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Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density

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ABSTRACT **cDNA encoding a receptor-like protein-tyrosine-phosphatase (PTP) termed DEP-1 was isolated from a HeLa cell library. The cDNA predicts an enzyme consisting of an extracellular segment containing eight fibronectin type III repeats, a single transmembrane segment, and a single intracellular PTP domain. Following expression of DEP-1 cDNA in COS cells a glycoprotein of 180 kDa was detected and PTP activity was demonstrated in immunocomplexes with a C-terminal peptide antiserum. Endogenous DEP-1 was detected in WI-38 human embryonic lung fibroblasts by immunoblotting and immunocomplex PTP assays. Immunoblot analysis of DEP-1 expression in WI-38 cells revealed dramatically increased levels and activity of the PTP in dense cultures relative to sparse cultures. Also, DEP-1 activity, detected in PTP assays of immunocomplexes, was increased in dense cell cultures. In contrast, the expression levels of PTP-1B did not change with cell density. This enhancement of DEP-1 expression with increasing cell density was also observed in another fibroblast cell line, AG1518. The increase in DEP-1 occurs gradually with increasing cell contact and is initiated before saturation cell density is reached. These observations suggest that DEP-1 may contribute to the mechanism of contact inhibition of cell growth.**

Protein tyrosine phosphorylation is an essential element of signal transduction pathways that control fundamental cellular processes, including growth and differentiation, the cell cycle, and cytoskeletal function (1). The phosphorylation state of tyrosine residues in a target substrate is governed by the coordinated action of both protein-tyrosine kinases (PTKs) and protein-tyrosine-phosphatases (PTPs). Like the PTKs, the PTPs represent a large and diverse family of enzymes found ubiquitously in eukaryotes (2). Each PTP contains at least one conserved domain of ≈240 aa that is characterized by the signature motif (I/V)HCXAGXGR(S/T)G. The structural diversity within the PTP family arises primarily from the variety of putative regulatory sequences linked to the catalytic domains.

Cytoplasmic PTPs possess a single catalytic domain flanked at either the N or the C terminus by regulatory sequences that include Src homology 2 domains, segments of homology to the cytoskeleton-associated protein band 4.1 or to lipid-binding proteins, and motifs that direct association with intracellular membranes. These structural features implicate subcellular compartmentalization in the control of catalytic function (reviewed in refs. 3 and 4). In addition, analogous to the PTKs, receptor-like PTPs have been identified, suggesting the potential for control of signal transduction events through ligand-modulated dephosphorylation of tyrosine residues in proteins. Generally the structure of receptor-like PTPs can be described in terms of an intracellular segment containing one or two phosphatase domains, a single transmembrane domain, and a variable extracellular segment. These receptor-like PTPs can be distinguished on the basis of the diversity of their extracellular segments, which presumably reflects an equivalent diversity in the variety of ligands to which they may respond (3). However, a common structural theme among receptor PTPs is the presence of motifs found in proteins involved in cell adhesion. Thus the extracellular segment of PTPβ (5) and SAP-1 (6) in humans and DPTP10D and -99A (7–9) and DPTP4E (10) in *Drosophila* are characterized by a repeated array of fibronectin type III (FNIII) motifs, while LAR (11), PTPμ (12), and PTPκ (13), among others, possess multiple FNIII motifs in combination with immunoglobulin (Ig)-like domains. Such an arrangement of FNIII and Ig-like motifs is also found in members of the Ig superfamily of cell adhesion molecules that includes neural cell adhesion molecule (NCAM) (14). As may be anticipated from the similarity to NCAM, it has been demonstrated that PTPμ can promote cell–cell aggregation through homophilic binding interactions; i.e., the ligand for PTPμ is another molecule of the same enzyme expressed on the surface of an apposing cell (15, 16). Similar observations have now also been made for PTPκ (17).

