Journal of Cellular Biochemistry 58:389–401 (1995)

Arrest at the G2/M Transition of the Cell Cycle by Protein-Tyrosine Phosphatase Inhibition: Studies on a Neuronal and a Glial Cell Line

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Abstract The addition of the peroxovanadium (pV) derivatives potassium bisperoxo(1,10-phenanthroline)oxovanadate(v) (bpV[phen]) or potassium bisperoxo(pyridine-2-carboxylato)oxovanadate(v) (bpV[pic]), both of which are potent inhibitors of protein tyrosine phosphatases (PTPs) [Posner et al. (1994): J Biol Chem 269:4596–4604], to the culture medium of neuroblastoma NB 41 and glioma C6 cells resulted in a marked decrease in their proliferation rates and a progressive accumulation at the G2/M transition of the cell cycle. The effect was dependent on dose, cell type, and the pV compound employed. Mean values of the RNA-to-DNA and RNA-to-protein ratios in NB cells treated for 48 h with increased doses of bpV[phen] showed that general synthetic functions were not altered, nor did we observe oxidative damage to DNA using a sensitive DNA-nick detection assay. No changes in the expression and localization of vimentin, a component of the intermediate filament cytoskeleton, were observed by indirect immunofluorescence, showing that treatment did not disturb the cytoskeleton network. Measurements of BrdU incorporation into newly synthesized DNA showed that cells treated were not totally arrested. Furthermore, cells arrested at G2/M were able to reenter the cycle rapidly after the release of inhibition. This progressive accumulation at G2/M coincided with the detection of tyrosine-phosphorylated $p34^{cdc2}$ and a dramatic reduction in its kinase activity toward histone H1 by 48 h of culture. Both compounds were equally potent in inhibiting the catalytic activity of a yeast and the structurally distant mouse cdc25B in vitro, suggesting that the augmented tyrosine phosphorylation of $p34^{cdc2}$ derived from the in vivo inhibition of cdc25. Their equal in vitro potency contrasted with the considerably greater potency of bpV[phen] in vivo, suggesting that factors regulating the intracellular access of these compounds to cdc25 might be critical in determining in vivo specificity. In conclusion the final consequence of long-term exposure to potent and structurally defined PTP inhibitors on two highly proliferative nerve cell lines is to restrict cell growth. The corresponding hyperphosphorylation and reduced activity of $p34^{cdc2}$ likely reflects the unusual sensitivity of cdc25 as an in vivo target for peroxovanadium compounds.

Key words: protein-tyrosine phosphatases, cdc25, peroxovanadium derivatives, cell-cycle, G2/M transition, nervous cell lines

Abbreviations used: bpV[phen], potassium bisperoxo(1,10-phenanthroline)oxovanadate(v); bpV[pic], potassium bisperoxo(pyridine-2-carboxylato)oxovanadate(v); BrdU, deoxybromouridine; CDKs, cyclins dependant kinases; FITC, fluorescein isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate buffered saline; PI, propidium iodide; PMSF, phenylmethylsulfonylfluoride; pNPP, para-nitrophenylphosphate; PTPs, protein tyrosine phosphatases; SDS-PAGE, sodium dodecylsulfate-polycrylamide gel electrophoresis; TBS, tris buffered saline.

Received December 6, 1994; accepted April 7, 1995.

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Peroxide(s) of vanadium (pV), a mixture of orthovanadate and hydrogen peroxide, is a strong insulin mimetic [Posner et al., 1990] and potent protein-tyrosine phosphatase inhibitor [Faure et al., 1992]. A number of structurally defined pV compounds, each containing an oxo ligand, one or two peroxoanions, and an ancillary ligand in the inner coordination sphere of V, were synthesized and characterized as greater than 95% pure by nuclear magnetic resonance. These compounds activate the insulin receptor kinase (IRK) and mimic insulin's biological action both in vitro and in vivo. Their potency as insulin mimetics is proportional to their potency as

PTP inhibitors [Posner et al., 1994]. Most synthetic pVs are stable in neutral aqueous solutions, rendering them attractive agents for studying transmembrane signalling in cultured cells.

