Protein Phosphatase Type 1 in Mammalian Cell Mitosis: Chromosomal Localization and Involvement in Mitotic Exit

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Abstract. We have examined the role of protein phosphatase type 1 (PP-1) in mammalian cell mitosis. Immunofluorescence using anti-PP-1 antibodies revealed that PP-1, which is mainly localized in the cytoplasm of G1 and S phase cells, accumulates in the nucleus during G2 phase and intensely colocalizes with individual chromosomes at mitosis. This increase in nuclear PP-1 in G2/M cells was confirmed by immunoblotting on subcellular fractions. Microinjection of neutralizing anti-PP-1 antibodies before division blocked cells at metaphase, whereas injection of PP-1 in one pole of an anaphase B cell accelerated cytokinesis and the reflattening of the injected cell. These results reveal a specific cell cycle-dependent redistribution of PP-1 and its involvement in reversing $p34^{cdc2}$-induced effects after mid-mitosis in mammalian cells.

THE phosphorylation and dephosphorylation of proteins plays a pivotal role in the transduction of hormonal signals and the regulation of a number of pathways of cellular metabolism (for reviews see references 2, 10). Recently attention has focused on the role of reversible phosphorylation in the regulation of the mammalian cell division cycle, leading to the identification of a protein kinase conserved in organisms from yeast to man (26, 27). This kinase, $p34^{cdc2}$, apparently plays a central role in the entry of cells into mitosis (27, 29). Through its phosphorylation of other cellular enzymes, such as the *src* tyrosine kinase, $p34^{cdc2}$ appears to modulate changes in cellular morphology and cytoskeletal organization accompanying the entry of cells into mitosis (24). While little is known of the other proteins that contribute to regulating the coordinate passage of cells through mitosis, the activation of $p34^{cdc2}$ kinase involves its dephosphorylation, underlining the importance of protein phosphatases which, by catalyzing specific dephosphorylations, may play an integral role in the regulation of cell division.

One of the most highly conserved enzymes in eucaryotes is protein phosphatase type 1 (PP-1).[1] Indeed, the products of the bimG gene of Aspergillus nidulans (12), and the $dis2^+$ gene and the $bws1^+$ gene of the fission yeast *Schizosaccharomyces pombe* (4, 31) encode proteins that are 86, 82, and 81% identical in amino acid sequence to rabbit skeletal muscle PP-1. Recent genetic studies in these lower eucaryotic organisms have shown that mutation in these PP-1-like genes causes mitotic defects in chromosome disjunction and the separation of nuclei during anaphase (4, 12, 31). These putative functions for PP-1 add to its previously demonstrated roles in the regulation of glycogen and cellular metabolism, the modulation of protein synthesis, and relaxation of smooth muscle (for reviews see references 2, 10). While the strict conservation of PP-1 is indicative of its role in the regulation of a diverse array of fundamental processes, we still do not know how one enzyme can orchestrate so many events. Biochemical analysis of the catalytic subunit of PP-1 reveals it can be purified as heterodimer with either of two proteins, the heat stable inhibitor 2 (15) or a glycogen-binding (G) component. PP-1 complexed with inhibitor 2 forms a soluble holoenzyme in which PP-1 activity is regulated through a reversible phosphorylation reaction (2, 10). Alternatively, a glycogen-bound holoenzyme identified as PP-1 complexed to the G component, which anchors PP-1 glycogen, is also regulated by phosphorylation (16, 17, 33). Other reports have demonstrated the specific binding of PP-1 to myofibrils (9) and muscle membranes (where the enzyme is in a form similar to that associated with glycogen; reference 9), and an association with chromatin (19, 23). Taken together these biochemical analyses show the existence of intracellular PP-1-binding proteins and provide the first clues that the intracellular distribution of PP-1 activity may be an important aspect of its regulation.

We have examined the distribution and potential role of PP-1 in the mammalian cell division cycle. Immunolocalization studies using affinity-purified antibodies specific for PP-1 reveal that the phosphatase, mainly cytoplasmic in G1 cells, progressively accumulates into the nucleus as cells progress through G2 and into mitosis. Such nuclear-localized PP-1 is tightly associated with condensed chromosomes during all stages of mitosis. Microinjection experi-

1. *Abbreviation used in this paper*: PP-1, protein phosphatase type 1.

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ments indicate an essential role for this nuclear localization in targeting PP-1 activity which appears to be required for the exit from mitosis in mammalian cells.

Materials and Methods

Phosphatase Purification and Anti-Type 1 Antibody

The 33-kD catalytic fragment of PP-1 was purified from rabbit skeletal muscle by the method of Brautigan et al. (5) with the addition of a final step of Mono-Q chromatography. This preparation is rendered fully active by treatment with Mn^{2+} and digestion with trypsin (5). The catalytic fragment was dialyzed immediately before use from a stock solution of 0.35 mg/ml into an injection solution containing 0.3 mg/ml rabbit anti-mouse antisera (to act as a marker for injected cells) in an injection buffer containing 100 mM K^+ glutamate, 39 mM K^+ citrate, 1.0 mM $MgSO_4$, pH 7.25, essentially as described elsewhere (13). Antibodies to the catalytic fragment of PP-1 were produced as described elsewhere (5),and dialyzed into 0.5× PBS for microinjection. These antibodies selectively inhibit PP-1 without inhibiting or immunoblotting the type 2A catalytic subunit (6). For control antiserum, preimmune sheep antibodies were used and dialyzed as described above. Synchronized REF-52 cells were immunoblotted with anti-PP-1 essentially by the techniques described elsewhere (6). Assessments of PP-1 activity in REF-52 cells were made as described before using phosphorylase A as a substrate (5), in the presence and absence of okadaic acid (3).

Microinjection and Cell Culture

Rat embryo fibroblast (REF-52) cells (28) were cultured in DME supplemented with 8% FCS in a humidified atmosphere containing 5% CO_2 essentially as described elsewhere (24). Cells were subcultured 2–3 d before use onto either 25-mm-diam glass coverslips (Schutt Laborteknik, Gottingen, Germany) for microinjection studies, or 12-mm glass coverslips for immunofluorescence. Synchronous cells were obtained by 36 h of serum deprivation and refeeding as described elsewhere (24). Mitotic cells were synchronized by serum deprivation and resynchronized at S phase using a 2-mM hydroxyurea block for 12 h. Hydroxyurea was removed by sequential washing in fresh DME for 1, 5, 10, and 15 min. Under these conditions, 95% of the cells enter S phase within the initial hour. Cells were microinjected with ultrafine (~0.2-μm outside diameter) microneedles for anaphase cells. Immediately after injection, mitotic cells were loaded into Dvorak-Stortler controlled environment chambers (Nicholson Precision Instruments Inc., Gaithersburg, MD) and observed on a heated stage on a Zeiss Axiophot photomicroscope.