Such data reinforce the concept that receptor PTPs may play a role in transducing signals initiated by cell–cell contact. For example, normal cells display density-dependent arrest of cell growth (18). Thus, as cultures approach confluence and adjacent cells touch each other, growth is inhibited. Since tyrosine phosphorylation triggered by growth factor receptor PTKs has been implicated in promoting cell growth and proliferation (1), PTPs, as the natural antagonists of PTK function, may exert a negative effect on such growth-promoting signals. We report the isolation of cDNA encoding a receptor-like PTP whose expression may contribute to the contact inhibition of cell growth. It comprises eight FNIII repeats in its extracellular segment, a single transmembrane domain, and a single intracellular catalytic domain. Intrinsic activity has been demonstrated. The most striking property of this enzyme is that in two distinct cells lines expression is enhanced ≥10-fold as cells approach confluence. We propose the name DEP-1 (high cell density-enhanced PTP 1) for this enzyme.§

MATERIALS AND METHODS

Isolation and Characterization of DEP-1 cDNA. Primers corresponding to amino acid sequences KCAQYWP and HCSAGIG from the conserved PTP domain were used in a polymerase chain reaction (PCR) with phage DNA of a HeLa cell cDNA library as template. A ≈300-bp PCR fragment was identified and used to screen a HeLa cell cDNA library

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Abbreviations: FNIII, fibronectin type III; NCAM, neural cell adhesion molecule; PTK, protein-tyrosine kinase; PTP, protein-tyrosine-phosphatase.

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§The sequence reported in this paper has been deposited in the GenBank database (accession no. U10886).

(Stratagene). Of 1.8×10^6 phage plaques screened (19), 24 positive clones were identified. The largest, a 5.1-kb cDNA, was cloned and sequenced by the dideoxy chain-termination method. The open reading frame was sequenced on both strands.

Cell Culture and Transfections. WI-38 human embryonic lung fibroblasts and COS transformed monkey kidney fibroblasts (ATCC) and AG1518 foreskin fibroblasts (Human Mutant Cell Repository, Camden, NJ) were grown in Dulbecco's modified Eagle's medium and Eagle's medium, respectively, containing 10% fetal bovine serum and supplemented with antibiotics. COS cells were transfected by a standard calcium phosphate method, with lysates prepared 48 hr after transfection.

Antibodies to DEP-1 and Immunoblotting and Immunocomplex Assays. Two peptides, corresponding to aa 1297–1315 (peptide CSH-241) and 1321–1334 (CSH-243) at the C terminus of DEP-1, were synthesized and coupled to rabbit serum albumin and polyclonal antisera were generated in rabbits by standard procedures. Cells were lysed in 20 mM Hepes, pH 7.5/0.5% sodium deoxycholate/0.5% Triton X-100/150 mM NaCl/1 mM benzamidine/1 mM dithiothreitol with aprotinin (5 μg/ml) and leupeptin (5 μg/ml). For immunoblotting, after semidry electrophoretic transfer to nitrocellulose, filters were blocked and then probed with affinity-purified anti-DEP-1 (CSH-243) antibody or with anti-PTP1B monoclonal antibody FG6. Immunoreactive species were detected by enhanced chemiluminescence (ECL; Amersham). For immunocomplex PTP assays, 60 μg of cell lysate was immunoprecipitated with 20 μl of anti-DEP-1 (CSH-243) antiserum (with or without pretreatment with antigen), preimmune serum, or 4 μg of anti-PTP1B monoclonal antibody FG6. Washed immunocomplexes were assayed for PTP activity in triplicate with 3 μM [^{32}P]phosphotyrosine-containing reduced, carboxamidomethylated, and maleylated lysozyme as substrate (20). Measurements of total PTP activity used 1–2 μg of cell lysate protein per assay. Protein concentration was determined by the method of Bradford (21).

