

Cytokinetic failure and asynchronous nuclear division in BHK cells overexpressing a truncated protein-tyrosine-phosphatase

(cell cycle/cytokinesis/syncytia/mitosis)

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ABSTRACT Previous work has shown that a T-cell protein-tyrosine-phosphatase truncated in its carboxyl-terminal domain (Δ C11.PTP) has full enzymatic activity but no longer localizes in the particulate fraction of the cell. Two baby hamster kidney (BHK) cell lines overexpressing the truncated protein are markedly multinucleate, a state likely caused by a failure in cytokinesis. Nuclei within syncytial cells overexpressing Δ C11.PTP display a remarkable asynchronous entry into mitosis. The effects require tyrosine phosphatase activity because expression of an inactive form of the truncated enzyme yields cells indistinguishable from the parental cell line. Redistribution of the enzyme from the particulate to the soluble fraction is apparently important to these observed effects because cells overexpressing the full-length, wild-type enzyme are morphologically similar to controls. Further, when these cells contain more than one nucleus, their syncytial nuclei undergo mitosis synchronously.

Protein-tyrosine-phosphatases (PTPs) represent a diverse family of enzymes entirely specific toward phosphotyrosyl residues. Both transmembrane, receptor-like PTPs and intracellular forms have been identified, and their primary structures have been determined mostly from cDNA sequence analyses (for reviews, see refs. 1-3). Little is known as to their precise roles in cell physiology, although the leukocyte common antigen CD45, a hematopoietic-specific 180- to 220-kDa receptor, was shown to be essential for coupling the T-cell receptor to the phosphatidylinositol-turnover pathway (4). CD45 is also required for *in vivo* tyrosine phosphorylation after T-cell activation (5).

The intracellular PTPs, varying from 40 to 120 kDa, that have been identified contain large regulatory regions extending from the catalytic core toward either the amino- or carboxyl-terminal ends of the molecule. A 48-kDa human T-cell enzyme (TC.PTP) displays a carboxyl-terminal extension of \approx 11 kDa that is responsible for its localization (6) and determines its specificity toward certain artificial substrates (7). Thus, the full-length TC.PTP localizes mainly in the particulate fraction when expressed in baby hamster kidney (BHK) cells and requires limited proteolysis for *in vitro* activity toward reduced, carboxamidomethylated, and maleylated lysozyme (7). By contrast, a 37-kDa carboxyl-terminal-truncated form of TC.PTP (designated Δ C11.PTP) localizes in the aqueous phase and is constitutively active toward most substrates *in vitro* (7).

In this study we report that while BHK cells overexpressing the wild-type TC.PTP exhibit a normal phenotype in terms of growth and morphology, clonal cell lines expressing the carboxyl-terminal truncated enzyme become highly multinucleate, apparently through a failure in cytokinesis. The data show that cytokinesis often fails in Δ C11.PTP-

containing cells because furrowing cannot be completed. Cells with syncytial nuclei exist in nature (8, 9), in certain pathological conditions (10), and have been generated by cell fusion (11, 12). Multinucleation can also be induced by cytochalasin through a failure to maintain the cytokinetic furrow (13). In all previous cases, the syncytial nuclei have entered S-phase and mitosis synchronously, indicating that S- and M-phase nuclei express dominant signals. Unexpectedly, nuclear division in syncytial BHK cells expressing Δ C11.PTP occurs asynchronously. These data suggest that tyrosine phosphorylation is involved, directly or indirectly, in both furrow development and the signaling events that coordinate nuclear division.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of TC.PTP in BHK Cells. Construction and transfection of the vector containing either TC.PTP or Δ C11.PTP cDNA were as described (6). An oligonucleotide (5'-GTGATCCACTCGAGTGCAGGCA-3') designed to abolish catalytic activity (Cys-175 \rightarrow Ser) was synthesized on an Applied Biosystems DNA synthesizer (Howard Hughes Medical Institute DNA synthesis facility) and used to prime an M13 single-stranded DNA template containing TC.PTP cDNA (6). The methods for preparing and isolating the mutant cDNA have been described elsewhere (6, 7). The mutation in the cDNA was verified by DNA sequence analysis of the region surrounding the mutation site and found to contain the correct point mutation as well as a premature stop codon located 18 base pairs (bp) after Gly-327. A 1.0-kilobase-pair (kbp) *Xba* I fragment containing the mutant cDNA was extracted from an agarose gel and ligated into the expression vector as described (6).