Immunofluorescence Analysis

For immunofluorescence studies, cells were fixed in 3.7% Formalin for 5 min and extracted with acetone (−20°C), and free protein binding sites were blocked with a brief incubation in PBS containing 0.1% BSA. Alternatively, cells were fixed in −20°C methanol or preextracted in 0.5% Triton before formalin fixation as described before (13). Synchronized cells were stained for distribution of the catalytic subunit of PP-1 using affinity-purified sheep antibodies specific for the catalytic fragment of PP-1 diluted 1:100 (5). After a 1-h incubation, cells were washed and the distribution of primary antibody was determined by staining cells with fluorescein-conjugated rabbit anti-sheep affinity-purified antibodies (13). Cells were stained for DNA with HOESCHT (1 μg/ml, bisbenzimide; Sigma Chemical Co., (La Verpilliere Cedex) France), mounted, and photographed as described elsewhere (25). Microinjected cells were fixed in Formalin as described above before staining for the distribution of the injected antibodies with fluorescein-conjugated goat anti-rabbit antibodies (24). Cells were stained for actin microfilaments as described elsewhere (13), before being mounted and photographed.

Cell Fractionation and Immunoblotting of Extracts from Synchronized Cells

Synchronized REF-52 cells growing on 60-mm dishes were treated with 20 μM cytochalasin B (Sigma Chemical Co.) for 30 min, before being washed once in 25°C PBS, pelleted at 3,000 *g*, and resuspended in 100 μl (per 60-mm dish of cells) of hypotonic buffer (20 mM Hepes, pH 7.2, 10 mM KAc, 1 mM MgAc, 1 mM DTT, 0.5 mM EDTA) containing 20 μM cytochalasin B. Cells were allowed to swell for 10 min on ice, before lysis by addition of NP-40 at 0.1% final and KAc at 100 mM. After 5 min in ice and vortex, nuclei and membranes were pelleted by centrifugation for 10 min at 8,000 *g*, resuspended in 100 μl of lysis buffer, and called nuclear fraction (N); the supernatant was the soluble cytoplasmic fraction (S).

For immunoblotting of these fractions with anti-PP-1, 50 μl of each fraction (equivalent to half a 60-mm dish of cells) was loaded on a 12.5% acrylamide gel for analysis by one-dimensional electrophoresis before transfer on nitrocellulose for immunoblotting with affinity-purified anti-PP-1 (diluted 1:200) and staining with anti-sheep peroxidase-conjugated antibodies and chloronapthol as described before (6).

Results

Immunolocalization of PP-1 in Rat Embryo Fibroblasts during the Cell Division Cycle

To examine the cytolocalization of PP-1 in mammalian cells, REF-52 cells (28) were synchronized by serum deprivation as described previously (8, 25). Under these conditions, upon serum stimulation, cells have a doubling time of 26–28 h with four phases corresponding to G1 (0–16 h), S phase (16–20 h), G2 (20–26 h), and mitosis (25–28 h) (24). Synchronized cells, fixed as described in Materials and Methods, were stained with a sheep polyclonal antibody affinity purified against a 33-kD catalytic fragment of rabbit skeletal muscle PP-1 (5). This antibody (anti-PP-1) has been shown to be specific for PP-1 and unreactive with phosphatase type 2A (5). Anti-PP-1 recognized a single protein of 36 kD in growing REF-52 cells (Fig. 1 *A*). Cells stained in G1 with this anti-PP-1 revealed a distribution for PP-1 mainly throughout the cytoplasm, with only little evidence of staining in the nucleus (Fig. 1, *B* and *C*). A similar distribution was also observed in quiescent cells, throughout the G1 period and using different methods of fixation of the cells (formalin or methanol) (data not shown). This localization, mainly cytoplasmic, is consistent with both its previous implication in glycogen metabolism (for review see reference 10) and in S6 dephosphorylation (32), and with our observation that PP-1 appears to play an essential role as the dominant phosphatase in the regulation of actomyosin contractility in rat embryo fibroblasts (13). As cells proceeded through S phase and into G2, there was a progressive increase of the phosphatase staining into the nucleus. Cells in S phase (18 h after refeeding) begin to show a low-level staining within the nucleus (Fig. 1, *D* and *E*) in addition to the bright cytoplasmic staining. During G2, the level of nuclear staining for PP-1 continued to increase until cells reached prophase, when the level of nuclear staining was considerably brighter than in the cytoplasm. Methanol fixation or brief extraction of cells with 0.1% Triton X-100 before formalin fixation provided higher contrast images of PP-1 nuclear staining. Fig. 1, *F* and *G*, shows phase-contrast and PP-1 staining of late G2 cells fixed in formalin after prior extraction in Triton X-100 (note the chromatin is beginning to condense in the cell on the right in *F*). Moreover, Fig. 1 *G* was printed approximately twice as darkly as the other fluorescence panels to compensate for the brightness of the staining within the nucleus of the prophase cell. At the onset of mitosis, nuclear PP-1 is specifically associated with the condensing chromatin (Fig. 2, *A* and *B*), and individual chromosomes are brilliantly stained at prometaphase (Fig. 2, *C* and *D*). After disassembly of the nuclear envelope, bright staining for PP-1 is still clearly discerned on the metaphase chromosomes together with some staining throughout the cell cytoplasm

