Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 154-161

EXPRESSION OF PTP35, THE MURINE HOMOLOGUE OF THE PROTEIN TYROSINE PHOSPHATASE-RELATED SEQUENCE IA-2, IS REGULATED DURING CELL GROWTH AND STIMULATED BY MITOGENS IN 3T3 FIBROBLASTS

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Received October 17, 1995

Summary: Protein tyrosine phosphatases (PTPases) have been implicated in the control of cell proliferation and differentiation. To isolate new members of this family potentially involved in cell growth regulation, we looked for PTPase sequences differently expressed in proliferating or quiescent NIH 3T3 fibroblasts. The full-length cDNA of one of these growth-regulated genes, named PTP35, was isolated from a 3T3 library and found to encode the murine IA-2 PTPase-related sequence. Endogenous PTP35 mRNA steady-state levels were found to be strictly regulated during cell growth in 3T3 fibroblasts, being high in actively cycling cells and barely detectable in density-arrested cells. Both PTP35 mRNA and protein levels could be induced in quiescent cells by mitogenic stimulation. The growth factor specificity and kinetics of this induction were analyzed in detail.

Protein Tyrosine Phosphatases (PTPases) are implicated in the positive as well as negative regulation of cell growth and differentiation by dephosphorylating specific substrates involved in the tyrosine kinase signal transduction pathways (1,2). As protein tyrosine phosphorylation represents a crucial event in normal cell growth, which must be strictly controlled to avoid abnormal proliferation leading to cell transformation, it could be expected that the activity and abundance of PTPases should be modulated in response to mitogens or treatments affecting the cell proliferative conditions. Accordingly, regulation at the mRNA level in different cell proliferative conditions has been reported for several PTPases and suggested to reflect an involvement of the correspondent enzymes with cell cycling, contact inhibition and development (3-11).

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Abbreviations: PTPase, protein tyrosine phosphatase; PCR, Polymerase Chain Reaction; bFGF, basic fibroblast growth factor; PDGF, platelet derived growth factor; EGF, epidermal growth factor; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

0006-291X/95 $12.00

With the purpose of isolating new members of the PTPase family potentially involved in the control of cell proliferation, we looked for expression-regulated genes in 3T3 fibroblasts, a model system for the study of cell-proliferation related genes (12). Several PTPase clones were isolated by PCR and tested for mRNA expression in growing and resting 3T3 cells. One of the growth-regulated genes, which we named PTP35, was obtained as a full-lenght clone from a 3T3 cDNA library and found to represent the murine homologue of the PTPase-related IA-2 gene (13-17). Here we analyze its regulation in fibroblasts, in which both the PTP35 mRNA and protein levels are greatly modulated during cell growth.

Materials and methods

Reagents: DMEM, FCS, gentamicin and nystatin were from GIBCO/BRL, recombinant human bFGF was produced in Pharmacia, Italy; recombinant human PDGF B/B was from Boehringer; recombinant human EGF was from Genzyme.

Polymerase Chain Reaction amplification of PTPase sequences: Total RNA from 3T3 fibroblasts was reverse-transcribed and amplified by PCR with Taq polymerase (Perkin-Elmer) and two primers encoding respectively the PTPase-conserved amino acids FWEMVWE or VHCSAG. The amplification products of about 360 bp were subcloned into vector pTZ19R (Pharmacia) and the recombinant plasmids were analyzed by restriction mapping and sequenced using the dideoxynucleotide chain termination method and Sequenase (United States Biochemical).

Screening of the 3T3 cell cDNA library: An NIH 3T3 fibroblasts cDNA Lambda Zap II library (Stratagene, catalogue n° 936305) was screened by overnight hybridization at 65 °C in 5 X SSC, 5 X Denhardt's, 0.1% SDS, 100 mg/ml salmon sperm DNA, using the PTP35 PCR-derived SalI-EcoRI fragment ^{32}P-radiolabeled with the Multiprime DNA Labelling System (Amersham). Washing was performed at 65 °C in 1 X SSC, 0.1% SDS., The pBluescript SK plasmids were excised from the Lambda Zap II positive clones as described by Stratagene and sequenced. Sequence analysis and databank searching were performed with Geneworks (Intelligenetics, CA) software packages.

