Elevation of membrane tyrosine phosphatase activity in density-dependent growth-arrested fibroblasts

(contact inhibition/cell proliferation/tyrosine dephosphorylation)

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ABSTRACT Swiss 3T3 cells harvested at high density contain a membrane protein-tyrosine-phosphatase (EC 3.1.3.48) whose specific activity is on average 8-fold higher than that of cells harvested at low or medium densities. Investigation of the conditions affecting this elevation of specific activity suggests that it is associated with density-dependent growth arrest. Fibroblasts in the exponentially doubling phase have a relatively low level of membrane phosphatase specific activity, which rises only as the rate of cell proliferation decreases and is maximal when cell growth is contact inhibited. These observations have been extended to BALB/c 3T3 fibroblasts and normal human diploid fibroblasts. The increase in membrane tyrosine phosphatase activity is coupled to density arrest and not to cellular quiescence in general, as no increase in phosphatase specific activity is detected when non-contact-inhibited cells are induced to arrest their growth through serum deprivation. The observed alterations in specific activity are attributable to a tyrosine phosphatase of Mw 37,000 that was purified and characterized from solubilized membrane fractions of Swiss 3T3 cells. A regulatory mechanism controlling tyrosine phosphatase activity may play a role in cell proliferation and growth arrest caused by cell contact.

Cellular phosphotyrosine levels are regulated by the relative activities of opposing protein-tyrosine kinases and phosphatases. The activation of receptor tyrosine kinases is mediated by interaction with their respective growth factors and usually induces mitogenesis and cell proliferation (1). In contrast, nothing is known about the in vivo regulation of protein-tyrosine-phosphatases (protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48). If these enzymes are involved in controlling tyrosine phosphorylation pathways, their activities, like those of the tyrosine kinases, should be modulated in response to specific cellular events.

Several protein-tyrosine-phosphatases have recently been identified by cDNA cloning and can be structurally classified as either nonreceptor phosphatases or putative receptor-linked enzymes (2-6). The finding that the extracellular domains of several receptor-like tyrosine phosphatases (LAR, DLAR, DPTP) are homologous to those of cell adhesion molecules such as neural cell adhesion molecule, fasciclin II, and L1 suggests that the interaction of like phosphatases on neighboring cells may serve as a signal to alter the catalytic activity of the intracellular portion of the phosphatase (2, 3). Streuli et al. (3) have suggested that this possible mechanism of receptor-like tyrosine phosphatase activation could counteract the effects of tyrosine kinases and thus play a role in the contact inhibition of cell growth. To test this hypothesis we examined whether tyrosine phosphatase activity is altered with respect to cell density. Here we report that contact-inhibited cells contain significantly higher levels of membrane-associated nonreceptor tyrosine phosphatase activity than do cells in the growth phase, and we propose that density-dependent inhibition of cell growth involves the regulated elevation of activity of this enzyme.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 cells (American Type Culture Collection CCL 92) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum (HyClone Laboratories). Cells were seeded at low density (3 x 10^4 cells per cm^2) on tissue culture dishes (Nunc) and grown to confluency with media changes every 2-3 days.

Preparation of Cytosolic and Particulate Extracts of Swiss 3T3 Cells. Cells (5 x 10^6) were washed three times with cold phosphate-buffered saline and harvested by scraping into buffer E (50 mM Hapes, pH 7.0/2 mM EDTA/2 mM EGTA/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/2 mM benzamidine/10 mM aprotinin per ml). After centrifugation (1000 x g, 10 min, 4°C) the cells were resuspended in 0.5 ml of buffer E, homogenized with 30 strokes in a Braun homogenizer, and centrifuged (100,000 x g, 30 min, 4°C) to obtain the cytosolic fraction. The pellet was resuspended in 0.4 ml of buffer E with 2% Triton X-100, incubated at 4°C for 1 hr, and centrifuged (100,000 x g, 30 min, 4°C) to obtain the solubilized membrane fraction. The protein concentration in the extracts was determined by the method of Bradford (7). Equivalent amounts of protein were obtained when equal numbers of cells were harvested at densities of 3 = 10^5 to 5 x 10^6 cells per cm^2.