Immunofluorescence Microscopy. Cells were grown on polylysine-coated glass coverslips for a minimum of 12 hr, fixed, and permeabilized as described (13). Cells prepared for immunolocalization of proliferating cell nuclear antigen (PCNA) were fixed according to ref. 14 by using 1% paraformaldehyde/phosphate-buffered saline (PBS) for 2 min, followed by -20°C methanol for 10 min and treatment with 0.5% Nonidet P-40 in PBS for 2 min. Washes, incubation with antibodies, staining with propidium iodide, and mounting of coverslips were as described (13).

Mouse anti- β tubulin (Eastacres Biologicals, Southbridge, MA), mouse anti-PCNA (19F4 ascites, Coulter) primary antibodies, and anti-telophase disc serum (13) were used at dilutions of 1:50, 1:200, and 1:500, respectively. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and fluores-

Abbreviations: DCB, dihydrocytochalasin B; PCNA, proliferating cell nuclear antigen; PTP, protein-tyrosine-phosphatase; DiOC₁₈, 3,3'-dioctadecyloxycarbocyanine perchlorate.

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cein isothiocyanate-conjugated goat anti-human IgG secondary antibodies (Tago) were applied to cells at 8.5 $\mu\text{g}/\text{ml}$.

Membranes were stained with an intercalating fluorescent membrane lipid, 3,3'-dioctadecyloxycarbocyanine perchlorate (DiOC₁₈; Molecular Probes) at 100 $\mu\text{g}/\text{ml}$, directly after fixation. Staining was done for 30 min at 37°C in PBS/3% bovine serum albumin/0.05% Tween 20/0.1% sodium azide, followed by a PBS wash, permeabilization, and counterstain with propidium iodide (13).

All samples were observed by using an MRC-500 laser scanning confocal apparatus (Bio-Rad) attached to a Nikon Optiphot microscope.

Immunoblot Analysis. Plates of BHK cells were grown to confluency, washed three times with PBS, scraped, and centrifuged for 3 min in a clinical centrifuge and extracted with a 3-fold excess (vol/vol) of a low-salt buffer [25 mM imidazole/2 mM EDTA/2 mM EGTA/8% sucrose (wt/vol)/1 mM benzamidine/aprotinin at 50 kallikrein units per ml/leupeptin at 5 $\mu\text{g}/\text{ml}$, pH 7.0], by using a Teflon Dounce homogenizer. The extracts were centrifuged at 100,000 $\times g$ for 20 min at 4°C. The pellets were resuspended in the same volume of buffer but containing 0.5% Triton X-100 and 0.5 M NaCl. The extraction procedure was then repeated, and the supernatants were saved for immunoprecipitation. A rabbit polyclonal anti-PTP antibody (5501) raised against purified $\Delta\text{C11.PTP}$ (7) was used for immunoprecipitation and detection in immunoblots of TC.PTP or $\Delta\text{C11.PTP}$. These procedures have been described elsewhere (6, 7).

