubation with RNase (20 μg/ml) at 37°C. The cells were stained with propidium iodide (50 μg/ml) for 30 min. and analyzed by flow cytometry.

RESULTS

Generation of retrovirus

To re-express PTPμ in LNCaP cells, we generated a tetracycline regulatable retrovirus encoding the PTPμ cDNA sequence tagged with the green fluorescence protein (GFP). To assess the role of PTPμ in the regulation of cadherin-mediated adhesion, we also generated tetracycline regulatable retroviruses encoding three mutant forms of PTPμ containing single amino acid mutations in the catalytic site. Mutation of the conserved cysteine residue PTPμC1095S (C-S) results in a catalytically inactive enzyme. The mutation of a conserved aspartate residue D1063A (D-A) is likely to create a substrate trap by affecting only V_{max} and not K_m (Flint et al., 1997; Garton et al., 1996). In addition, mutation of a conserved arginine R1101M (R-M) residue is likely to decrease both K_m and V_{max} of the enzyme which may act in a "dominant negative" fashion (Neel and Tonks, 1997). To determine whether the intracellular PTP domains of PTPμ were required to affect cadherin-dependent adhesion, we constructed a retrovirus encoding for the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (PTPμ-extra).

Re-expression of PTPμ

Wild type PTPμ (PTPμWT) was re-expressed in the LNCaP cells, and the expression was confirmed by immunoblot analysis. LNCaP cells infected with retrovirus containing an empty vector did not express PTPμ (Fig. 1, Vec), whereas cells infected with retrovirus containing PTPμWT (Fig 1, WT) expressed both the full length protein (200 kDa) as well as the proteolytically processed forms (100 kDa) (Brady-Kalnay and Tonks, 1994). The re-expression of the mutant forms of PTPμ was also confirmed by immunoblot analysis of lysates from LNCaP cells (Fig. 1, D-A, C-S and R-M, respectively). PTPμ-extra was detected as a single protein of approximately 100 kDa (Fig. 1, Extra). As a control, the expression of PTPμ in normal prostate epithelial cells is shown (Fig. 1 NPr).

To ensure an appropriate subcellular localization of the mutant forms of PTPμ, we analyzed the expression of PTPμGFP by fluorescence microscopy five days after retroviral infection. Control cells infected with a virus containing an empty vector did not show any fluorescence (Fig. 2A and B). In contrast , fluorescence microscopy revealed that between 70-90 % of the LNCaP cells expressed PTPμWT, and that PTPμ was primarily localized to the plasma membrane as previously shown (Fig. 2C and D;

Hellberg et al., 2000). Re-expression of the D-A (Fig. 2E and F) the C-S (Fig. 4G and H) and the R-M mutants (Fig. 4I and J) showed that all three mutants were expressed at a similar level as PTPμWT. In addition, all three mutants showed the same pattern of localization as PTPμWT, demonstrating that the expression and intracellular localization is not affected by the change in catalytic activity or substrate binding.

Re-expression of PTPμ induced adhesion to purified PTPμ.

PTPμ has been shown to mediate homophilic cell-cell adhesion via its extracellular Ig domain (Brady-Kalnay et al., 1993; Gebbink et al., 1993). To verify that the mutant forms of PTPμ were able to mediate homophilic binding in LNCaP cells, we employed an *in vitro* adhesion assay where purified recombinant PTPμ was immobilized on nitrocellulose-coated coverslips. As expected, cells infected with an empty vector did not adhere to PTPμ (Fig. 3A) since these cells do not express PTPμ. Re-expression of PTPμWT induced LNCaP adhesion to purified PTPμ (Fig. 3E), as did re-expression of the D-A (Fig. 3I), the C-S (Fig. 3M) and the R-M (Fig. 3Q) mutant forms of PTPμ. Quantitation of the adhesion assays showed that the number of cells that adhered to purified PTPμ was significantly greater for cells infected with WT and mutant forms of PTPμ as compared to cells infected with vector only (Fig. 4A, Table I). However, there was no significant difference between cells expressing PTPμWT compared to the mutant forms of PTPμ in their ability to adhere to purified PTPμ (Fig. 4A, Table I). Taken together, these data confirm that the re-expressed PTPμ is capable of mediating homophilic binding, and that the phosphatase activity is not necessary for this adhesion to occur, as previously demonstrated (Brady-Kalnay et al., 1993). As an internal control in each experiment, cells were allowed to adhere to laminin. Adhesion to extracellular matrix proteins such as laminin is mediated through integrin receptors. Since there is no evidence indicating that PTPμ regulates integrin function, LNCaP adhesion to laminin should not be affected by the re-expression of PTPμ. As expected, LNCaP cells infected with an empty vector adhere to laminin (Fig. 3B), and this adhesion was not altered by re-expressing WT (Fig. 3F) or mutant forms of PTPμ (Fig. 3J, N and R respectively; Fig. 4B, Table I). None of the retroviral infected cells adhered to nitrocellulose coated with BSA only (data not shown).