Purification of the Tyrosine Phosphatase. Cytosolic (150 μg) and membrane (200 μg) proteins were diluted to a volume of 1 ml with buffer A (50 mM Tris-HCl, pH 7.5/20 mM 2-mercaptoethanol/0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) and loaded at a flow rate of 0.5 ml/min onto a Mono Q HR 5/5 column (Pharmacia) equilibrated in buffer A. Fractions were collected at 1-min intervals. The column was washed for 10 min with buffer A, and proteins were eluted with linear gradients of 0–0.5 M NaCl (30 min) and 0.5–1.0 M NaCl (5 min) followed by 1.0 M NaCl (5 min), all in buffer A. The peak fractions of activity obtained from ion-exchange chromatography of cytosolic or solubilized membrane extracts of confluent cells were applied to a Superose 12 column (Pharmacia) equilibrated and run (0.2 ml/min) in buffer A containing 0.2 M NaCl. The gel filtration column was calibrated with ferritin, aldolase, bovine serum albumin, ovalbumin, and chymotrypsin as molecular weight markers, and blue dextran and acetone were used to determine the void and total column volumes, respectively.

Assay of Tyrosine Phosphatase Activity. Portions of cytosolic and solubilized membrane extracts were assayed at 30°C

Abbreviations: CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; RR-src peptide, RRLIEDAEYARG.

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6996
in 250-µl reaction mixtures containing 50 mM 2-(N-morpholino)ethanesulfonic acid at pH 6.0, bovine serum albumin at 1 mg/ml, 0.5 mM dithiothreitol, 0.01% CHAPS, and 40 µM 32P-labeled RRLIAEYAARG (RR-src peptide) (8). Samples were removed at 2, 4, 6, and 10 min to verify that dephosphorylation was linear with respect to time and were processed as described (8) for determination of specific activity. Tyrosine phosphatase specific activity is expressed as pmol of phosphate released per min per mg of protein. Fractions (10 µl) from ion-exchange or gel-filtration columns were assayed as described above but for 30 min in a 50-µl reaction mixture containing 20 µM 32P-labeled RR-src peptide.

RESULTS

Effect of Cell Density on Tyrosine Phosphatase Specific Activity. Swiss 3T3 fibroblasts (5 × 10⁴ cells) were harvested at low, medium, and high cell density (Fig. 1 A–C). Cell lysates were prepared and separated into cytosolic and membrane fractions and assayed for tyrosine phosphatase activity towards RR-src peptide with the tyrosine residue phosphorylated. The tyrosine phosphatase specific activity of the cytosolic fraction of high-density cells was on average 2-fold higher than that of cytosolic fractions of low- or medium-density cells (Fig. 1D). The tyrosine phosphatase specific activity of membrane-associated fractions from high-density cells was on average 8-fold higher than that of the corresponding fractions of low- or medium-density cells, suggesting that a membrane phosphatase may be a major target for regulation under these conditions (Fig. 1E). Similar increases were also observed with BALB/c 3T3 and primary human skin fibroblasts.

Mixing experiments were carried out to check whether the increased specific activity of tyrosine phosphatase in fractions from high-density cells was an artifact of post-harvesting events. Swiss 3T3 cells were plated at low density and equal numbers were harvested at medium (6–8 × 10⁴ cells/cm²) or high (5 × 10⁴ cells/cm²) density. Cell lysates were diluted with buffer E to equalize protein concentrations and then mixed in various ratios (medium-density cell lysate to high-density cell lysate: 0:1, 1:3, 1:1, 3:1, and 1:0) and processed to obtain cytosolic or solubilized membrane fractions. When assayed, the phosphatase specific activities in the mixed fractions were additive —i.e., there was no enhancement or inhibition exerted by one part of the mixed sample upon the combined total (data not shown). This is as would be predicted if the respective phosphatase activities in the cells at different densities are determined by cellular rather than postlysis events.

Another possibility that could explain the observed elevation of tyrosine phosphatase specific activity in cells at high density is that we are selectively assaying only the releasable portion of phosphatase present and this portion could increase at high density. Aside from the cytosol and solubilized membrane fractions, there remains only the Triton X-100-insoluble membrane fraction as a source of additional activity. Tyrosine phosphatase activity has been reported to be associated with this latter fraction from placenta, and although resistant to Triton X-100, it can be released with KCl/CHAPS (9). Extraction by 0.6 M KCl/1.0% CHAPS was carried out on the Triton X-100-insoluble membrane fractions from cells at medium and high densities. The tyrosine phosphatase activity released by KCl/CHAPS treatment from the high-density cell material has about the same fold higher specific activity than that released from the medium-density cell material as does the Triton X-100-extractable phosphatase from these samples (data not shown). It therefore appears that the observed increase in membrane-associated phosphatase activity in high-density cells is not due to reduced solubility of the corresponding enzyme in membranes of cells at medium density.