Two-Dimensional Gel Analysis of Phosphotyrosine Proteins. Nearly confluent populations of BHK cells were treated for 24 hr with 5 μM dihydrocytochalasin B (DCB), followed by 12 hr with nocodazole at 0.06 $\mu\text{g}/\text{ml}$ before labeling. All cells, including those that became detached, were collected, washed with phosphate-free medium, and incubated for 4 hr at 37°C in labeling medium [Dulbecco's modified Eagle's medium/10% dialyzed fetal calf serum containing 1 mCi of ³²PO₄ (3000 Ci/mmol; 1 Ci = 37 GBq)]. Cells were rinsed once with PBS (37°C) and frozen immediately in liquid nitrogen. To obtain phosphotyrosine-containing proteins the frozen cells were extracted as described in ref. 6, and 0.5 mg of protein extract, at a concentration of 1 mg/ml, was treated with 20 μl of anti-phosphotyrosine antibodies cross-linked to agarose beads (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitation was done for 16 hr at 4°C with continuous rotation. The beads were washed twice at 4°C with the extraction buffer and once with 25 mM imidazole (pH 7.2) and 1.0 mM Na₃VO₄. The immunoprecipitates were treated for 2 min at 4°C with 3 μl of RNase/DNase (1 mg/ml) followed by 3 min at 37°C in 40 μl of 0.3% SDS. Two-dimensional analysis of the samples was performed on the immunoprecipitates as described (15).

RESULTS

Multinucleation Phenotype in BHK Cells Overexpressing $\Delta\text{C11.PTP}$. Several independent lines of $\Delta\text{C11.PTP}$ -transfected BHK cells are clearly distinct in morphology from controls containing the vector alone or those overexpressing the 48-kDa wild-type TC.PTP. The most striking alteration, a highly multinucleate state, was observed in two established cell lines designated as IC6 and 3A3. Phase contrast (data not shown) and confocal microscopy (Fig. 1) reveals that 60% and 28% of IC6 cells and 3A3 cell lines, respectively, contain multiple nuclei (Table 1). By contrast, binucleate and multinucleate cells are rarely seen (<10%) in control cells or in cells overexpressing TC.PTP (Table 1).

To demonstrate the multinucleated state unequivocally, cells were labeled with DiOC₁₈, a membrane-intercalating fluorescent lipid that marks endoplasmic reticulum and the plasma membrane. With this stain, one can readily distin-

guish between two closely apposed cells (Fig. 1C) and cells that contain multiple nuclei within one contiguous cytoplasm, such as IC6 (Fig. 1A). In contrast, cells overexpressing TC.PTP (Fig. 1B) and control cells (Fig. 1C) are largely mononucleate.

In a control experiment, an inactive form of $\Delta\text{C11.PTP}$ (CS- $\Delta\text{C11.PTP}$) was transfected into BHK cells, and numerous methotrexate-resistant colonies containing the mutant were selected. As shown for one representative cell line analyzed by confocal microscopy, the level of multinucleation was indistinguishable from control cells (Table 1). Of a total of 13 independent methotrexate-resistant colonies selected, none exhibited >8% binucleate phenotype.

Defective Cytokinesis in BHK Cells Overexpressing $\Delta\text{C11.PTP}$. In normal cells during late mitosis, a telophase disc forms at the spindle equator and completely bisects the cell undergoing cleavage. This element contains a 60-kDa protein, designated TD-60 (13). Antibodies to TD-60 were used to monitor cleavage-furrow formation and progression in $\Delta\text{C11.PTP}$ and control cells (Fig. 2). In the controls, the disc coincides with the cleavage furrow throughout telophase (Fig. 2A). By contrast, in $\Delta\text{C11.PTP}$ cells, the telophase disc is seen in a variety of constricted diameters, and yet membrane furrowing is either absent or incomplete (Fig. 2B and C), suggesting that multinucleation results from a defect in cytokinesis.

Asynchronous Mitosis in Multinucleate $\Delta\text{C11.PTP}$ Cells. Unexpectedly, the syncytial nuclei of $\Delta\text{C11.PTP}$ -containing cells appear to enter mitosis asynchronously (Table 1). In one example, a cell is shown in which a set of mitotic chromosomes coexists with two interphase nuclei in a contiguous cytoplasm (Fig. 3A). When cells are optically sectioned along the vertical Z axis by confocal microscopy, there is no apparent boundary between the mitotic and interphase domains of the cell (data not shown). All BHK cell lines overexpressing $\Delta\text{C11.PTP}$ were asynchronous, regardless of whether they exhibited a high or low level of multinucleation. Conversely, those cells overexpressing the full-length enzyme and rendered multinucleate with DCB are completely synchronous in mitosis (Fig. 3B).