Re-expression of PTPμ restored cadherin-mediated adhesion.

To investigate whether PTPμ plays a role in cadherin-mediated adhesion in LNCaP cells, we immobilized purified recombinant E-cadherin and N-cadherin on the nitrocellulose-coated coverslips. Despite the fact that these cells express both E- and N-cadherin as well as α-, ß-, γ- catenin and p120 (Hellberg et al., 2000a; Hellberg et al., 2000b), LNCaP cells infected with an empty vector did not adhere to either E-cadherin (Fig. 3C) or N-cadherin (Fig. 3D). Re-expression of PTPμWT restored the ability of LNCaP cells to adhere to both types of cadherin (Fig. 3G and H, respectively). Quantitation of the adhesion assays shows that the number of cells infected with PTPμWT that adhered to either E-cadherin or N-cadherin was significantly higher than the number of cells infected with vector only (Fig. 3C and D, respectively; Table I). These data illustrate that expression of PTPμ is necessary for cadherin-mediated adhesion in LNCaP cells.

Re-expression of the C-S mutant has been shown to restore cadherin-mediated adhesion in LNCaP cells (Hellberg et al., 2000; Hellberg and Brady-Kalnay, 2000). To determine whether the D-A and R-M mutants also restored cadherin-mediated adhesion, we repeated the adhesion assays with cells expressing the mutant forms of PTPμ. Expression of the D-A mutant restored both E-cadherin and N-cadherin-mediated adhesion (Fig. 3K and L, respectively), as did re-expression of the C-S mutant (Fig. 3O and P), and the R-M mutant (Fig. 3S and T). As seen in fig 4C and D, re-expression of WT or mutant forms of PTPμ induced a significant adhesion to both E-cadherin and N-cadherin as compared to LNCaP cells infected with an empty vector. In contrast, there was no significant difference in adhesion between cells infected with PTPμWT compared to cells infected with any of the mutant forms of PTPμ. The statistical analysis for LNCaP cell adhesion to E- and N-cadherin is summarized in Table I. Expression of PTPμ-extra induced LNCaP adhesion to purified recombinant PTPμ (Fig. 3U), confirming that the intracellular domains are not required for PTPμ to mediate homophilic binding (Brady-Kalnay et al., 1993). Intriguingly, PTPμ-extra did not restore LNCaP adhesion to recombinant E-cadherin (Fig. 3W), demonstrating that one or both of the intracellular domains of PTPμ is necessary for E-cadherin-mediated adhesion. However, PTPμ extra did restore N-cadherin-mediated adhesion (Fig. 3X). As expected, the expression of PTPμ extra did not affect LNCaP adhesion to laminin (Fig. 3V).

Wild type but not mutant forms of PTPμ regulate LNCaP cell growth.

To identify a physiological role for the catalytic activity of PTPμ, we investigated how the re-expression of PTPμWT affects LNCaP cell growth. LNCaP cells infected with PTPμWT grew at a slower rate than cells infected with an empty vector, and they also displayed a lower saturation density (Fig. 5). Re-expression of either the D-A, the C-S or the R-M mutant forms of PTPμ did not affect the growth rate of LNCaP cells as compared to cells infected with an empty vector (Fig. 5), clearly demonstrating that the catalytic activity of PTPμ is required for negative growth regulation. We then performed a DNA cell cycle analysis. Preliminary results show a small increase in the number of cells in G0/G1 phase of the cell cycle for cells expressing PTPμWT compared to cells infected with an empty vector or the mutant forms of PTPμ (data not shown). Together, these results indicate that PTPμ is involved in the negative regulation of cell growth.

DISCUSSION

In this study, we show that PTPμ excerts a dual role in cadherin-mediated adhesion and signal transduction. The first pathway, leading to E-cadherin-mediated adhesion, is dependent on the presence of the phosphatase domains of PTPμ but independent of its catalytic activity. The other pathway, which regulates cell growth, is dependent on the phosphatase activity of PTPμ and occurs independently of cadherin-mediated cell-cell adhesion.