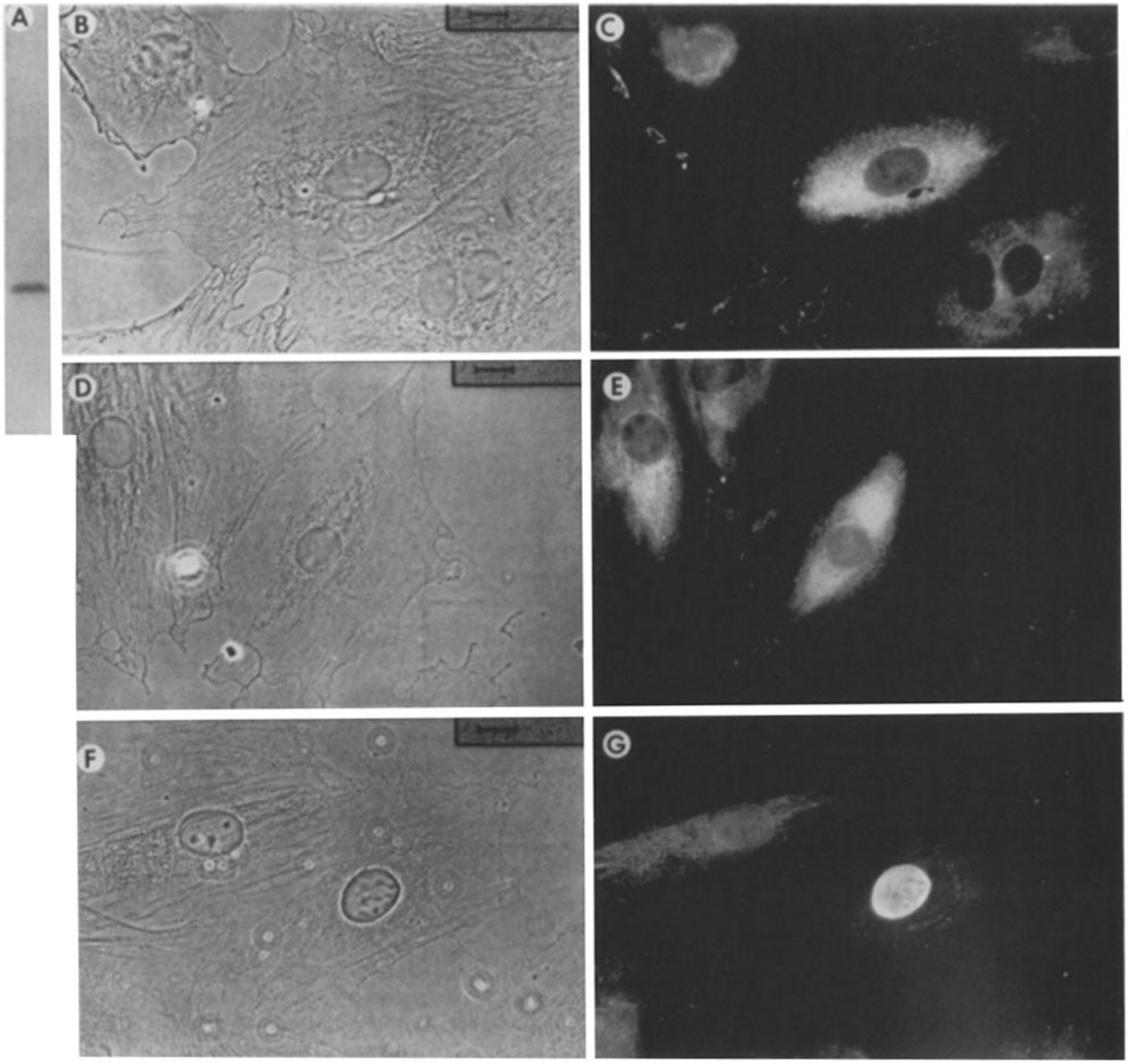

Figure 1. Immunolocalization of PP-1 through the cell cycle of mammalian fibroblasts. Rat embryo fibroblasts growing on glass coverslips were synchronized through 36 h of serum deprivation as described in Materials and Methods. At different times thereafter, cells were fixed and processed for immunofluorescence using an affinity-purified sheep anti-PP-1 which recognizes a single protein at 36 kD in extracts of randomly growing REF-52 cells (*A*). Shown are phase-contrast images (*B, D,* and *F*) and fluorescence micrographs (*C, E,* and *G*) of cells at different times in the cell cycle. *B* and *C* show PP-1 distribution in G1 cells; *D* and *E* show its distribution in S phase cells; and *F* and *G* show its distribution in late G2 with one prophase cell in the center of the image. Bars, 5 μm.

(Fig. 2 *E*). This bright staining of the chromosomes remains through anaphase (Fig. 2 *F*) and early telophase (Fig. 2 *G*) with the chromatin still being clearly stained against the cellular background. This nuclear staining disappears only at late telophase, when the chromatin is decondensed and the nucleus has regained its interphase morphology (~1 h after reformation of the nuclear envelope), leaving cells stained mainly in the cytoplasm like G1 cells (Fig. 1 *C*). Such images of chromatin staining at mitosis were specific to the use of anti-PP-1 antibody since this staining pattern was abolished by preincubation of the anti-PP-1 antibodies with an excess of PP-1 (Fig. 2 *H*, staining of a prometaphase cell). Furthermore, this association of PP-1 staining with the chromosomes of mitotic cells was abolished by treatment of the fixed cells with DNase 1 (protease free, 0.5 mg/ml for 10 min) before immunostaining. Such treatment resulted in a diffuse staining for PP-1 throughout the cytoplasm of mitotic cells (data not shown). These results show a cell cycle–dependent change in PP-1 distribution, with a gradual increase in nuclear PP-1 from the end of G1–S, culminating at mitosis with an intense staining associated with the condensed chromosomes.