RESULTS

Isolation and Characterization of DEP-1 cDNA. A fragment encoding a segment of the catalytic domain of a putative PTP was identified by PCR amplification of phage DNA from a HeLa cell cDNA library. This PCR fragment was used to screen a HeLa cell cDNA library. A phage clone containing a 5.1-kb insert with an open reading frame of 4011 nt encoding a receptor-like PTP of 1337 aa was isolated (Fig. 1*A*). The proposed initiating ATG codon is flanked by a purine (G) at the −3 position and is thus in agreement with the Kozak rules for initiation (22). There is an in-frame stop codon ≈290 bp upstream of the predicted initiation site. The initiating methionine is followed by a hydrophobic sequence that may serve as a signal sequence. Based on the statistical analysis of known cleavage sites for the signal peptidase (23), the mature N terminus has been assigned to Gly^{37}. A second hydrophobic region is found between aa 977 and 996 and is followed by a series of predominantly basic residues, a feature characteristic of a stop-transfer sequence. Thus, a putative mature extracellular segment of 940 aa and an intracellular portion of 341 aa are predicted by the cDNA sequence (Fig. 1). The extracellular portion is largely composed of eight FNIII domains, whereas the intracellular segment contains a single PTP domain spanning aa 1060–1296 (Fig. 1). Thirty-four potential sites for N-linked glycosylation were identified in the extracellular segment. Sequence comparisons of the catalytic domain of DEP-1 with other PTPs revealed that the closest relationships are to PTPβ (5) and SAP-1 (6) (55% and 46% identity in the catalytic domains, respectively).

Transient Expression of DEP-1 in COS Cells. To study the protein product of the DEP-1 cDNA, the 5.1-kb *Eco*RI insert was cloned into the expression vector pMT2 (41) and the construct was transfected into COS cells. Affinity-purified anti-CSH-243 antibodies, raised against a C-terminal peptide of DEP-1, detected a protein of ≈180 kDa in lysates of DEP-1-transfected cells. This protein was not detected when antibodies had been preincubated with the peptide conjugate used for immunization (Fig. 2*A*). Treatment of transfected cells with tunicamycin reduced the apparent size of the DEP-1 immunoreactive species to 140 kDa, indicating that the 180-kDa form is glycosylated. Furthermore when immunocomplexes were assayed for PTP activity, ≥10-fold higher activity was detected in anti-CSH-243 immunocomplexes from the transfected cells than in the immunocomplexes from the untransfected cells (Fig. 2*B*). Most of this activity was absent in immunocomplexes derived from immunoprecipitations using antiserum preincubated with the peptide conjugate or preimmune serum. Thus, DEP-1 cDNA encodes a 180-kDa glycoprotein with intrinsic PTP activity.

Cell Density-Dependent Expression of Endogenous DEP-1 in WI-38 and AG1518 Cells. Northern and immunoblot analyses indicate that DEP-1 is broadly expressed (data not shown). Of those cell lines tested, WI-38 cells, a diploid fetal lung fibroblast-like cell line with finite lifespan, showed the highest level of protein expression (data not shown). DEP-1 was recovered from a membrane-enriched fraction of lysates of WI-38 cells as a protein of ≈180 kDa recognized specifically by the anti-peptide antiserum (data not shown).

There have been reports of increased PTP activity in lysates of cells from dense relative to sparse cell cultures (24). Therefore WI-38 cell lysates from sparse (<7000 cells per cm^2) or dense (>25,000 cells per cm^2) cultures were compared by immunoblotting for their content of DEP-1. DEP-1 expression was at least 10-fold higher in the dense cell cultures (Fig. 3*A*). Similar results were obtained with a second anti-peptide antiserum against DEP-1 (data not shown). These effects on DEP-1 expression were not restricted to WI-38 cells. Similar results were obtained in the human foreskin fibroblast cell line AG1518 (Fig. 3*A*). When the amount of PTP1B, a ubiquitously expressed cytoplasmic enzyme that is the major PTP in many tissues and cell lines, was compared in sparse and dense cells, no difference was observed (Fig. 3*B*). DEP-1 and PTP1B immunocomplexes, as well as total cell lysates, from sparse and dense cell cultures were also analyzed in PTP assays (Fig. 3*C*). In agreement with the findings from the immunoblotting experiments, DEP-1 activity was increased in the dense cell cultures. The observed increase in activity in DEP-1 immunoprecipitates was not as great as the increase observed by comparative immunoblotting of lysates of sparse and dense cell cultures, because we were unable to precipitate all of the DEP-1 with this antibody. No difference was observed in activity of PTP1B in immunoprecipitates or in total PTP activity in cell lysates from sparse and dense cell cultures (Fig. 3*C*).