To test whether mitotic asynchrony correlates with $\Delta\text{C11.PTP}$ activity, 13 clonal lines that express the inactive enzyme (CS- $\Delta\text{C11.PTP}$) were analyzed. None of these, including the small number of naturally occurring binucleate cells or multinucleate cells induced by DCB treatment, showed any asynchrony (Table 1). This result indicates that the activity of the truncated enzyme is responsible for the observed nuclear asynchrony.

Asynchrony also extends to the cytoplasmic domain surrounding each nucleus as illustrated in Fig. 4. A single cell contains a mitotic spindle immediately adjacent to an interphase microtubule array (Fig. 4); their corresponding chromosomes and nucleus are also shown (Fig. 4'). The image represents a 0.3- μm horizontal optical section of the cell, eliminating the possibility that this image represents one microtubule array overlying the other.

Although, typically, $\Delta\text{C11.PTP}$ syncytial cells display only one nucleus in mitosis at any one time, other nuclei in the cell are also cycling. Nuclei at distinct stages of interphase can coexist with mitotic chromosomes in a single cell. For example, a cell is shown where one nucleus is in S phase, as demonstrated by the presence of PCNA (Fig. 5) (16, 17), and the other is in mitosis, as is evident from the presence of condensed chromosomes (Fig. 5'). These data suggest that the mitotic dominant signal does not induce the premature chromosome condensation of S-phase nuclei normally characteristic of syncytial cells (11).

Subcellular Localization of Full-Length and Truncated PTP May Be Responsible for Multinucleation and Asynchrony. It has been previously shown that the overexpressed full-length