The LNCaP cells provide an excellent model for studying the role of PTPμ in the regulation of cell growth. Unlike normal prostate epithelial cells, this cell line does not express PTPμ, and they do not display contact inhibition of growth. Despite the fact that they express E-cadherin and N-cadherin as well as α-, ß-, γ-catenin and p120, they were found to be deficient in cadherin-mediated adhesion. Re-expression of PTPμWT restored this adhesion, demonstrating a functional role for PTPμ in cadherin-mediated adhesion. LNCaP cells expressing PTPμWT displayed a decrease in their growth rate, indicating that PTPμ may act as a growth suppressor. Re-expression of the catalytically inactive mutants were found to restore cadherin-mediated adhesion without affecting the growth rate of the LNCaP cells. Taken together, these data indicate that PTPμ, through its catalytic acitvity, regulates cell growth in prostate carcinoma cells.

The mechanism whereby PTPμ regulates cell growth is unknown. Previous studies have shown that expression of both PTPμ and the receptor phosphatase DEP-1 are upregulated in cells grown at high density (Gebbink et al., 1995; Ostman et al., 1994). It has been suggested that the increased phosphatase expression regulates the ability of growth factor receptors to respond to their ligands. Mink lung cells grown at low confluency responded to EGF stimulation by a 4-fold higher phosphorylation of the EGF-receptor than did confluent cells (Sorby and Ostman, 1996). In addition, overexpression of CD45 was found to attenuate PDGF-receptor signaling (Mooney et al., 1992; Way and Mooney, 1993). Indeed, PTPμ has been found in a complex containing the HGF receptor (Hiscox and Jiang, 1999). Therefore, it is possible that normally occurring upregulation of PTPμ when cells are grown at high density could mediate contact inhibition of growth by dephosphorylating growth factor receptors and/or their substrates.

Alternatively, PTPμ may exert its growth inhibitory effects through its effects on cadherin-mediated adhesion and signal transduction. Several studies have suggested a role for E-cadherin as a growth suppressor (Hermiston and Gordon, 1995; Miyaki et al., 1995; St.Croix, 1998). A recent study also suggested that overexpression of N-cadherin may negatively regulate growth (Levenberg et al., 1999). Alterations in the function of the E-cadherin/catenin adhesion system occur frequently in a wide variety of human carcinomas (Birchmeier and Behrens, 1994; Takeichi, 1993). Since PTPμ is found in complex with classical cadherins (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995; Hiscox and Jiang, 1998) it is possible that a loss of expression or function of PTPμ may result in decreased cadherin-dependent adhesion and loss of growth suppression in human carcinomas.

The mechanisms underlying cadherin-mediated contact inhibition of growth are not understood, but it has been speculated that it may occur through inhibition of mitogenic signals initiated from growth factor receptors (St.Croix, 1998). Growth factor receptors associate with the cadherin/catenin complex (Hiscox and Jiang, 1999; Hoschuetzky et al., 1994; Kanai et al., 1995). In addition to PTPμ, several other phosphatases, including PTPκ and PTP1B, have been shown to interact with cadherins and catenins (Aicher et al., 1997; Balsamo et al., 1996; Cheng et al., 1997; Fuchs et al., 1996; Kypta et al., 1996). The association of the cadherins with both kinases and phosphatases indicates a critical role for dynamic tyrosine phosphorylation in both cadherin-mediated adhesion as well as in the signal transduction pathways that occur downstream of cadherin-mediated adhesion. It is possible that PTPμ negatively regulates cell growth by dephosphorylating growth factor receptors that are associated with the cadherin/catenin complex.

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LEGENDS TO THE FIGURES

Figure 1. Re-expression of PTPμ in LNCaP cells. LNCaP cells were infected with retrovirus containing an empty vector (VEC), PTPμWT, PTPμ-extra (Extra) or the mutant forms of PTPμ containing a single point mutation in the catalytic site; D-A, C-S, R-M for five days. The cells were lysed, and 30 μg of lysate were separated by 6 % SDS-PAGE, transferred to nitrocellulose and blotted with a monoclonal antibody (SK7) to PTPμ. As a comparison, 30 μg of lysate from normal prostate epithelial cells (NPr) is shown.

Figure 2. Re-expression of GFP-tagged WT and mutant forms of PTPμ. LNCaP cells were infected with retrovirus containing an empty vector (A and B), PTPμWT (C and D) or mutant forms of PTPμ containing a single point mutation in the catalytic site; D-A (E and F), C-S (G and H) or R-M (I and J). Five days after infection, the expression of GFP-tagged proteins was visualized by fluorescence microscopy. Representative phase contrast (A, C, E, G and I) and fluorescent (B, D, F, H and J) images are shown.