PP-1 Immunoblotting of Subcellular Fractions

To confirm that the marked increase in nuclear PP-1 at the

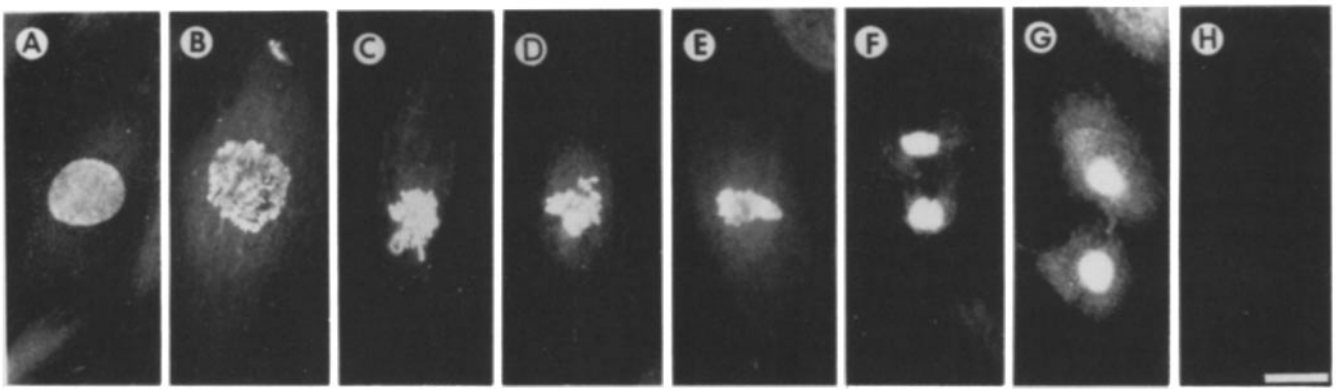

Figure 2. Immunolocalization of PP-1 in mitotic mammalian cells. Synchronized rat embryo fibroblasts growing on glass coverslips were fixed in methanol as they reached mitosis (mitotic index ~15–20%) and stained with affinity-purified anti-PP-1 antibodies as described for Fig. 1. Fluorescence micrographs of individual cells stained at various stages of mitosis: (*A* and *B*) in early and late prophase, respectively; (*C* and *D*) in prometaphase; (*E*) in metaphase; (*F*) in anaphase; and (*G*) in telophase. (*H*) Staining of a mitotic with the same anti-PP-1 serum but after preincubation of the antibody with purified PP-1. Bar, 7 μm.

end of G2 and during mitosis observed by immunofluorescence truly reflected an increase in PP-1 catalytic subunit abundance in the nuclei, as opposed to the possible unmasking of an epitope through differential association with regulatory subunits, we performed fractionation of synchronized cells for analysis by Western immunoblotting. Cells were grown on 60-mm plates and synchronized as described above. Plates of cells were scraped and incubated in a small volume of hypotonic buffer before lysis with NP-40 (0.1%) and preparation of the nuclear fractions as described in Materials and Methods to obtain pelleted nuclear fractions and cytoplasmic soluble fractions. Before harvesting, cells were treated with 20 μM cytochalasin B to disassemble the actin microfilament networks and thus release the PP-1 bound to actomyosin into the soluble fraction. Fig. 3 shows the Western blot from G1 and G2/M nuclear and soluble fractions. In G1 cells, immunoblotting reveals a 36-kD protein in the soluble fraction which is detected similarly in the cytoplasmic soluble extract from G2/M cells (Fig. 3, lanes *G1S* and *G2S*). In the nuclear fractions, G1 cells show only a faint staining of the 36-kD band (Fig. 3, lane *G1N*) whereas a much stronger signal is detected at 36 kD in G2/M cell nuclei (Fig. 3, lane *G2N*), even though the sample loading per lane was the same. (Fractions recovered from half a 60-mm dish of confluent REF-52 cells—i.e., 4–5 × 10^6 cells—were loaded per lane; the equivalence of the loading was also checked through red Ponceau staining of the blot before immunoblotting.) These data show that an increased association of PP-1 with the insoluble fraction occurs during the G2 period of the cell cycle and thus confirm our immunocytolocalization observation.

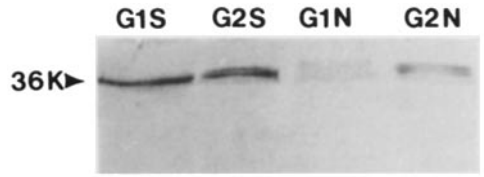

Figure 3. Evidence for a nuclear accumulation of PP-1 in G2 cells. To assay the relative abundance and activity of PP-1 in nuclear and cytoskeletal compartments of G1 and G2 cells, synchronized cells were harvested and subcellular fractions prepared as described in Materials and Methods. The figure shows immunoblotting analysis of the different fractions from G1 and late G2 cells, using anti-PP-1 affinity-purified antibodies. S and N refer to the cytoplasmic (soluble) and nuclear (insoluble) fractions, respectively.

Metaphase Arrest after Phosphatase Inhibition

To investigate the potential role of PP-1 during mitosis, since it appeared that PP-1 underwent nuclear localization during G2 and at mitosis, we examined how neutralizing PP-1 activity by microinjection of anti-PP-1 antibodies would affect the undergoing of mitosis in rat embryo fibroblasts. To obtain high proportions of mitotic cells, REF-52 cells were synchronized by serum starvation and used 24 h after refeeding. Cells selected in the earliest phases of mitosis were microinjected with either control antiserum (Fig. 4, *A* and *B*) or 0.5 mg/ml affinity-purified anti-PP-1 antibody diluted into 0.5× PBS (Fig. 4, *C* and *D*). Cells were injected with antibodies as they entered mitosis, and fixed 90 min afterwards. Under usual circumstances, most cells have entered telophase by this time. Microinjection of the control antibodies (stained with fluorescein-conjugated anti-sheep antibodies; Fig. 4 *B*) had no discernible effect on the transition of the cells through to telophase, and a pair of brightly stained reflattening cells is clearly visible in the marker antibodies stained image (Fig. 4 *B*). In the phase image (Fig. 4 *A*), the nuclei in the injected control cells are already discernible, implying that the envelope is reforming; this indicates that injection of the control antiserum did not disrupt the progression of the cells through mitosis. Indeed, cells injected with control antiserum continued through at least another cell division cycle without apparent deleterious effects (data not shown). In contrast, cells injected at the start of mitosis with inhibitory anti-PP-1 antibodies arrested at metaphase (Fig. 4, *C* and *D*). Both the phase image (Fig. 4 *C*) and the chromatin staining with HOECHST (Fig. 4, inset *d*) show that the injected cell (stained for the injected antibodies, Fig. 4 *D*) was arrested at metaphase with the chromatin clearly aligned across the center of the cell. None of the cells injected with anti-PP-1 before mitosis (detected by immunofluorescence staining of injected antibodies) completed mitosis but instead all arrested at metaphase (~20 cells injected per experiment, per-