Northern analysis: Northern blots were performed using total RNA from proliferating or quiescent cells and the PTP35 PCR-derived SalI-EcoRI fragment ^{32}P-labelled with Multiprime DNA Labelling System (Amersham) as a probe. . Hybridization was performed overnight at 42° in 5 X SSC, 1 X Denhardt, 0.1% SDS, 50% formamide, 250 mg/ml salmon sperm. Washing was performed in 0.2 X SSC, 0.1 % SDS. Equal loading of all the lanes was verified by filter rehibridization with a mouse β-actin or a human GAPDH probe.

Cell culture and treatment: NIH 3T3 fibroblasts (ATCC CRL 1658) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) foetal calf serum (FCS), nystatin (10000 U/l) and gentamicin (50 μg/l). For Northern analysis during cell growth, 3T3 cells were seeded at low density (10^3 cells per cm^2) and grown to confluence (4 x 10^4 cells per cm^2) with media changes every other day. Each day, cells were trypsinized and counted or harvested for RNA preparation. For analysis of mRNA induction, 3T3 cells were seeded at semiconfluence (3 x 10^4 cells per cm^2) in DMEM + 10% FCS and then incubated in DMEM + 0.4 % FCS for 24 h. Quiescent cells were then treated with 30 ng/ml of bFGF, PDGF B/B , EGF or FCS (10% v/v) in the same medium and harvested at the indicated time points.

Protein analysis: bFGF-treated fibroblasts from a 100 mm dish were lysed with 900 ml of lysis buffer (25 mM Hepes pH 7.4, 10% glycerol, 5 mM $MgCl_2$, 50 mM NaF, 2 mM PMSF, 1 mM EGTA, 100 mM NaCl, 40 μg/ml benzamidin, 1% Triton X-100) and submitted to immunoprecipitation followed by SDS-PAGE and immunoblotting according to standard procedures, using anti-PTP35 antibodies obtained against the recombinant PTP35 intracellular domain.

Results

Degenerated primers corresponding to conserved sequences in the PTPase catalytic domains were used to amplify total RNA from NIH 3T3 cells by PCR. Expression of several of these PTPase-related sequences was analyzed by Northern blot hybridization with RNA from actively growing or density-arrested 3T3 cells, looking for growth-regulated genes. One of these sequences, which we named PTP35, hybridized to an mRNA of about 3.8 kb which could be detected only in exponentially growing cells (Fig. 1). None of the other 3T3-derived PTPase sequences including PTP1B (18), which is shown in Figure 1 as a negative control, were found to be so strictly regulated at the expression level during cell proliferation. To isolate a complete PTP35 cDNA, an NIH 3T3 cells library was screened using the PCR fragment as a probe, obtaining a full-length sequence encoding a transmembrane protein with a single intracellular domain homologous to PTPases. Search in GenBank data base revealed full length homology of the PTP35 cDNA to the PTPase-related IA-2 sequence, which was considered so far a tissue-specific gene expressed only in pancreas and brain (13-17).

Since PTP35 was selected because it was expressed only in proliferating cells, the regulation of PTP35 mRNA expression during growth of 3T3 cells was analyzed in a time-course experiment (Fig. 2). Northern blot analysis of mRNA steady-state levels obtained from cells at different days of culture showed that PTP35 expression was strictly correlated with cell growth. Maximum levels were observed in exponentially growing cells (days 1 to 4) and decreased until little or no expression was observed in confluent, quiescent cells. A similar pattern of PTP35 growth-regulated expression was also observed in freshly isolated rat smooth muscle cells (data

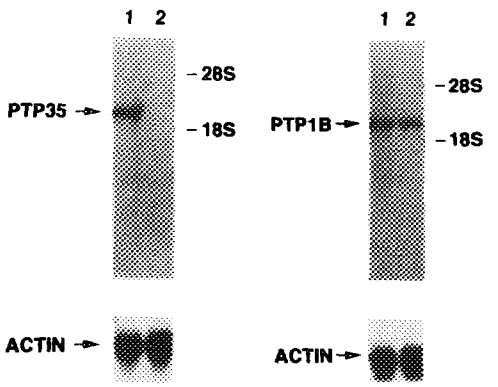

Figure 1. *Expression of PTP35 and PTP1B mRNA in growing or quiescent NIH 3T3 cells.* Northern blot hybridization of total RNA from NIH 3T3 fibroblasts grown to about 50% confluence (4 X 10^3 cells/cm^2) (lane 1) or to 100% confluence (4 X 10^4 cells/cm^2) (lane 2). Hybridization was performed in parallel with a PCR-derived probe for PTP35 or PTP1B. Equal loading of the lanes was verified by rehibridization of the filters with an actin probe.