Finally, to investigate the kinetics of the density-dependent up-regulation of DEP-1 expression, lysates of cells at intermediate cell densities were included in the analysis. The highest expression was found in cells at saturation density, but at intermediate densities an increase in expression relative to sparse cell cultures was also observed (Fig. 4). Thus, the up-regulation of DEP-1 is initiated before saturation density is reached and is therefore not solely a consequence of growth arrest.

DISCUSSION

Unlike most receptor PTPs, which possess a tandem repeat of two catalytic domains, DEP-1 contains only one PTP

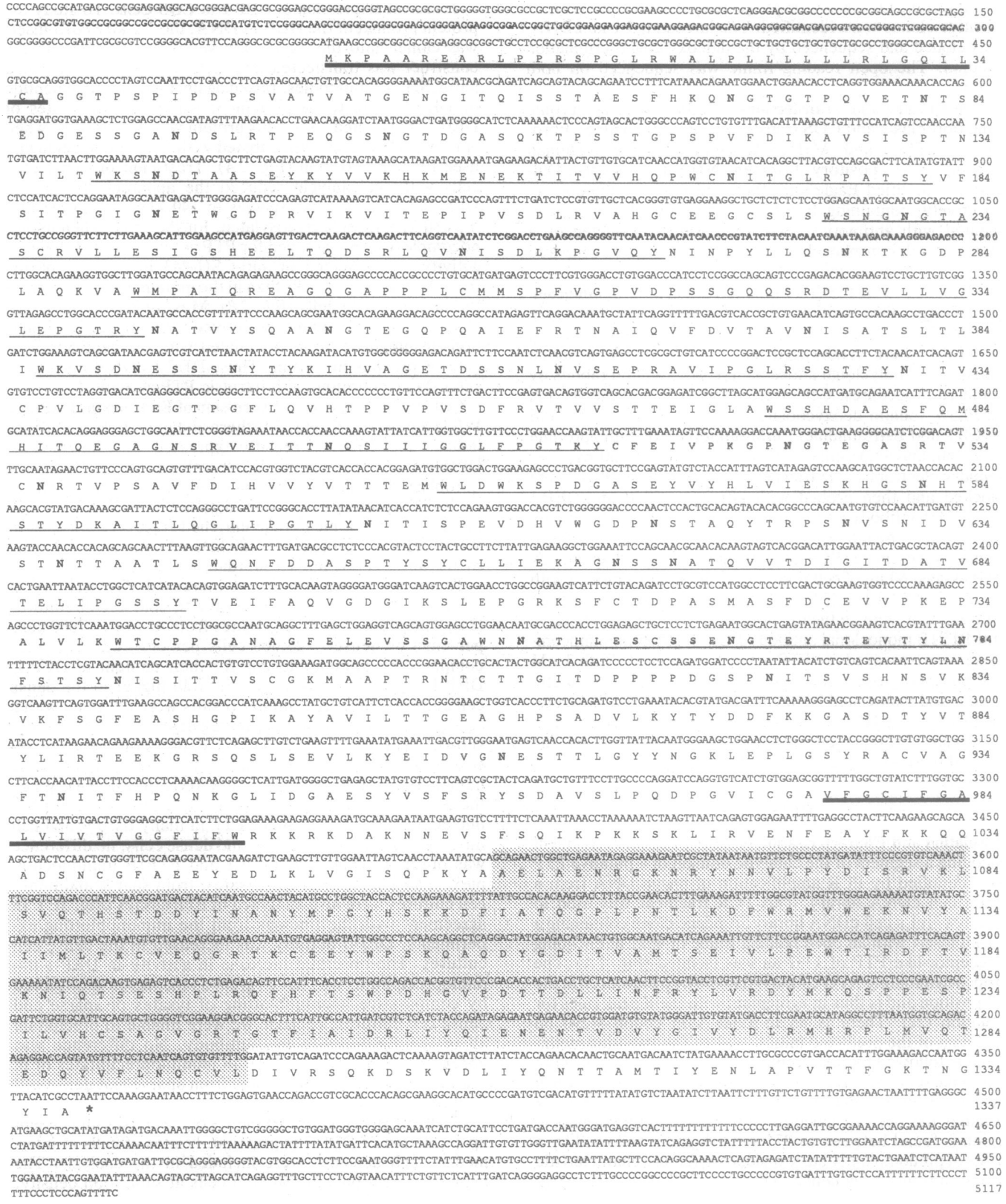

FIG. 1. **Nucleotide and predicted amino acid sequence of DEP-1. Shading defines the conserved catalytic domain that is homologous to other members of the PTP family. The putative signal and transmembrane sequences are underlined in bold. A segment of each predicted FNIII motif is presented as the intervening sequence between the invariant tryptophan and tyrosine residues found toward the N and C termini, respectively, of the hydrophobic core of the domain underlined. Potential asparagine (N) glycosylation sites are in bold type.**