Figure 3. LNCaP adhesion to purified recombinant proteins. LNCaP cells were infected with retrovirus containing an empty vector (A-D), PTPμWT (E-H), or mutant forms of PTPμ containing a single point mutation in the catalytic site; D-A (I-L), C-S (M-P), R-M (Q-T) or PTPμ-extra (U-X). Five days after infection, the cells were incubated with coverslips spotted with purified recombinant PTPμ (A, E, I, M, Q and U), laminin (B, F, J, N, R and V), E-cadherin (C, G, K, O, S and W) or N-cadherin (D, H, L, P, T and X). Adherent cells were visualized by dark-field microscopy and photographed using a 35 mm camera.

Figure 4. Quantitation of LNCaP adhesion to purified recombinant proteins. To quantify the number of adherent cells from the experiments shown in Figure 3, the 35 mm negatives from six experiments were scanned and the digitized images were analyzed using the Metamorph image analysis program. To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data is presented as mean ± SEM. **A.** Adhesion to PTPμ. **B.** Adhesion to laminin. **C.** Adhesion to E-cadherin. **D.** Adhesion to N-cadherin. For statistical analysis see Table I.

Fig. 5 Re-expression of PTPμWT decreases the LNCaP growth rate. LNCaP cells were plated at a density of 40,000 cells/well in 12 well plates. The following day, one well for each plate was trypsinized and counted giving the number of adherent cells at time 0. The remaining cells were infected with retrovirus containing an empty vector, PTPμWT, or mutant forms of PTPμ containing a single point mutation in the catalytic site; D-A, C-S or R-M. The cells in one well were trypsinizedand counted each day. The data is given as percent of cells on the day of infection, and is given as the mean +/- SEM.

Table I. Statistical analysis of LNCaP adhesion to purified recombinant proteins. The data shown in figure 4 is presented as mean number of adherent cells ± SEM. For each substrate, Student's t test was used to compare the number of cells infected with PTPμ to the number of adherent cells infected with an empty vector.