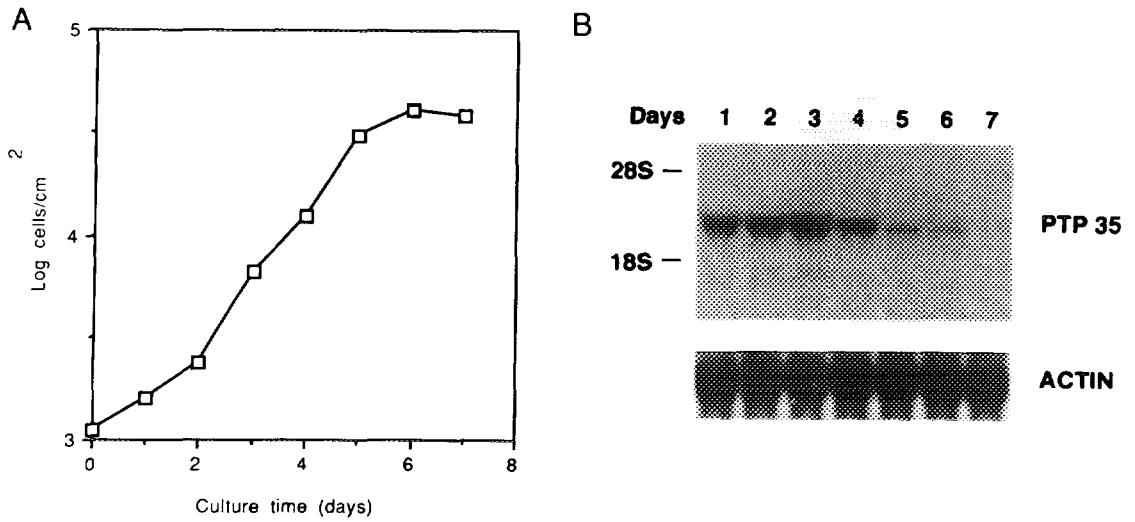

Figure 2. (A) *Growth curve of NIH 3T3 cells.* 3T3 cells were seeded at 10^3 cells/cm^2 and cultured in DMEM medium supplemented with 10% FCS. Each day, cells were trypsinized and counted. (B) *Time-course of PTP35 mRNA levels during cell growth.* 20 μg of total RNA from cells harvested at each day of culture were analyzed by Northern blot hybridization. Equal loading of all lanes was verified by rehybridization with an actin probe.

not shown). When we used spontaneously transformed 3T3 fibroblasts, which had lost contact inhibition, expression of PTP35 mRNA was observed also in overconfluent cultures, probably as a consequence of their inability to reach complete quiescency (data not shown).

To verify if mitogenic stimulation could induce expression of PTP35 mRNA in quiescent cells, 3T3 fibroblasts were plated to confluency, serum starved for 24 hours and treated for the indicated time with different mitogenic stimuli. Northern blot analysis of total RNA revealed that while all the factors tested were able to induce *c-fos* mRNA in quiescent 3T3 cells after 30', only basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF) and foetal calf serum (FCS) were able to induce expression of PTP35 mRNA (Fig. 3). bFGF was repeatedly found to induce the biggest and more sustained effect, while FCS had a weaker effect which declined more rapidly. Epidermal Growth Factor (EGF) and insulin, alone or in combination, were not able to produce this effect, at least in comparable doses. These findings may be explained by differences in the potency of the mitogens tested or in the activation of specific intracellular signalling pathways.