domain in its intracellular segment. It therefore joins an expanding group of such receptors that includes PTPβ (5) and SAP-1 (6) from humans and DPTP10D (8, 9) and DPTP4E (10) from *Drosophila*. These enzymes also share a similar organization of their extracellular segments, which comprise a repeated array of multiple FNIII motifs. These would be expected to form a rod-like structure projecting from the surface of the cell (25).

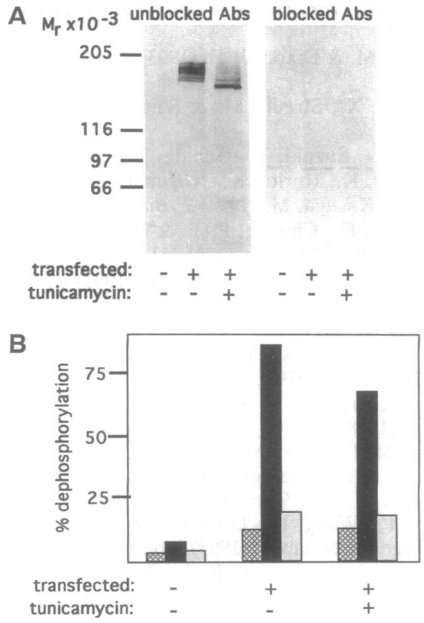

FIG. 2. Transient expression of DEP-1. COS cells were mock-transfected or transfected with pMT2-DEP-1 and treated with tunicamycin (0.2 μg/ml) or left untreated (*A*). Ten micrograms of cell lysate protein was analyzed by immunoblotting using affinity-purified anti-CSH-243 antibodies (Abs) in the absence (unblocked) or presence (blocked) of competing antigen. (*B*) For immunocomplex PTP assays, 60 μg of COS cell lysate was immunoprecipitated with preimmune serum (cross-hatched bars) or anti-CSH-243 antiserum in the absence (solid bars) or presence (stippled bars) of competing antigen. These data are representative of three such experiments.

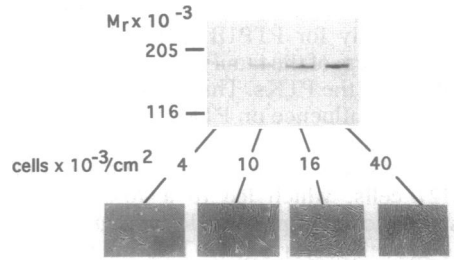

FIG. 4. Analysis of DEP-1 expression at various cell densities. WI-38 cells were harvested at the indicated cell densities and lysates (10 μg) were analyzed by immunoblotting with affinity-purified anti-CSH-243 antibodies. Photomicrographs of cell cultures at the various cell densities are shown below.