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Figure 1

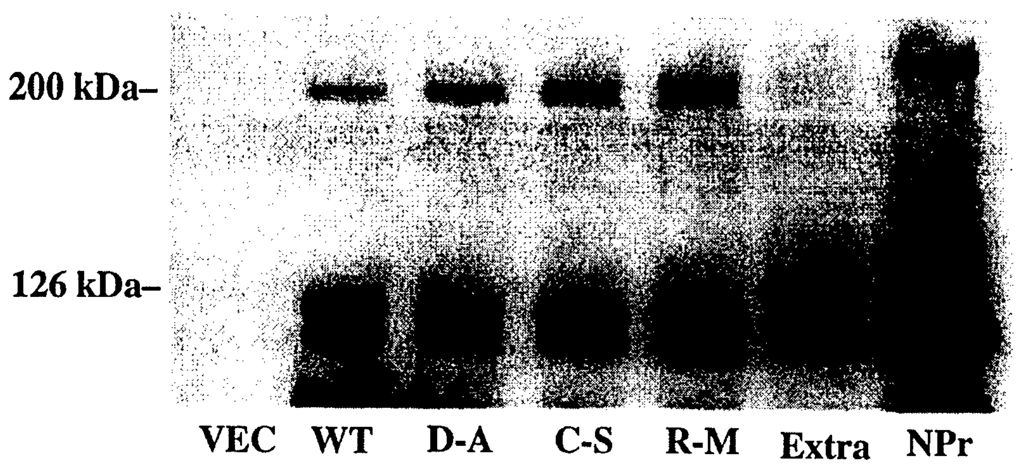

Figure 2

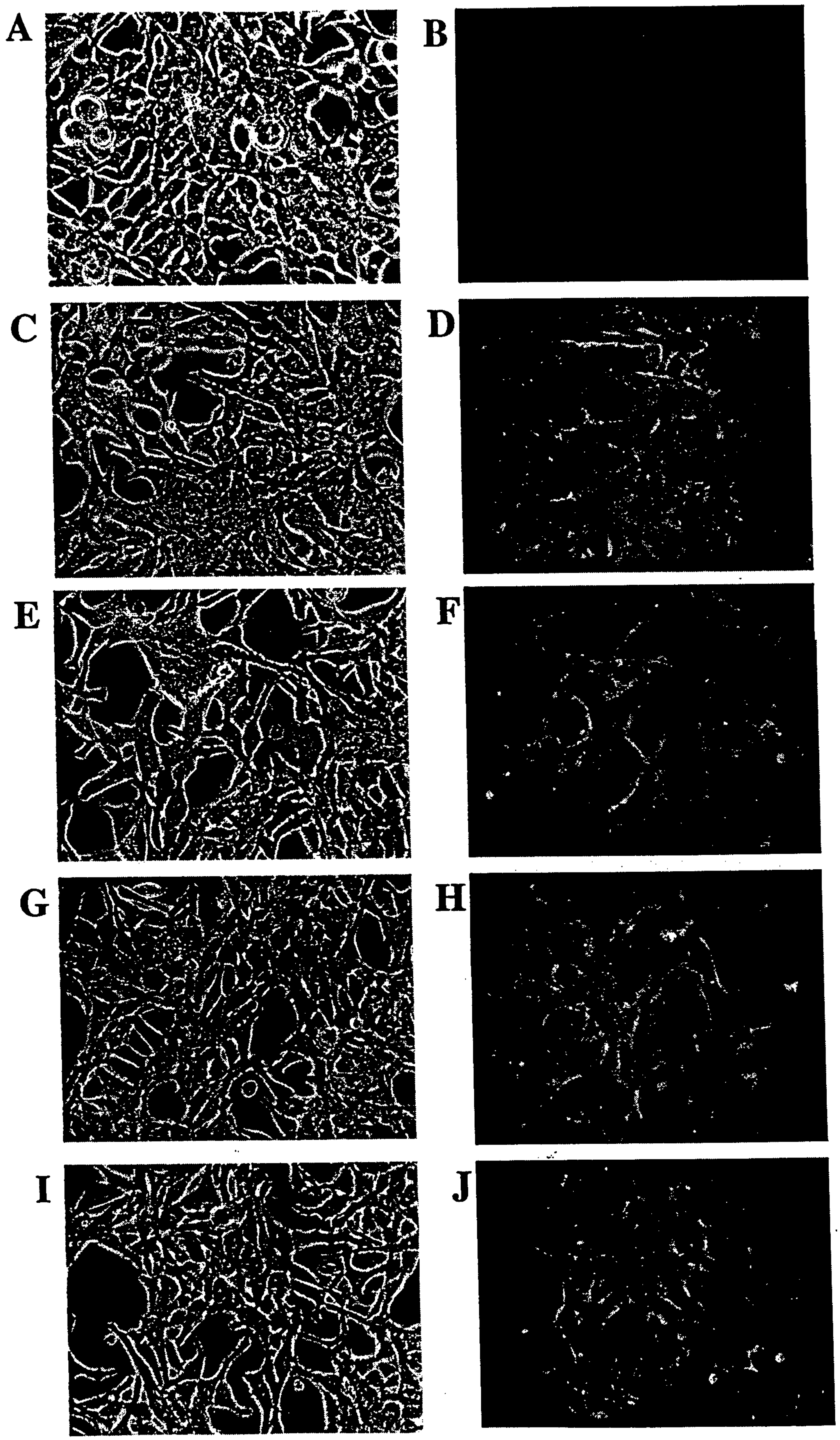