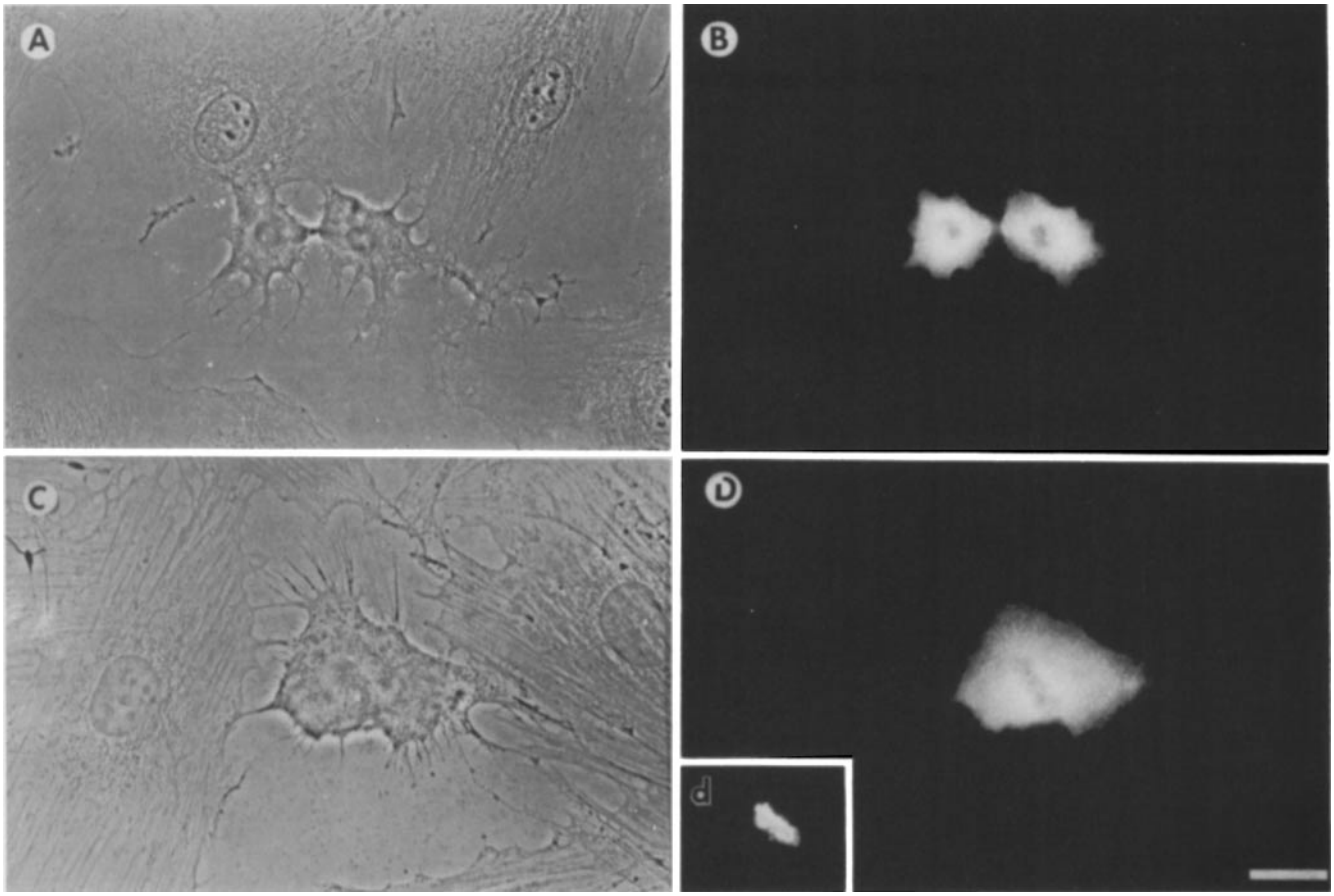

Figure 4. Microinjection of antibodies which neutralize PP-1 into late G2 cells blocks the cells at mitotic metaphase. To examine if inhibiting PP-1 affects mitotic transition, rat embryo fibroblasts growing on glass coverslips were serum synchronized and then resynchronized at S phase using hydroxyurea. 8 h after release from the hydroxyurea block, as most cells were about to enter mitosis, early prophase cells were microinjected with 0.5 mg/ml of affinity-purified sheep anti-PP-1. Cells were fixed 45 min after injection and stained for the injected antibody. Shown are phase-contrast (*A* and *C*) and fluorescence micrographs showing the distribution of the injected antibody (*B* and *D*) to identify injected cells. *A* and *B* show a cell injected with a control antibody solution alone and *C* and *D* show a cell injected with anti-PP-1. Bar, 7 μm.

formed four times). This inhibitory effect of anti-PP-1 antibody was abolished by treating with 1 mM DTT or boiling the antibody solution before injection or by preincubating the antibody with an excess of PP-1, showing that the effect of mitotic arrest at metaphase was specific to anti-PP-1 antibodies.

Microinjection of PP-1 and Anti-PP-1 in Anaphase Cells

To examine the role of PP-1 in the events subsequent to metaphase, cells were allowed to proceed through to late anaphase A–early anaphase B before we injected one of the two sister cells. Under normal conditions sister halves of the same mitotic cell form mirror images of one another as they reflatten during telophase, making it simple to assess any possible changes induced by microinjection. This technique allows a direct comparison of the consequent behavior of the two halves of the same mitotic cell thus providing an endogenous synchronized control. Anaphase cells were injected with either neutralizing anti-PP-1 antibodies or control antiserum and fixed 45 min after microinjection, by which time mitosis was normally completed and the cells were in telophase and completely flat. With control antiserum (Fig. 5, *A–C*), microinjection of one half of the anaphase pair did not interfere with the transition through and completion of mitosis. Indeed, as shown in Fig. 4 *A*, both halves of the injected mitotic cell (the half which was injected is stained for antibody in Fig. 5 *B*) show a similar morphology and actin staining (Fig. 5 *C*). From this we conclude that this microinjection technique provides a feasible way to assess the effects of anti-PP-1 in anaphase/telophase transition. In marked contrast, the sister half of an anaphase cell injected with anti-PP-1 antibodies (Fig. 5, *D–F*; marked by antibody staining in Fig. 5 *E*) clearly arrested with a rounded phenotype which differs considerably from the flattened morphology of the uninjected sister half (Fig. 5 *D*; note that the injected cell has been left for 1.5 h after injection and the uninjected sister cell is clearly in late telophase). The cell which was injected with anti-PP-1 antibodies still closely resembled an anaphase B cell. The cell was compact, tightly rounded with condensed chromatin, and stayed arrested at the stage for more than 24 h. When cells were injected with anti-PP-1 antibody in both halves of the anaphase cell (which usually occurred if cells were injected too early in anaphase A) neither half flattened down, whereas injection of control antiserum