Relationship of Tyrosine Phosphatase Activity to Cell Density and Growth Rate. The observed alterations in tyrosine phosphatase activity could reflect either the cell density or the rate of cell growth. To distinguish between these possibilities, fractionated lysates were made at different times from Swiss 3T3 cells seeded at low density and grown to confluency. Little to no changes in phosphatase specific activity were observed in cytosolic (data not shown) or membrane fractions during the first 3 1/2 days in culture, when the cells were doubling in number every 24 hr.
after, tyrosine phosphatase specific activity began to increase as the rate of cell division decreased (Fig. 2A). As cells approached confluence (5 1/2 days), cytosolic phosphatase specific activity was 1.5-fold higher (data not shown) and membrane-associated phosphatase specific activity was 5-fold higher than when the cells were in the growth phase over the first 3 1/2 days of culture. At confluence (7 1/2 days), cytosolic specific activity had increased 2-fold (data not shown), while a 9-fold increase in membrane tyrosine phosphatase specific activity was observed. These elevated specific activities were maintained for at least another 2 days after confluency. Since confluent cultures of fibroblasts were used to seed the plates at low cell density, tyrosine phosphatase activity can also be rapidly altered in the opposite manner, that is, changing from high to low within 12 hr. The phosphatase specific activity rises as the rate of cell division falls (Fig. 2A) and is not directly proportional to increasing cell density (Fig. 2B). Instead, a sharp increase in membrane phosphatase specific activity occurs when the fibroblasts become contact inhibited at a density of $5 \times 10^4$ cells per cm$^2$ (Fig. 2B).

**Effect of Cell Quiescence on Tyrosine Phosphatase Specific Activity.** The results above suggest that the increase in tyrosine phosphatase specific activity is associated with density-dependent growth arrest and/or reduced rates of cell proliferation. To determine whether a similar rise in phosphatase specific activity occurs in cells induced to arrest their growth by conditions other than contact inhibition, Swiss 3T3 fibroblasts were induced to become quiescent by changing the media on logarithmically growing cells (3 1/2-day culture) to media containing low serum (0.5%). The membrane-associated phosphatase activity was then assayed every 24 hr over a further 5-day period. Cell number and density remained unchanged and no sustained increase in phosphatase specific activity was detected compared with a 6-fold increase in specific activity in control cells maintained with 10% serum (Fig. 3). Thus, the elevated tyrosine phosphatase specific activity in density-dependent growth-arrested cells is not generally associated with a decrease in the rate of cell proliferation or with all types of growth arrest.

**Purification and Characterization of the Tyrosine Phosphatase.** To characterize the phosphatase responsible for this activity, cytosolic and solubilized membrane fractions were fractionated by fast protein liquid chromatography on Mono Q. A single peak of phosphatase activity was detected in cytosolic and membrane-derived samples from lag, doubling, and confluent cells (Fig. 4). The increase in peak phosphatase activity from confluent cells corresponds to that measured in the original cytosolic and membrane fractions. The elution position of this activity did not vary with the type of sample

![Graph A](image1.png)

**Fig. 2.** Relationship of membrane tyrosine phosphatase specific activity to the rate of cell growth and to cell density. The logarithmic scales are base 10. (A) Cells were seeded at low density and grown to confluence. At the times indicated, cells were harvested and membrane-associated tyrosine phosphatase specific activity was determined. Data are from one experiment and all values were determined in duplicate. (B) As in A. Each point represents at least a duplicate determination from one experiment. The points are from three independently conducted experiments.
Fig. 3. Effect of low serum concentration on cell growth and membrane-associated tyrosine phosphatase specific activity. The experiment was carried out as described in the legend to Fig. 2. At 3½ days (arrow) the serum in the medium was reduced from 10% to 0.5% (●). For comparison, a parallel set of cells was maintained in medium with 10% serum (○). Membrane-associated tyrosine phosphatase specific activity was determined in fractions prepared at the times indicated from cells switched at 3½ days to medium with 0.5% serum (■) or maintained in 10% serum (○).