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Figure 3

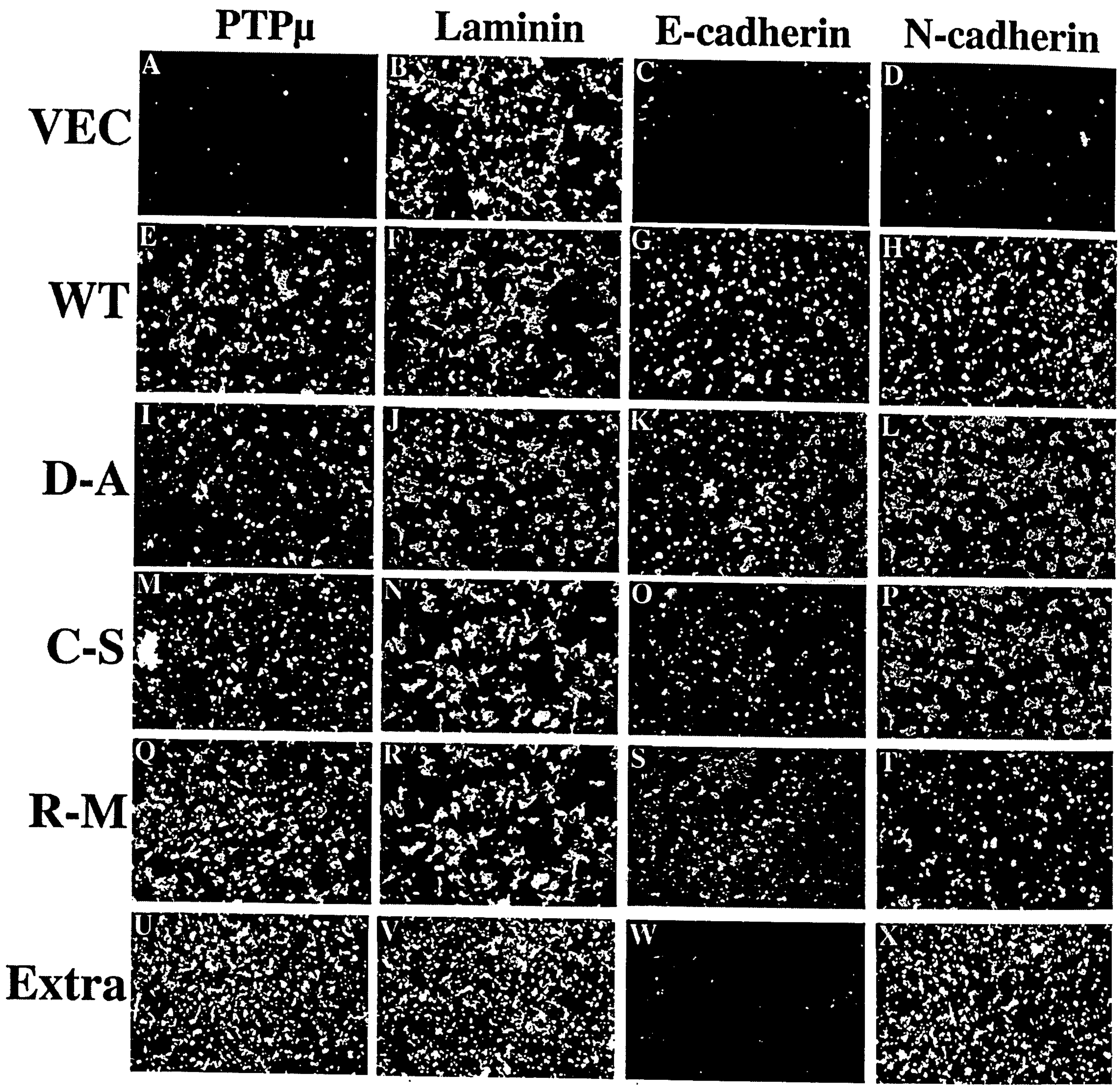

Figure 4

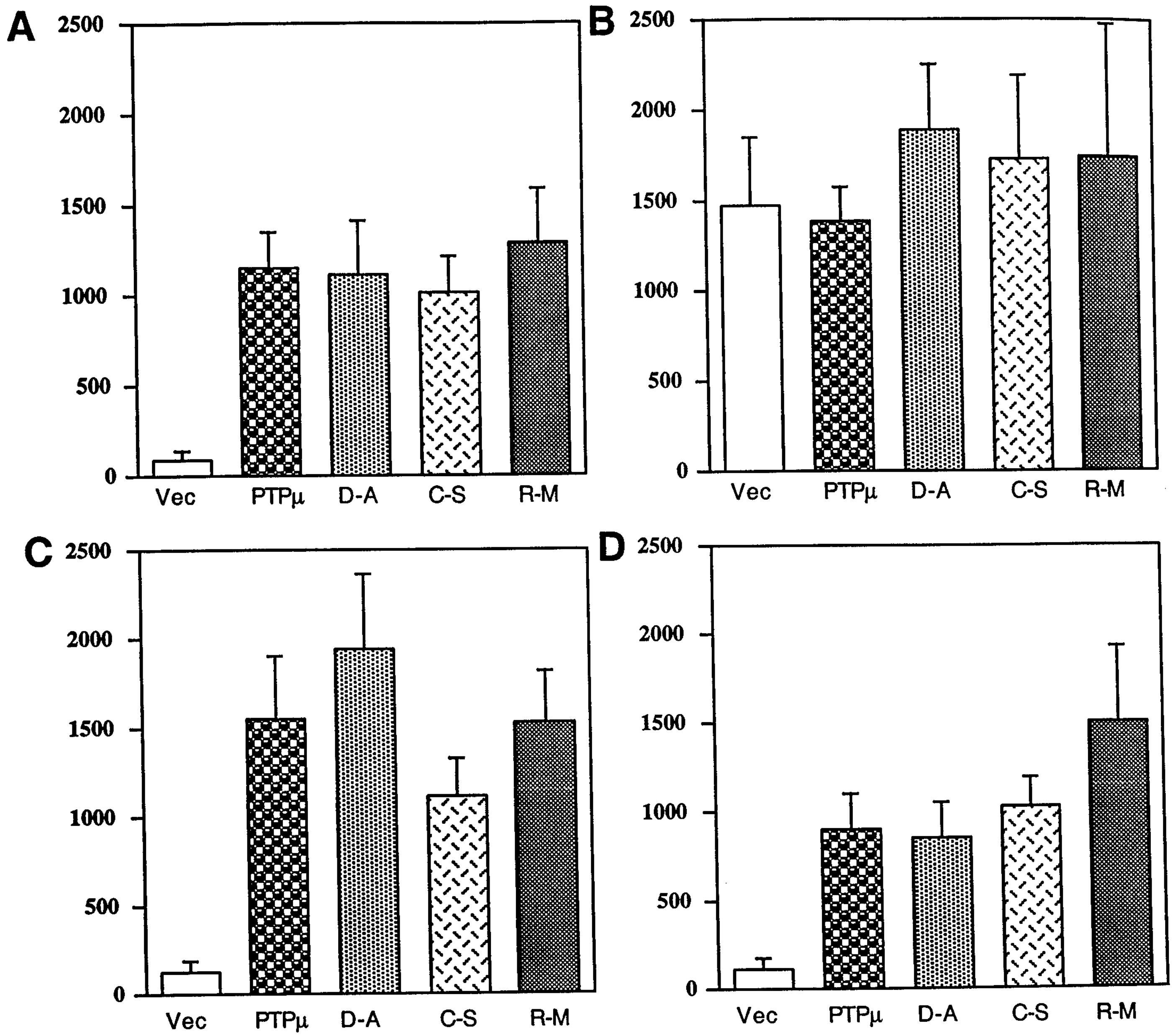