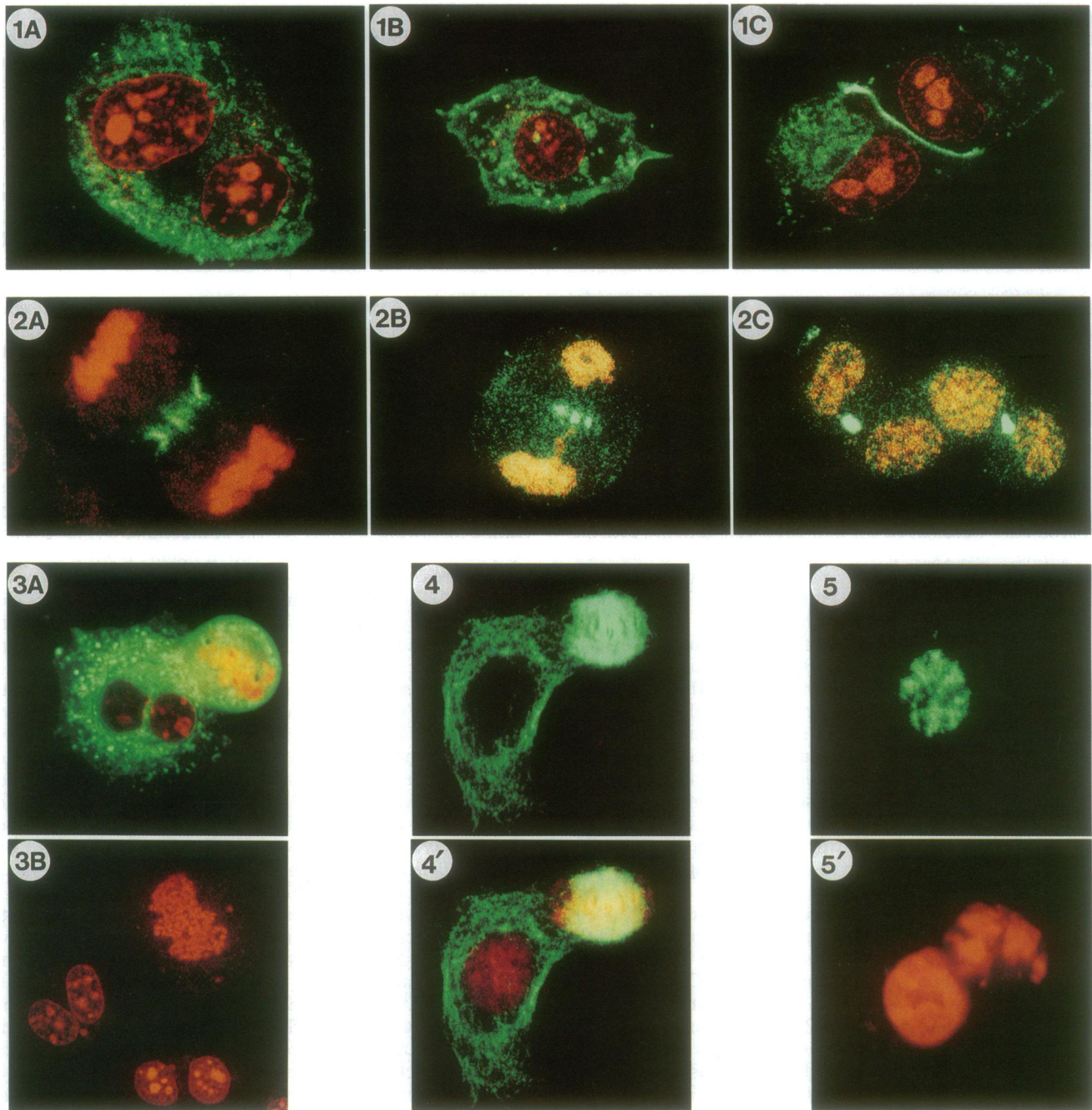


FIG. 1. Cells overexpressing Δ C11.PTP cDNA become multinucleate. Multinucleation was determined by epifluorescence analysis using the membrane marker DiOC₁₈ (green) and the DNA stain propidium iodide (red). (A) A multinucleate Δ C11.PTP-containing cell is distinguished by the presence of two or more nuclei (red) within a single contiguous cytoplasm (green). In contrast, cells overexpressing TC.PTP (B) or the vector alone (C) typically exhibit only one nucleus. DiOC₁₈ highlights the membrane barrier between two adjoining cells (C).

Fig. 2. Multinucleation results from a failure in cytokinesis. BHK Δ C11.PTP cells were synchronized in mitosis by release from nocodazole blockage. After 60 min of recovery, cells were fixed and probed with antiserum to TD-60, which serves as a telophase marker (13). (A) The telophase disc (green, white where intense) normally remains associated with the plasma membrane throughout furrowing and becomes increasingly constricted. In Δ C11.PTP cells, the disc is frequently seen as either partially (B) or fully (C) constricted but disjoined from the plasma membrane, which exhibits little or no furrowing. Cells are counterstained with propidium iodide (red or orange). The cell border is made evident by TD-60 background (green).

Fig. 3. Asynchronous entry of Δ C11.PTP syncytial nuclei into mitosis. (A) Multinucleated Δ C11.PTP cells generally enter mitosis asynchronously, as demonstrated by prophase chromosomes (red) and two interphase nuclei (red) within the same cytoplasm stained with DiOC₁₈ (green). Cells were blocked 16 hr in nocodazole at 0.06 μ g/ml and released for 60 min before fixation and staining. (B) Multinucleate cells expressing the full-length PTP and containing two to four nuclei after 5 μ M DCB treatment enter mitosis synchronously. One cell with three sets of condensed chromosomes and two binucleate cells are shown. Multinucleate TC.PTP cells were generated by 24- to 48-hr blockage with 5 μ M DCB and were further treated with nocodazole at 0.06 μ g/ml for the last 12 hr.