The kinetics of PTP35 mRNA induction following bFGF treatment were then analyzed in a time-course experiment using 3T3 cells synchronized by a 24 hours serum starvation. As shown in Figure 4A, PTP35 mRNA steady-state levels were dramatically increased after bFGF stimulation and reached a peak 24 hours after treatment. The low levels of PTP35 mRNA observed in the

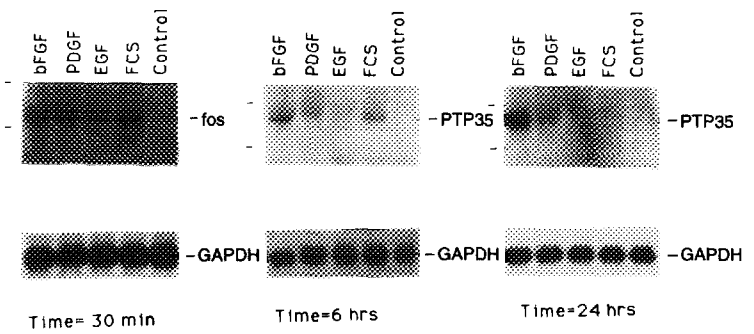

Figure 3. *Expression of PTP35 mRNA after NIH 3T3 stimulation with different mitogens.* Quiescent NIH 3T3 cells were serum starved for 24 hours and then treated with 30 ng/ml of bFGF, PDGF, EGF or with 10% fetal calf serum. At 30 minutes, 6 hrs or 24 hrs after treatment, cells were lysed, total RNA was prepared and analyzed in Northern blot, using a *c-fos* or a PTP35 probe, as indicated. To verify equal loading of all lanes, the same filters were rehybridized with a GAPDH probe.

untreated cells at early times were not reproducibly observed in most replicate experiments, PTP35 expression in serum starved cells being generally undetectable by Northern hybridization.

To study the effect of the mRNA regulation on the PTP35 protein levels, we immunoprecipitated total lysates of cells stimulated with bFGF using affinity-purified anti-PTP35 antibodies produced against the recombinant intracellular domain of the protein expressed in *E. coli*. As shown in Figure 4B, a protein of about 105 kDa, corresponding to the predicted size of the PTP35 protein, could be immunoprecipitated from 3T3 cell lysates using these antibodies. The protein was not detected when the immunoprecipitation was performed in the presence of an excess recombinant PTP35 intracellular domain, or when the preimmune serum was used (Data not shown). Its apparent size compared with the expected protein molecular weight would suggest that this protein is not extensively glycosylated *in vivo* in 3T3 cells. As shown in fig. 4B, the PTP35 protein is barely detectable in quiescent cells but is induced after bFGF treatment, reaching a maximum at 24 hours and staying high for several hours thereafter. The steady-state levels of the PTP35 protein following bFGF treatment are in agreement with the pattern of mRNA induction shown in Figure 4A, suggesting that the dramatic changes observed at the mRNA level regulate the availability of the PTP35 protein.

Discussion

Several reports demonstrate that PTPases play an important role both in the positive and negative regulation of cell growth (19-24), suggesting that elucidation of the mechanisms modulating the activity of specific PTPases will provide fundamental insight into the cell proliferation and transformation pathways.

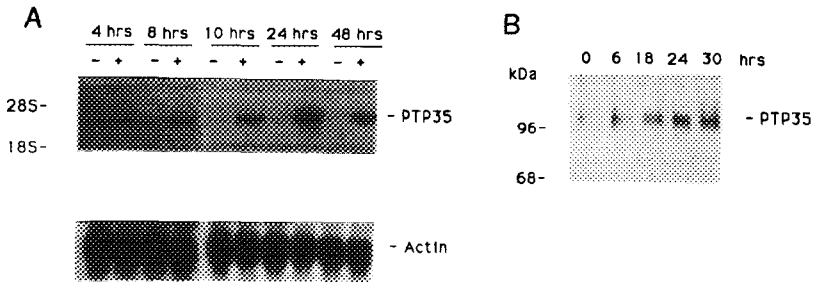

Figure 4. *Kinetic analysis of PTP35 mRNA and protein induction in 3T3 cells after mitogenic stimulation with bFGF.* (A) Northern blot time-course analysis of total RNA from cells untreated (-) or stimulated with 30 ng/ml of bFGF (+) at different times after treatment. Approximately 20 μg of total cellular RNA per lane was used. Equal loading of all lanes was verified by rehybridization with an actin probe (lower panel). (B) Western blot kinetic analysis of PTP35 protein expression after bFGF treatment. Cells lysates were prepared from the stimulated 3T3 cells at different times and equal amount of proteins were immunoprecipitated with affinity-purified anti-PTP35 antibodies. The immunoprecipitated samples were loaded on a 7,5% SDS-polyacrilamide gel, blotted onto nitrocellulose, incubated with the same antibodies and subsequently with a peroxidase-conjugated second antibody.