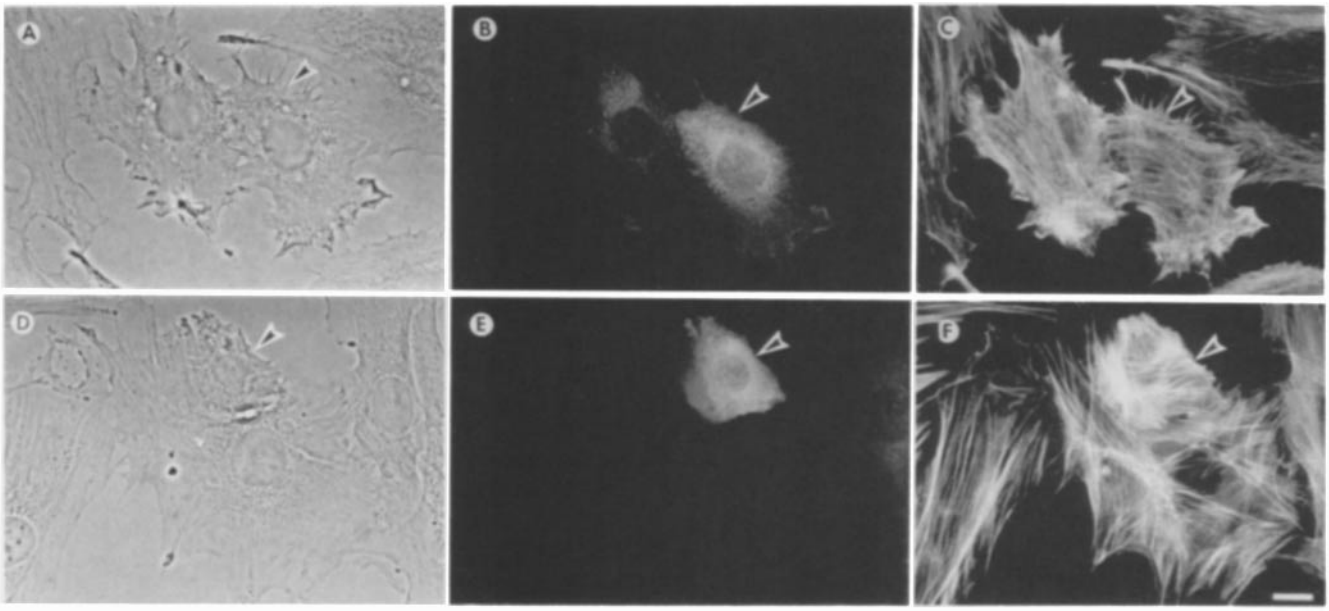

Figure 5. Inhibition of PP-1 activity in anaphase A cells arrests further proceeding and prevents anaphase B and telophase transition. Synchronized rat embryo fibroblasts in late G2 were allowed to progress into mitosis. Selected cells were microinjected into one half as the cells progressed into anaphase B (when the two sister cells begin to form and move poleward), with either control preimmune serum or affinity-purified anti-PP-1. Cells were allowed to continue through anaphase and telophase for 50–90 min before fixation and staining for the distribution of the injected antibody (using fluorescein anti-sheep antisera) and F-actin using rhodamine phalloidin. Shown are phase-contrast (*A* and *D*) and fluorescence micrographs showing the distribution of the injected antibody (*B* and *E*) and F-actin (*C* and *F*). *A–C* show a cell injected with control antibodies and *D–F* show a cell injected with anti-PP-1 antibodies. Bar, 4 μm.

in this case did not hinder mitosis which proceeded normally, with both halves becoming fully flattened (data not shown). Again, if the antibody was denatured (by boiling or DTT treatment) these effects on mitosis were not observed (data not shown). These data show that inhibiting PP-1 activity during anaphase prevents completion of cell division, implying that PP-1 still plays an integral role at this stage in mitosis.

The conclusion that PP-1 plays an essential role in the completion of anaphase/telophase was reinforced by modifying the experiment described for Fig. 5 by injecting the active phosphatase enzyme, PP-1, into the separating halves of anaphase cells. Again, microinjection solutions contained an inert antibody to allow identification of microinjected cells. As shown in Fig. 6, *A–C*, after injection of PP-1 into one half of an anaphase cell, the injected half (*arrowheads*) begins to show flattening ahead of the associated uninjected half such that by 15 min, the injected cell is visibly much larger than the uninjected sister cell. If this is allowed to go to completion, the microinjected cell (marked by antibody staining in Fig. 6 *E*) proceeds to complete mitosis within 15–25 min, well in advance of the other uninjected half (Fig. 6 *D*). In these cases, the injected sister cell became completely flat with a reassembled nuclear envelope, differing markedly from the uninjected sister cell which was still rounded with condensed chromatin.

These data reinforce our observation with anti-PP-1 antibodies, that PP-1 plays an essential role in the completion of mammalian cell division. The observation that increasing the level of PP-1 at anaphase markedly accelerated the spreading of telophase cells strongly supports the conclusion that PP-1 activity plays a key role in the exit from mitosis and must be tightly regulated during the early stages in the mitotic cell. In this respect the targeting of PP-1 in the nucleus of prophase cells and onto the condensed chromatin of mitotic cells may be implicit in such a regulatory process.

Discussion

We have examined both the intracellular distribution of PP-1 and its participation in the mammalian cell division cycle, particularly in mitosis. Immunofluorescence staining of synchronized rat embryo fibroblasts revealed PP-1 is differentially localized during the cell cycle, and undergoes a marked relocation into the nucleus at the end of the G2 phase, culminating in intense staining of the condensed chromosomes during mitosis. This increase in nuclear PP-1 at the G2/M transition was also observed by immunoblotting on fractionated cells. Experiments using microinjection of anti-PP-1 or PP-1 show clearly the continuous involvement of PP-1 after mid-mitosis in the events required for the completion of cell division from metaphase onward. Specific inhibition of PP-1 activity in mitotic cells, through microinjection of neutralizing antibodies directed against PP-1, blocked cells in metaphase when injected in late G2/early prophase cells, and stopped the progression from anaphase to telophase when injected into an anaphase cell. Conversely, microinjection of the free PP-1 catalytic fragment into one pole of an anaphase cell accelerated the reflattening and completion of telophase of the daughter cell from the injected half.