FNIII motifs were originally described in the extracellular matrix protein fibronectin and comprise ≈90 residues (26). The three-dimensional structure of two such domains has been determined and shown to fold with a topology similar to that of Ig domains (25, 27). It consists of seven β-strands forming a sandwich of two antiparallel β-sheets—one with three strands and the other with four. Comparison of the sequences of FNIII motifs illustrates several highly conserved residues, in particular hydrophobic residues, such as the invariant tryptophan and tyrosine (residues 22 and 68 respectively in the 10th FNIII motif of fibronectin) that lie at the N- and C-terminal ends of the core. In addition, there is a highly conserved loop linking β-strands E and F which has a consensus sequence GLXPG (25, 27). While these features are conserved in the predicted FNIII motifs of DEP-1, the sequence conservation following the invariant tyrosine, equivalent to residue 68 in the F strand of the 10th FNIII motif of fibronectin, is less pronounced. The greatest variation would be in the G strand, which contributes to the four-stranded sheet. While secondary-structure predictions suggest that some of the sequences following the invariant tyrosine residue of the predicted FNIII motifs of DEP-1 may form β-strands, the precise nature of the fold in these cases remains unclear. Nevertheless, with the exception of the third FNIII motif, there are sufficient residues to allow for domains of ≥85 residues.

FNIII motifs have been detected in >50 different eukaryotic proteins, both extracellular and intracellular, and have even been observed in prokaryotes (28). Most of these domains have no established function. However in fibronectin itself, which contains 15–17 such motifs, some roles have been ascribed (26). The best characterized interaction with fibronectin involves FNIII motif 10, which is the major site for cell adhesion. FNIII motif 10 contains the cell attachment-promoting sequence RGD, which promotes adhesion by binding to members of the integrin superfamily, a process that is augmented by interactions between heparin binding sites in fibronectin and cell surface proteoglycans. Although none of the FNIII motifs in DEP-1 contains an RGD sequence, which would normally be located on the loop between the F and G strands (25, 27), an XGD sequence is found on the expected loop in five of these motifs. It is possible that the extracellular segment of this PTP may participate in cell:cell interactions as a heterophilic adhesion molecule.

Great progress has been made in defining the mitogenic signaling pathways initiated by the binding of growth factors to their cognate receptor PTKs or triggered by the aberrant activation of oncogenic PTKs (29, 30). In contrast, the precise mechanisms underlying the inhibition of cell growth that occurs as normal cells approach confluence remain to be ascertained. The involvement of surface glycoproteins such as NCAM (31) and contactinhibin (32) has been proposed but their precise role is unclear. In light of the reversibility of tyrosine phosphorylation *in vivo*, the PTPs may function as natural antagonists of PTK action to keep the kinase activity in check and prevent uncontrolled growth and proliferation.