Fig. 4. Asynchrony of microtubules in cytoplasmic domains of Δ C11.PTP cells. A mitotic spindle and an interphase microtubule array (both green) detected with anti-tubulin antibody are found in the same cell, indicating cytoplasmic compartmentalization of the cytoskeleton (Fig. 4). Double labeling with propidium iodide shows both mitotic and interphase chromatin (Fig. 4'). The image represents a 0.3- μ m section.

Fig. 5. S-phase and mitotic nuclei coexist within syncytial Δ C11.PTP cells. A Δ C11.PTP cell with one mitotic and one interphase nucleus is shown. (Fig. 5) The interphase nucleus is in S phase, as determined by staining for PCNA (green) (16, 17). (Fig. 5') Staining with propidium iodide (red) illustrates juxtaposition of the mitotic chromosomes and the nucleus. Cells were blocked with nocodazole at 0.06 μ g/ml for 12-16 hr before immunofluorescent staining.

Table 1. Asynchronous nuclear division of multinucleated cells containing the truncated T-cell PTP

	Cells with two or more nuclei, %	Bi/multinucleated cells with asynchronous nuclei, %
Δ C11.PTP		
IC6	61.0 + 2.9	80.8
3A3	28.4 + 1.9	*
TC.PTP	9.6 + 3.0	0.0 [†]
Control (vector only)	7.9 + 2.2	0.0 [†]
CS Δ C11.PTP	7.5 + 2.0	0.0 [†]

Cells expressing the truncated T-cell PTP (Δ C11.PTP) are predominantly multinucleate; although most of these cells have 2 nuclei, cells with 3–10 nuclei are common (data not shown). In contrast, multinucleation is a low-frequency event in cells overexpressing the full-length T-cell PTP cDNA (TC.PTP) or the vector alone. Most multinucleated Δ C11.PTP cells have asynchronous nuclei, so that condensed chromosomes occur in the same cytoplasm with interphase nuclei. For each cell type, three counts of 100 cells were made by epifluorescence of propidium iodide-stained nuclei. Continuity of cytoplasm was established by staining cytoplasmic RNA with propidium iodide and by phase-contrast microscopy (data not shown). *Asynchrony is present but has not been statistically determined. [†]Asynchrony was quantitated for populations in which multinucleation was induced by 48-hr treatment with 5 μ M DCB.

TC.PTP and Δ C11.PTP distribute primarily to the particulate and soluble fractions of BHK cells, respectively, as determined by their activities (6). We have further examined the intracellular localization of these enzymes by immunoblot analyses. Extracts from the soluble and particulate fractions were immunoprecipitated with a polyclonal antibody raised against purified Δ C11.PTP (7) and probed with the same antibody (Fig. 6). Both Δ C11.PTP and CS Δ C11.PTP were readily extracted in the aqueous phase with low-salt buffers, whereas almost all of the TC.PTP was particulate (Fig. 6). The data indicate that the phenotype of enhanced multinucleation and asynchronous nuclear division correlates with the localization of PTP activity within the soluble cytoplasmic fraction.

Tyrosine Phosphorylation in PTP-Overexpressing Cells. Anti-phosphotyrosine immunoprecipitates from extracts of