Differential Localization of PP-1 during the Mammalian Cell Cycle

Using affinity-purified anti-PP-1 antibodies, we have determined distinct changes in locale of PP-1 as cells proceed

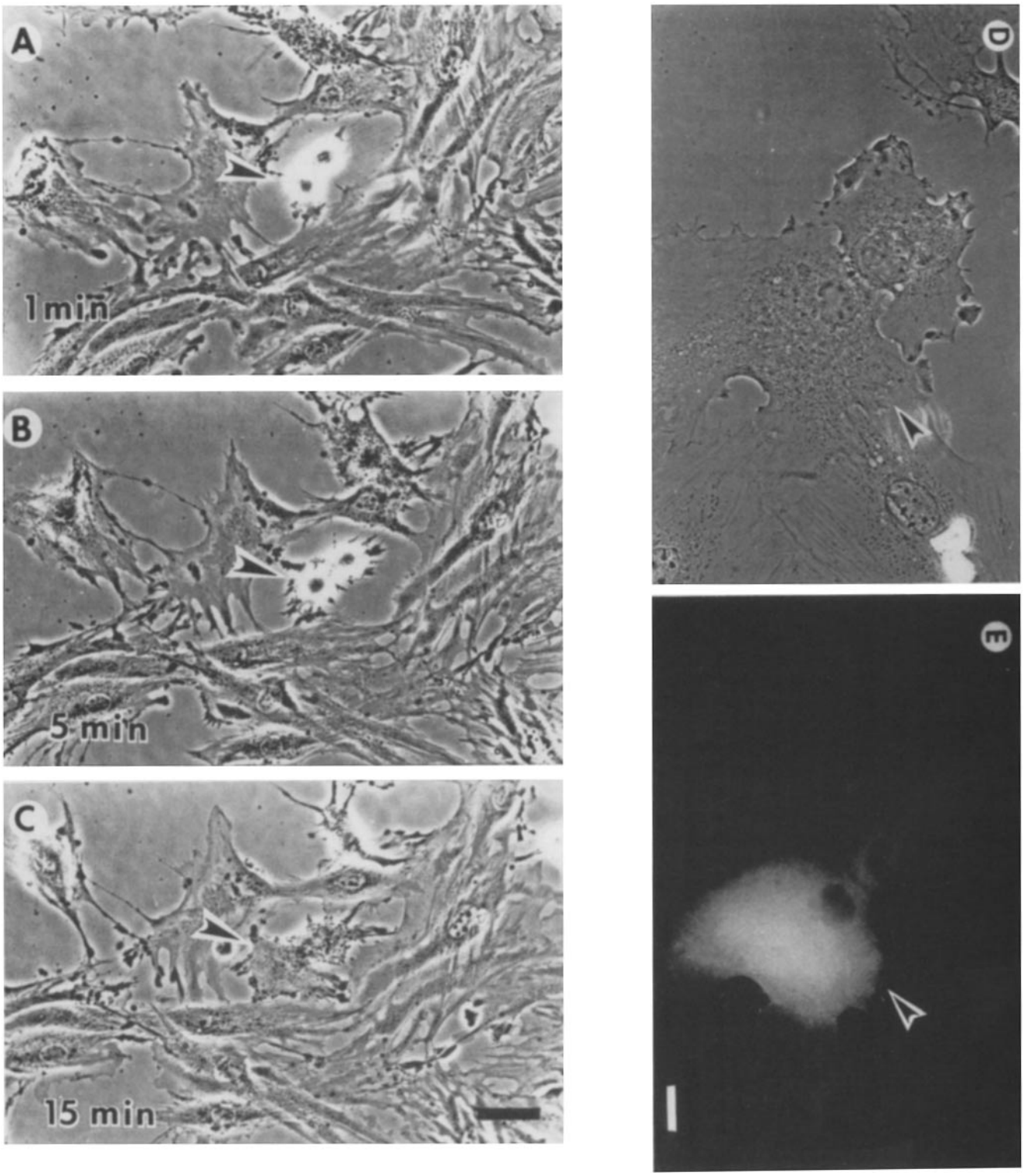

Figure 6. Microinjection of PP-1 during anaphase A accelerates the rate of exit of cells through mitosis. Rat embryo fibroblasts synchronized through serum starvation and hydroxyurea were allowed to proceed into mitosis before selected cells were microinjected into one pole of an anaphase B cell. Cells were injected with 0.5 mg/ml of purified catalytic fragment of PP-1 and photographed on the microscope stage. Shown are phase-contrast micrographs of living cells immediately after injection (*A*, 1 min; *B*, 5 min; *C*, 15 min); and cells fixed 20 min after injection in *D* and *E*. *E* shows the injected cell as detected by staining for the presence of the marker antibody. The injected cell is indicated in each panel (*arrowhead*). Bars: (*C*) 10 μm; (*E*) 4 μm.

through their division cycle. Our observation that in quiescent or G1 cells the phosphatase was localized principally in the cytoplasm is in agreement with its previous implication in glycogen metabolism (for review see reference 10), its previous implication in the modulation of S6 phosphorylation (32), and with our previous study concluding that PP-1 has an active role in the maintenance of actomyosin dynamics of interphase fibroblast contractility (13). Throughout G2, the staining for PP-1 progressively increased within the nucleus, culminating in the bright staining for PP-1 on condensed chromosomes. PP-1 has been previously reported to bind to chromatin and be released by 0.5 M NaCl or DNase digestion but no relation to the cell cycle was examined (19, 23). Furthermore, this differential location of PP-1 during the cell cycle was confirmed by immunoblotting on subcellular fractions which showed a progressive increase in PP-1 associated with the nuclear fraction as cells progressed toward G2/M.