Figure 5

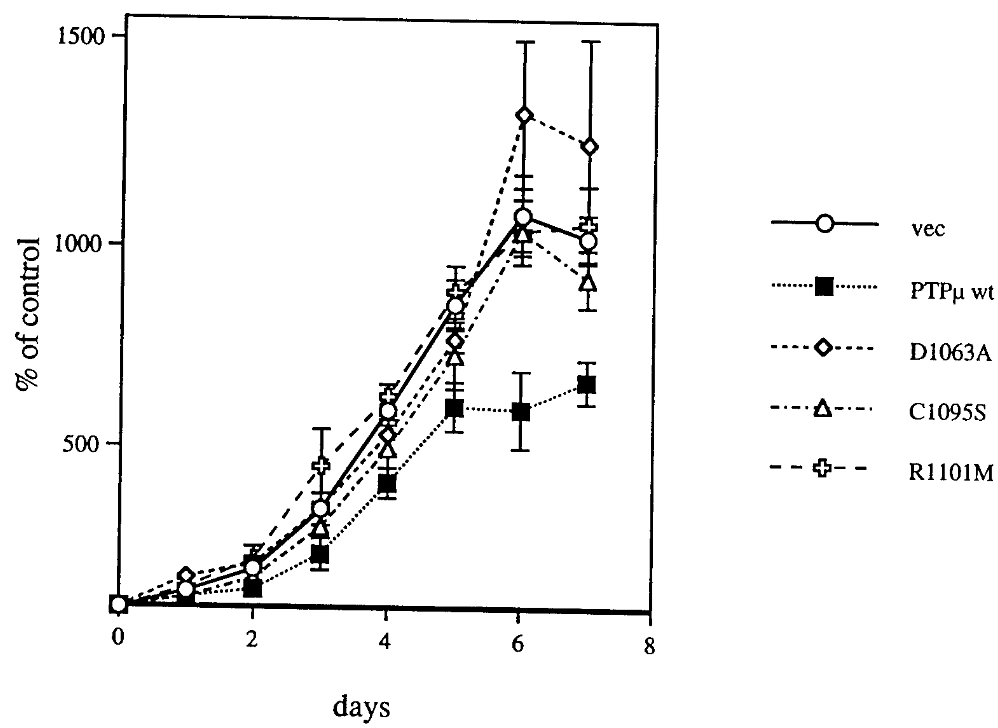
1500
1000
500
% of control
0
2
4
6
8
days
vec
PTPμ wt
D1063A
C1095S
R1101M