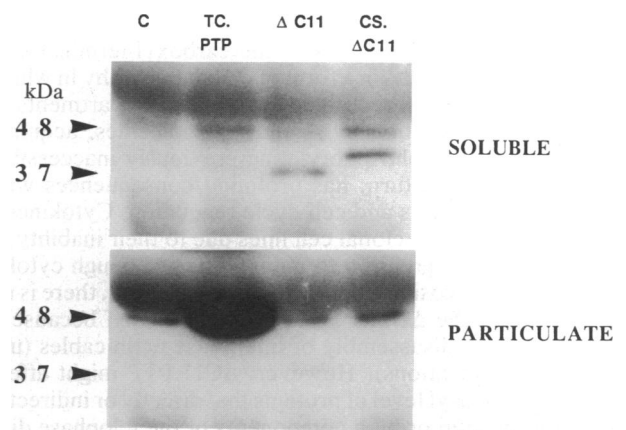


FIG. 6. Expression and compartmentalization of PTP in different BHK cell lines. Soluble and particulate fractions were obtained by a low-salt cell extraction followed by high-speed centrifugation and solubilization of the pellet in a high-salt/detergent buffer. PTP was immunoprecipitated with anti-PTP (5501) from these extracts, after adjustment to equivalent protein concentrations, and subjected to immunoblot analysis. Blots were probed by using the same PTP antibody and ¹²⁵I-labeled protein A. Extracts from cells expressing the vector alone (lane C), full-length TC.PTP, active-truncated Δ C11.PTP (lane Δ C11), or inactive-truncated CS- Δ C11.PTP were analyzed. The molecular masses of the proteins were determined from prestained markers (Amersham). CS- Δ C11.PTP encodes an inactive 40-kDa carboxyl-terminal truncated species recognized by antibody 5501. The slightly higher molecular mass of this species results from the addition of six amino acid residues, bearing no sequence homology to PTPs, at its carboxyl-terminal end (after Gly-327).

BHK cells ³²P-labeled *in vivo* were analyzed by two-dimensional gel electrophoresis (Fig. 7). The cells were treated with DCB to generate uniformly multinucleate populations and with nocodazole to arrest cells at mitosis. Fig. 7 shows that the level of tyrosine phosphorylation of several proteins either increases or decreases relative to controls (closed and open arrows, respectively), depending on whether the full-length or truncated PTP is being expressed.