Fig. 4. Ion-exchange chromatography of cytosolic (A–C) and membrane (D–F) fractions from cells harvested at different phases of growth. Tyrosine phosphatase activity profiles are shown for fractions from cells harvested at lag phase (A and D), exponentially doubling phase (B and E), and confluency (C and F). Cells were seeded at low density (3.1 \times 10^3 cells per cm^2) and equal numbers of cells were harvested in the lag phase (12 hr after plating), in the exponentially doubling phase (42 hr after plating), and at confluency (7½ days after plating).
DISCUSSION

Little is known about cellular events or conditions that may contribute to alterations in tyrosine phosphorylation. These enzymes have been implicated in several key processes such as the regulation of the cell cycle through action on the mitotic control element M





phosphatase. The apparent molecular weight of this phosphatase was estimated by gel filtration chromatography to be 37,000.

This increase in phosphatase specific activity is not simply related to increasing cell-cell contact, since the specific activity of this membrane enzyme remains relatively low during the exponential phase of growth when cell density is rapidly rising. Activity begins to increase only as the rate of cell proliferation decreases in cells approaching saturation density, and it is highest when cell growth is arrested at confluence (Fig. 2). However, an elevated tyrosine phosphatase activity is not invariably associated with decreased rates of cell proliferation and quiescence, as no such increase is detected when subconfluent cells in the doubling phase are grown arrested by serum depletion (Fig. 3). This is not surprising because growth arrest due to removal of mitogenic stimuli involves events distinct from those occurring to negatively regulate the growth of confluent cultures in the presence of serum. The observed time course and conditions under which we observe elevation of phosphatase activity suggest the existence of a regulatory mechanism that maintains membrane tyrosine phosphatase activity at basal levels during cell proliferation and increases this activity under certain conditions of growth arrest. This is consistent with the observation of Karelund (17) that the tyrosine phosphatase inhibitor vanadate has no effect on cell growth rate during the exponential phase of growth but can induce cells to overcome contact inhibition. The potential ability of tyrosine phosphatases to reverse the cellular activities and effects of growth factor receptor and oncogene tyrosine kinases identifies these phosphatases as candidate antioncogenes and inhibitors of cell growth. An increase in tyrosine phosphatase activity such as we have described could alter the balance between tyrosine phosphorylation and dephosphorylation reactions in favor of the latter and thus may represent an event that contributes to the negative regulation of cell growth.

Fractionation of solubilized membrane extracts of Swiss 3T3 cells shows that an enzyme of Mr ~37,000 manifests the elevated tyrosine phosphatase activity of density-arrested cells. A soluble form of this phosphatase is also present in cytosolic extracts (Fig. 4). While it is possible that receptor-like tyrosine phosphatases with homology to cell adhesion molecules are involved in cell-cell signaling and contact inhibition of cell growth (the original hypothesis we set out to investigate), the size of the membrane phosphatase and the existence of a soluble counterpart suggest that the enzyme described belongs to a family of low-molecular-weight non-receptor tyrosine phosphatases. Hence, it is unlikely that this phosphatase itself generates a transmembrane signal. Several particulate nonreceptor tyrosine phosphatases in this molecular weight range have been reported (9, 18, 19), and certain of these enzymes are also found to have soluble forms. A clear difference exists between the membrane-associated and soluble forms of the Mr 37,000 phosphatase, since in density-arrested fibroblasts the membrane enzyme undergoes a significant 8-fold increase in specific activity, whereas the soluble enzyme increases only about 2-fold. Thus the membrane-associated phosphatase may be a particular target for regulation under these conditions. It is attractive to speculate that the nonreceptor tyrosine phosphatases could be activated by the binding of extracellular factors to cell surface molecules. A possible pathway for such modulation is exemplified by the association of the nonreceptor tyrosine kinase lck with the intracellular portion of the transmembrane T-cell surface antigen CD4. CD4 interacts with class II major histocompatibility complex determinants expressed on antigen-presenting cells and transduces an intracellular signal that may be mediated by the linked tyrosine kinase (20–23). Whether the increased activity of this tyrosine phosphatase in confluent cells is due to activation of existing molecules or to increased synthesis of new enzyme requires further investigation. Elucidation of the regulatory controls governing tyrosine phosphatase activity will provide insight into the mechanisms of cell proliferation and transformation.

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