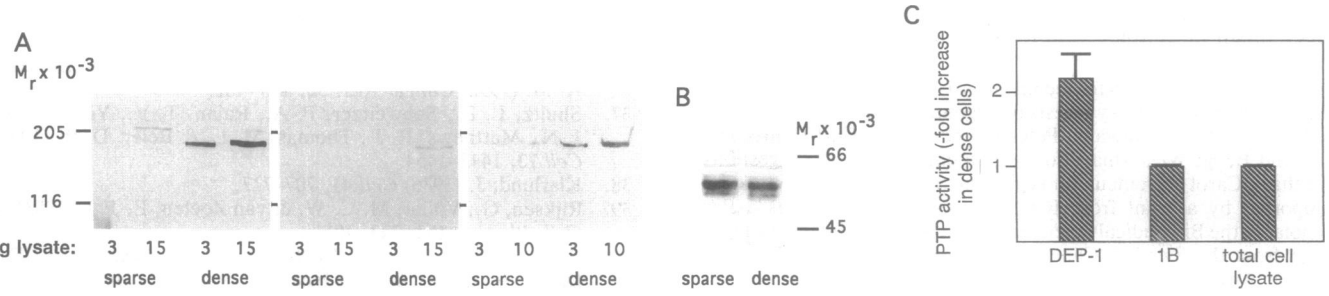

FIG. 3. Comparison of expression of DEP-1 and PTP1B in sparse and dense cells. (*A*) Indicated amounts of WI-38 (*Left* and *Center*) or AG1518 (*Right*) cell lysates from sparse or dense cultures were analyzed by immunoblotting with affinity-purified anti-CSH-243 antibodies in the absence (*Left* and *Right*) or presence (*Center*) of competing antigen. (*B*) Five micrograms of WI-38 cell lysates was analyzed by immunoblotting with the anti-PTP1B monoclonal antibody FG6. (*C*) Immunocomplex PTP assays of DEP-1 and PTP1B and total activity in cell lysates from sparse and dense cultures presented as mean ± SEM for 12 (DEP-1), 7 (PTP1B), and 8 (total cell lysate) determinations.

In fact, the specific activity of PTPs measured *in vitro*, as illustrated originally for PTP1B (33) and subsequently for many other members of the family, is 1–3 orders of magnitude in excess of that of the PTKs. This implies that the PTPs may exert a dominant influence on PTKs *in vivo*. The ability of a receptor PTP to counteract the growth-stimulatory potential of receptor PTKs was illustrated by expression of CD45 in murine C127 cells, which led to a 70% decrease in the mitogenesis induced by platelet-derived growth factor and insulin-like growth factor I (34). This inhibition correlated with a decrease in phosphotyrosine in a subset of proteins, including the receptor PTKs themselves, whose phosphorylation on tyrosine residues was induced by growth factor binding. Similarly overexpression of PTP1B antagonizes the oncogenic potential of v-Src (35) and Neu (36). Furthermore, mutations in the gene for HCP, a Src homology 2 domain-containing PTP, cause the severe immune dysfunction characteristic of the motheaten phenotype in mice (37). Under normal conditions HCP may act as a suppressor of PTK signaling pathways—e.g., the receptor for colony-stimulating factor 1.

PTPs have been directly linked to density-dependent arrest of cell growth. Treatment of NRK cells with vanadate, a potent inhibitor of PTPs, was able to overcome density-dependent growth inhibition and stimulate anchorage-independent proliferation, a hallmark of transformed cells (38, 39). This generation of a transformed morphology was accompanied by increased cellular phosphotyrosine (38). Based on data such as these, it has been suggested that some PTPs may be the products of tumor-suppressor genes—i.e., their deletion or mutation may contribute to the increase in phosphotyrosine that is associated with certain neoplasias. Mutations observed in the gene for RPTPγ in murine L cells would be consistent with this hypothesis (40). In 3T3 cells the activity of a PTP in the membrane fraction is enhanced 8-fold in cells harvested at high density compared with low- or medium-density cultures (24). This increase was not seen in subconfluent cultures brought to arrest by serum deprivation. No information was presented to indicate the identity of the PTP involved in this effect and whether the elevated activity was the result of changes in specific activity or expression levels. However its apparent size on gel filtration, 37 kDa, suggests that it may be derived from a larger enzyme by proteolysis.

In this paper we describe a receptor-like PTP, DEP-1, whose expression is induced in two distinct lines as cells approach confluence and come into contact with each other. Thus the enhanced expression of the phosphatase, possibly coupled with fine tuning of the activity through ligand binding to the extracellular segment, could be envisaged as promoting net dephosphorylation of proteins in the membrane, thus countering the effects of the growth-promoting PTKs. The mechanism by which this induction of DEP-1 expression is achieved remains unclear. However, the broad distribution of DEP-1 suggests that it may be involved in a general mechanism of contact inhibition of cell growth.

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