With an approach aimed at the isolation of new PTPase genes involved in the regulation of cell growth we cloned a PTPase-related cDNA, which we called PTP35, representing the murine homologue of the IA-2 sequence (13,14). Interestingly, this gene has a very restricted pattern of expression in adult tissues, mainly limited to brain and pancreas (13), where it was very recently identified as a major Insulin Dependent Diabetes Mellitus autoantigen (17). Here we provide the evidence that this gene is also very abundantly expressed in mouse fibroblasts and rat smooth muscle cells, which are not of neuroectodermal origin. Expression in these cell types could explain the weak signal observed by Northern analysis with the IA-2 probe in some murine gastrointestinal tissues (14). Moreover, we show that both its mRNA and protein levels are tightly regulated during cell proliferation and are induced by stimulation with different mitogens.

Previous examples of PTPase regulation during cell growth include the mRPTP-s and DEP1 PTPases, whose mRNA levels are increased at high cell density or by serum starvation, suggesting that they may be involved in contact inhibition and establishment of cell quiescence (3,4). On the other side, serum and mitogenic stimuli have been shown to induce a family of PTPases representing early response genes, including the murine 3CH134/ERP gene and its human homologue CL100 (5-9). The latter have been demonstrated to be responsible for specific tyrosine dephosphorylation and inhibition of the MAP kinase, suggesting that the mRNA levels increase following mitogenic stimuli reflects the need for the timely presence of a negative regulator of cell growth (10,11). Based on the kinetics of its induction, PTP35 is not an early gene, being maximally expressed in the late phase of the cell cycle. Since the peak of DNA

synthesis following treatment of 3T3 cells with bFGF is centered around 18-20 hours (A. Isacchi, unpublished results), cells maximally expressing PTP35 should be in the late S-G2 phase. This rather unusual timing of induction, which has not been reported for other PTPases, may reflect the existence of a specific ligand or substrate which is also present only at particular times, depending on the cell proliferative state. A full understanding of the PTP35 biological role is hampered by the observation that despite its high homology (35-40 %) to PTPases it does not seem to possess intrinsic activity, probably due to a few specific amino acid changes in conserved residues (14). While the lack of activity observed so far with recombinant proteins could be related to incorrect folding in *E. coli* or to the necessity of post-translational modifications, we tested the endogenous PTP35 protein immunoprecipitated from 3T3 cells in an in vitro PTPase assay, using ^{32}P-labeled myelin basic protein as a substrate, without finding any specific catalytic activity associated with the PTP35 protein (A. Isacchi, unpublished results).

Although its biological function has not been yet fully understood, an inhibitory role has been proposed for the rat homologue of the PTP35 protein, suggesting that it may interfere with the action of active PTPases by competing with a common substrate (16). Expression of a catalitically inactive Syp phosphatase in 3T3 cells has been reported to block insulin action by competing with its endogenous counterpart (25), and a similar competitive mechanism has been proposed to explain the existence and biological significance of catalitically inert kinases (26, 27). Similarly, PTP35 may function *in vivo* by signal attenuation and its specificity of action could be regulated by interactions with its extracellular domain, by extreme selectivity in substrate binding or by its regulated availability during the cell cycle, which may reflect an involvement in specific proliferative events. More experiments will be needed to confirm this hypothesis, including the analysis of the growth features of stable 3T3 clones overexpressing the PTP35 protein in a countinuous, deregulated manner throughout the cell cycle.

In pancreas, use of monoclonal islet-cell-specific antibodies has shown quantitatively changed antigen expression depending on the functional state of the β-cell (28,29), while delayed expression of an antigene during pancreatic β-cells development has been shown to cause a failure to establish self-tolerance, resulting in the production of autoimmune lesions in the pancreatic islets (30,31). Although we have not examined the regulation of expression of PTP35 in pancreas, its recognition as an IDDM autoantigen (15,17) might also be related to modulation of its expression, for instance during pancreas development.

Acknowledgments

We thank Dr. Paola Gnocchi and Dr. Annamaria Isetta for the production of polyclonal antibodies against PTP35, and Jan Malisko for the oligonucleotides synthesis.

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