One of the intriguing questions raised by our observation concerns the processes regulating this increase in nuclear location of PP-1. Proteins the size of PP-1 (35 kD) have been shown to freely diffuse into the nucleus where they require binding to an immobile component to maintain nuclear localization. Alternatively, proteins above 60 kD require specific sequences, usually comprised of several contiguous lysines or arginines for translocation into the nucleus. A distinguishing feature of the PP-1 catalytic subunit (not present in the type 2A phosphatase) is a cluster of basic residues located at the carboxyl terminus. Although not seemingly required for nuclear relocation, this feature is conserved in PP-1 from mammals, *Drosophila* (11), and fungi (*Aspergillus nidulans, Schizosaccharomyces pombe*; references 4, 12, 30). Additionally, the relocation mechanism could involve the reversible phosphorylation of the PP-1 catalytic subunit or a specific binding subunit in the nucleus by a cell cycle–dependent kinase activity. However, no evidence yet exists for the in vivo phosphorylation of the PP-1 36-kD catalytic subunit. In vitro, PP-1 has been reported to be phosphorylated at a COOH-terminal site by the protein tyrosine kinase $pp60^{v\text{-}src}$, a phosphorylation which decreases PP-1 activity (20). PP-1 could also be released from a cytoplasmic site in response to an increased phosphorylation of some of its regulatory subunits. It has been shown that PP-1 dissociates from the G subunit that anchors it to glycogen when this G subunit becomes phosphorylated by the cAMP-dependent protein kinase (A-kinase) in vitro (16, 17), a reaction which may involve other kinases (14). In this respect, other protein kinases, the activity of which fluctuates during the cell division cycle, such as $p34^{cdc2}$, c-mos, c-src, c-abl, and also the proteins of the cyclin family which accumulate as the cell proceeds to mitosis, are good potential candidates to regulate the binding of PP-1 to either cytoplasmic or nuclear sites. Additionally, PP-1 may bind to one or more specific proteins in the nucleus after their cell cycle–dependent phosphorylation, forming a PP-1 binding site on the chromosomes. Indeed, we have already observed that PP-1 binds with a low K_m ($\sim$1 μM) specifically to histone H1 phosphorylated by cdc2 kinase (but not to unphosphorylated H1 or phosphorylated by other kinases) (Brautigan, D. L., manuscript in preparation). Since histone H1 phosphorylation is known to gradually augment throughout the cell cycle particularly during G2, a mechanism involving PP-1 binding to H1 may account for the gradual increase of PP-1 in the nuclear fraction.

Potential Function of PP-1 after Mid-Mitosis in Metaphase and Anaphase

The question arises, What function does nuclear relocation of PP-1 at mitosis perform? Mitosis involves profound changes in cell morphology; in particular, chromatin condensation, disassembly of the cytoskeletal networks, and breakdown of the nuclear envelope. Most of these changes—which occur in cells as they enter mitosis and appear to involve a marked increase in phosphorylation of proteins (including the microtubule-associated proteins, vimentin, histones, the nuclear lamins, *src*, and the cyclins)—have to be reversed after mid-mitosis after the metaphase–anaphase transition.

The results of the microinjection studies we have done with PP-1 and neutralizing antibodies to PP-1 reveal an essential role for this enzyme in the completion of mitosis from the metaphase stage and still during the transition from anaphase to telophase. Our observation that anti–PP-1 injected before mitosis blocked cells at metaphase suggests that PP-1 has its earliest function in the transition of cells through metaphase into anaphase. This is in agreement with previous genetic data from both *Aspergillus nidulans* (12) and yeast (4, 22, 30) which revealed that PP-1 affected spindle organization and nuclear separation. More recently, a study on PP-1 mutant from *Drosophila* described similar perturbations with cells bearing metaphase-like overcondensed chromosomes (1). Based on the block at metaphase with a spindle-like structure we observed, one might speculate that the molecular motors responsible for chromatid separation are under regulation by phosphorylation and their dephosphorylation by PP-1 might be integral to this process. Indeed, this hypothesis recently found strong support with the study by Hyman and Mitchison (18) showing that the polarity of the movement of microtubules on the kinetochores of isolated chromosomes can be reversed in a phosphorylation-dependent manner. Under conditions in which dephosphorylation is inhibited, microtubules are induced to move by the plus-end (toward the metaphase plate), rather than minus-end movement (toward the spindle poles), occurring when dephosphorylation is allowed or phosphorylation is inhibited. This implies that the molecular motors in the kinetochores, responsible for the direction of chromosome movement, are under control by phosphorylation/dephosphorylation. In this respect, there are homologies between at least two of the potential microtubule motor proteins, dynein and kinesin, and the actomyosin phosphoprotein myosin, which we demonstrated to be a preferential substrate for PP-1 in vivo (13), and has recently been shown to be also involved in cytokinesis in *Drosophila* (21). It will be interesting to examine if either of the microtubule motor proteins or other microtubule-associated proteins are also substrates of PP-1.

Downstream of the metaphase block induced with anti–PP-1 injection, we observed that the flattening of mitotic cells through anaphase B and telophase, leading to the morphology of interphase cells, is accelerated by microinjection of PP-1 and blocked by neutralizing anti–PP-1 antibodies. This

strongly supports the view that PP-1 is a key effector in the process of mitotic exit in mammalian cells. While the specific functions of PP-1 in mitotic exit remain unknown, we might speculate that PP-1 is probably involved in the dephosphorylation of a variety of mitosis-specific phosphoproteins, particularly those substrates for $p34^{cdc2}$ or the kinases activated downstream of it.

For example, the reflattening of mitotic cells probably involves the dephosphorylation of intermediate filament proteins which become highly phosphorylated at mitosis and we have observed that vimentin can be dephosphorylated in vivo by PP-1 (7). The nuclear lamins A and C also appear to be dephosphorylated by PP-1 in vivo (7); and while they are dephosphorylated during envelope reassembly, the phosphatase responsible remains to be identified. Our observations strongly suggest that PP-1 may play a role in this process since half an anaphase A cell injected with anti-PP-1 was blocked in anaphase and failed to reform an envelope which had clearly reformed in the uninjected sister cell.

However, all these potential substrates are dephosphorylated only after the metaphase–anaphase transition. The fact that we observed an intense staining for PP-1 on the chromatin as soon as cells enter prophase, while microinjected antibodies to PP-1 only blocked cells at metaphase, suggests that PP-1 activity must be tightly regulated up to this stage. In light of our recent finding showing an increase of inhibitor 2—one of the two specific heat-stable inhibitors for PP-1, at S phase and at mitosis (8)—it is tempting to speculate an involvement of inhibitor 2 in the downregulation of the chromatin-bound PP-1 up to metaphase. However, although we observed that inhibitor 2 was localized in both the nucleus and cytoplasm in prophase cells, it was never associated with the chromatin. Alternatively, downregulation of PP-1 might involve either a direct phosphorylation by $p34^{cdc2}$, or PP-1 binding to cyclin subunits (which get rapidly degraded at metaphase). However, further studies of the potential interactions of PP-1 with cdc2 subunits will be required to document any of these possibilities.

Our immunofluorescence and biochemical studies of the intracellular distribution of PP-1 shows that it undergoes a concerted reorganization during the mammalian cell cycle, while microinjection studies provided direct evidence that PP-1 regulates a number of events especially in the progression through mitosis, after the metaphase stage. Together, these results support the idea that the changes in PP-1 distribution at mitosis play an important role in modulating the activity of this enzyme and suggest that PP-1 might be a major phosphatase responsible for dephosphorylating $p34^{cdc2}$ kinase sites, an hypothesis that now awaits more detailed examination.

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