Table I. Statistical Analysis of LNCaP Adhesion to Purified Proteins

	Substrate							
	PTPμ		Laminin		E-cadherin		N-cadherin	
Virus type	Adherent cells	*P	Adherent cells	*P	Adherent cells	*P	Adherent cells	*P
Vector	95.5 ± 45.7	-	1483.4 ± 368.2	-	132.7 ± 62.8	-	118.0 ± 63.7	-
PTPμwtGFP	1150.0 ± 210.8	0.0006	1391.8 ± 192.7	0.8311	1561.5 ± 350.0	0.0024	905.3 ± 202.0	0.0040
D1063A	1122.2 ± 298.4	0.0067	1897.4 ± 360.9	0.4452	1943.3 ± 422.4	0.0017	859.3 ± 190.8	0.0042
C1095S	1022.5 ± 192.0	0.0008	1736.2 ± 463.0	0.6804	1117.8 ± 210.9	0.0012	1026.2 ± 169.8	0.0005
R1101M	1287.8 ± 305.6	0.0032	1739.0 ± 741.5	0.7654	1526.5 ± 269.3	0.0010	1504.0 ± 427.8	0.0094

The data from six adhesion assays were subjected to statistical analysis. The data is given as number of adherent cells ± SEM. The P values were obtained by students t test, 99% confidence interval.

GORDON CONFERENCE ON CELL CONTACT AND ADHESION-1999

Expression of the Receptor Protein Tyrosine Phosphatase, PTPμ, is necessary for Cadherin-mediated adhesion in Prostate carcinoma cells.

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The receptor tyrosine phosphatase PTPμ has previously been shown to interact with both the N- and E-cadherin adhesion complexes. Recent work has shown that PTPμ regulates N-cadherin mediated neurite outgrowth, but little is known about the molecular mechanism underlying these events. To investigate the role of PTPμ expression and signal transduction in the regulation of cadherin function, we employed a prostate carcinoma cell system that expresses normal levels of N- and E-cadherin as well as α-, ß- and γ-catenin, but has downregulated expression of endogenous PTPμ. However, cadherin-dependent adhesion was defective in this prostate cancer cell line. Using an *in vitro* adhesion assay, we observed that these cells adhere to laminin but not to purified recombinant PTPμ, N-cadherin or E-cadherin. Full length PTPμ tagged with the green fluorescent protein (GFP) was re-expressed in the cells using a retroviral/tetracycline-repressible system. This system yielded an efficiency of at least 90%, as determined by fluorescence microscopy. Re-expression of PTPμGFP restored cell adhesion to recombinant PTPμ as well as to E-cadherin and N-cadherin, indicating that PTPμ expression is necessary for cadherin-mediated cell adhesion in this system. To investigate the role of PTPμ phosphatase activity in cadherin-mediated adhesion, we re-expressed mutant forms of PTPμ with reduced catalytic activity or reduced substrate binding. The effect of these mutants on PTPμ and cadherin-dependent adhesion is currently being tested.

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CADHERIN-MEDIATED ADHESION IS DEPENDENT ON THE EXPRESSION OF THE RECEPTOR PROTEIN TYROSINE KINASE PTPμ IN PROSTATE CARCINOMA CELLS

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The receptor tyrosine phosphatase PTPμ has been shown to interact with both the N- and E-cadherin adhesion complex, indicating that PTPμ could participate in signaling events following cadherin-mediated adhesion. This hypothesis is supported by the recent finding that PTPμ regulates N-cadherin mediated neurite outgrowth. To further investigate the role of PTPμ expression and signal transduction in the regulation of cadherin function, we employed a prostate carcinoma cell system that expresses normal levels of N- and E-cadherin as well as α-, ß- and γ-catenin, but has downregulated expression of endogenous PTPμ. However, cadherin-dependent adhesion was defective in this prostate cancer cell line. Using an *in vitro* adhesion assay, we observed that these cells show normal integrin-mediated adhesion to laminin, whereas they do not adhere to purified recombinant PTPμ, N-cadherin or E-cadherin. Full length PTPμ tagged with the green fluorescent protein (GFP) was re-expressed in the cells using a retroviral/tetracycline-repressible system. This system yielded an efficiency of at least 90%, as determined by fluorescence microscopy. Re-expression of PTPμGFP restored cell adhesion to recombinant PTPμ as well as to E-cadherin and N-cadherin, indicating that PTPμ expression is necessary for cadherin-mediated cell adhesion in this system. To investigate the role of PTPμ phosphatase activity in cadherin-mediated adhesion, we re-expressed mutant forms of PTPμ with reduced catalytic activity or reduced substrate binding. The effect of these mutants on prostate carcinoma cell adhesion to PTPμ as well as N- and E- cadherin will be presented.

This work was supported by a DOD grant (DAMD 17-98-1-8586)