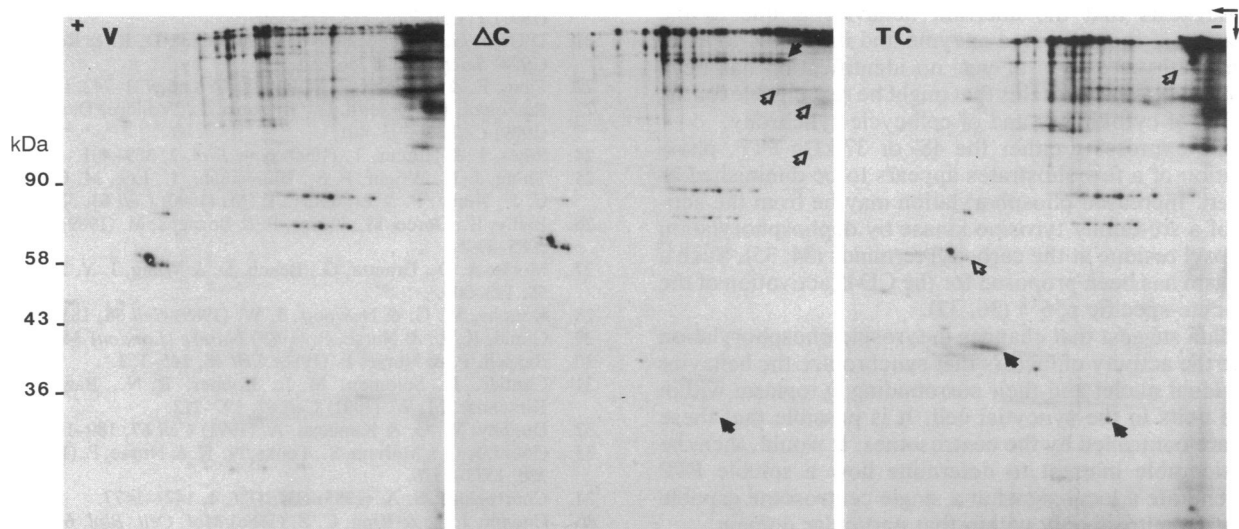


FIG. 7. Two-dimensional gel analysis of ³²P-labeled phosphotyrosyl proteins immunoprecipitated from BHK cells. Cells were labeled for 4 hr with ³²P; total extracts, equalized for protein concentrations, were immunoprecipitated by using an anti-phosphotyrosine antibody. Precipitated proteins were separated by isoelectric focusing (pH 3–10) in the first dimension followed by a 10% Laemmli gel for the second dimension. The gels were dried and exposed to film for 7 days at –70°C with DuPont intensifier screens. Immunoprecipitated proteins from cells containing vector control (V); active, truncated Δ C11.PTP (Δ C), and full-length TC.PTP (TC) are shown. Open or closed arrows indicate those proteins with reduced or enhanced level of phosphorylation, respectively, compared with controls. Molecular sizes of standard proteins are shown. The possibility that ³²P-labeled serine/threonine-containing proteins coprecipitated with proteins phosphorylated on tyrosine cannot be excluded.

DISCUSSION

This study indicates that loss of the carboxyl-terminal segment of TC.PTP results in a form of cellular anarchy in which the enzyme, no longer restricted to specific compartments or subjected to modulation by regulatory molecules, acquires the capability of dephosphorylating previously inaccessible substrates. This, in turn, has profound consequences with respect to cytokinesis and cell-cycle regulation. Cytokinesis seems to fail in these clonal cell lines due to their inability to sustain furrow progression to completion. Although cytokinesis is an actomyosin-dependent process (18–20), there is no indication that the Δ C11.PTP acts on actin itself because it does not induce disassembly of interphase actin cables (unpublished observations). However, Δ C11.PTP might affect the phosphotyrosyl level of proteins that directly or indirectly interact with actin or with components of the telophase disc that align with the cytokinetic furrow (13).

A surprising observation is that all multinucleated Δ C11.PTP cell lines exhibit a striking asynchrony in mitosis: interphase and mitotic nuclei with their corresponding microtubule arrays coexist in distinct domains within the syncytial cell. Under normal conditions, the onset of mitosis occurs simultaneously in various compartments of the cell, including the nucleus and the cytoplasm. Specific signals must coordinate this event, initiated by factors such as p34^{cdc2}, a protein kinase required for both G1/S (21, 22) and G2/M transitions (23–25) in mammalian cells. p34^{cdc2} is partially localized to the centrosome during late G2 (23, 26); therefore, the possibility exists that the centrosome acts to control mitotic entry of the proximal nucleus. Induction of mitosis would, thus, appear to involve domains of the cell, centered on individual nuclei, acting as independent and integrated units.

p34^{cdc2} is activated by tyrosine dephosphorylation during the G2/M transition (27–29); in *Saccharomyces pombe*, the reaction is brought about by p80^{cdc25} (30–32). A ts-p80^{cdc25-22} mutant fails to divide at the restrictive temperature but is rescued by overexpression of TC.PTP (33), indicating a role for tyrosine phosphatases in cell-cycle regulation. Cell-cycle signals are disrupted in BHK cells containing the truncated PTP but are not disrupted in those cells overexpressing the full-length enzyme or the inactive truncated mutant. This result suggests that the aberrant behavior is due to the localization of the truncated enzyme and its access to inappropriate substrates. As of yet, no identification has been made of those target proteins that might be responsible for the regulation of cytokinesis and of cell-cycle synchrony.

In cells expressing either the 48- or 37-kDa PTP, phosphorylation of a few substrates appears to be diminished or enhanced. Increased phosphorylation may be from the activation of a src-family tyrosine kinase by dephosphorylation of a tyrosyl residue at the carboxyl terminus (34, 35). Such a mechanism has been proposed for the CD45 activation of the lymphocyte-specific p56^{lck} (36, 37).

The data suggest that changes in tyrosine phosphorylation regulate the activity of factors that synchronize the behavior of individual nuclei and their surrounding cytoplasm within discrete units in the syncytial cell. It is possible that these events are controlled by the centrosomes. It would, then, be of considerable interest to determine how a soluble PTP could generate a local signal at a single centrosome capable of initiating mitosis only within that particular domain.

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