Vanadate, a known PTP inhibitor [Gordon, 1991], stimulates proliferation in yeast [Willsky et al., 1984] and mammalian cells [Klarlund, 1985]. Indeed, augmented tyrosine phosphorylation has been associated with the transformed state and the progression to neoplasia [Jove and Hanafusa, 1987]. By contrast, exposure of mouse 3T3 fibroblast to vanadate was shown to prevent tyrosine dephosphorylation of $p34^{cdc2}$ and the entry of cells into mitosis [Morla et al., 1989]. It was thus of interest to evaluate the effects of pV compounds whose PTP inhibitory potencies are much greater than that of vanadate [Posner et al., 1994]. In this study we show that treatment of two cell lines derived from mouse brain tissue with the pV derivatives, bpV-[phen] and bpV[pic], progressively stopped the proliferation of these transformed cells. This occurred consequent to a reversible block at the G2/M transition of the mitotic cycle, apparently because of the inhibition of $p34^{cdc2}$.

MATERIALS AND METHODS

Cell Culture

Neuroblastoma (NB 41A3, ATCC CCl 147) and glioma cells (C6, ATCC CCL107) were cultured at 37°C in a 5% CO_2 atmosphere in Costar 75-cm^2 flasks with 10 ml of Ham's F10 medium containing 12.5% horse serum, 2.5% fetal calf serum supplemented with 6 mM $NaHCO_3$, 100 IU penicillin/ml, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Gibco, BRL, Gaithersburg, MD). Peroxovanadium derivatives bpV-[phen] or bpV[pic] were synthesized and purified as previously described [Posner et al., 1994], dissolved in Ham's F10 medium, pH 7.4, to a final concentration of 1 mM. The solutions were sterilized by filtration through 0.22-μm, 25-mm nylon filters (Cameo 25N, Micron Separation Inc).

Flow Cytometry

Westborough MA cells were suspended by trypsinization, centrifuged (10 min, 600*g*), resuspended in 300 μl of cold phosphate-buffered saline (PBS), and immediately fixed by adding of 700 μl of cold ethanol (−20°C). For DNA analysis, the fixed cells were centrifuged and washed with PBS, pH 7.2, before resuspension and incubation in PBS containing RNAse (40 U/ml/10^6 cells) for 30 min at 37°C. The cells were centrifuged at 600*g* for 2 min, resuspended in 500 μl of PBS containing the DNA dye propidium iodide (50 μg/ml) (PI, Calbiochem-Behring Corp., San Diego, CA), and incubated for 30 min on ice. Analysis of DNA content was done using a Coulter EPICS 753 pulse cytometer (Coulter, Hialeah, FL) equipped with two INNOVA 90 argon ion lasers (Coherent Laser Products Division, Palo Alto, CA). The cells were excited at 488 nm (400 mW), and the red fluorescence (total DNA) was recorded to be greater than 590 nm. The percentages of cells in G0-G1, S, and G2M phases were calculated from the resulting DNA content histograms using the PARA1 program (Coulter). Debris and doublets were eliminated by plotting integrated red fluorescence vs. peak red fluorescence and by setting a bit map around the events on the diagonal (G0-G1, S, G2M singlets) [Crissman et al., 1985a].

Measurements of RNA in individual cells were done by resuspending the fixed cells in PBS containing Hoechst 33342 (HO, Calbiochem-Behring Corp.) (0.5 μg/ml) and staining for 30 min at 37°C. The tubes were then chilled on ice for 5 min and an equal volume of cold PBS staining solution containing HO (0.5 μg/ml) and pyronin Y (PY, Calbiochem-Behring Corp.) (2.0 μg/ml) was added to the cell suspensions. After 5 min of incubation on ice, the cells (7.5×10^5/ml) were analyzed in the dye solution. Laser emissions were 50 mW at 350 nm (HO) and 400 mW at 488 nm (PY); fluorescence emissions were between 400 and 450 nm for the HO and above 570 nm for the PY; 70 to 80% of the PY fluorescence was sensitive to ribonuclease A. Ten thousand events were acquired on a single-parameter 256-channel integrated fluorescence histogram (DNA and RNA content) or on a dual-parameter 64-channel integrated fluorescence histogram (RNA vs. DNA) [Crissman et al., 1985a,b).

Proteins in individual cells were measured by staining with HO (0.5 μg/ml) and fluorescein isothiocyanate (FITC, 0.1 μg/ml). Fluorescence emissions were between 400 and 450 nm for the HO and between 515 and 535 nm for the FITC [Crissman et al., 1985a,b]. To standardize the relative fluorescence intensity between the different samples, C6 cells from one control stock were added (10% of total cells) as an internal control for NB cells; conversely, NB cells were used as an internal control in C6 experiments

(NB41 cells consistently contained 2.5-fold more DNA than C6 cells). Cell viability was estimated by incubation (5 min, 37°C) in the presence of PI at a concentration of 0.5 μg/ml of culture medium. Analysis was done at 488 nm (400 mW), and viable and nonviable cells were distinguished by measuring the fluorescence of propidium iodide using a 590-nm filter [Sasaki et al., 1987].

The incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA was done according to Ormerod and Kubbies [1992]. Control cells or cells treated with peroxovanadiums were cultivated for 24 h in the presence of 10 μM BrdU. The cells were harvested and fixed as described above and incubated in PBS containing HO (1.2 μg/ml) at 37°C for 30 min. The tubes were chilled on ice, and an equal volume of cold PBS containing HO (1.2 μg/ml) and PI (4 μg/ml) was added. The incubation was continued for 30 min on ice. The quenching of the PI fluorescence by the incorporated BrdU was measured by UV excitation. A bivariate cytogram of red (PI > 610 nm) vs. blue (HO 408–505) fluorescence was recorded. DNA strand breaks were tentatively detected as previously described [Mirault et al., 1991], with modifications [Tremblay and Mirault, 1992].

Immunoblotting and Phosphorylation of p34^{cdc2}

Cells were rapidly rinsed in Ham's F10 solution containing 5 μM bpV[phen] and solubilized in 1 ml of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.4 mM Tris-HCl, pH 6.8). The samples were immediately boiled for 5 min and after one freeze-thaw cycle centrifuged 30 min at 12,000*g*; then the pellet was removed. Proteins were measured directly on the supernatant after a trichloracetic acid precipitation step [Lowry et al., 1951]. SDS-PAGE (50 μg protein, 4% stacking and 12.5% resolving gel) was done under reducing conditions [Laemmli, 1970]. Electrophoretic transfer of separated proteins from SDS gels to a nitrocellulose membrane (BA-S 85, Schleicher and Schuell, Keene, NH) was performed essentially as described by Burnette [1970]. The transfer was carried out overnight at 4°C at 200 mA in a buffer containing 20% methanol, 50 mM glycine, 50 mM Tris-HCl, pH 8.0. The membrane was blocked in PBS buffer containing 5% filtered nonfat milk for 1 h at room temperature. This blocking solution was then exchanged for 10 ml of affinity-purified rabbit antimouse C-terminal p34^{cdc2} antibody (Upstate Biotechnology Inc., Lake Placid, NY; 1/5,000 dilution in PBS containing 3% BSA) and gently shaken for 1 h at room temperature, followed by three 15-min washes with 50 ml of PBS containing 0.05% Tween 20. The blots were then revealed by using an enhanced chemiluminescence (ECL) kit according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

For immunoprecipitation experiments, the cells were cultivated in two Costar 75-cm^2 flasks as described and rapidly rinsed at the appropriate time with 10 ml Ham's F10 medium containing 5 μM of bpV[phen]. The cells were solubilized in 0.5 ml per flask in lysis buffer (50 mM Tris-HCL, pH 8.0, 0.15 M NaCL, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM benzamidine, 17 μg/ml PMSF, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin, 1 mM orthovanadate) for 30 min at 4°C and centrifuged at 12,000*g* for 10 min. The supernatant (0.5 ml per flask) was added to 4.5 ml of diluent (10 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 1 mM benzamidine, 17 μg/ml PMSF, 1 mM orthovanadate), and incubated at 4°C for 18 h with 1 μg affinity-purified antiphosphotyrosine antibody [Faure et al., 1992] followed by a 30-min incubation with 30 μl protein A-agarose beads (Boehringer Mannheim, Indianapolis, IN). The pooled beads were washed twice in 1.5 ml of solution A (1% Triton-100, 0.1% SDS, 50 mM Hepes, pH 7.4), twice in solution B (0.1% Triton-X100, 50 mM Hepes, pH 7.4), and finally suspended in Laemmli sample buffer. The proteins were then separated, transferred, and probed with antimouse C-terminal p34^{cdc} antibody as described.

Histone H1 Kinase Activity

Cells were lysed and immunoprecipitated as described except the primary antibody was exchanged for 1 μg of an antihuman p34^{cdc2} carboxyterminal antibody (Leinco Technologies Inc., St. Louis, MO). The agarose beads were washed once with 2 ml of kinase assay buffer (50 mM Hepes, pH 7.4, 40 mM $MgCl_2$, 0.1 mM orthovanadate).

Aliquots of immunoprecipitates (20 μl) were added to a reaction mixture containing 50 mM Hepes, pH 7.4, 40 mM $MgCl_2$, 25 μM [γ-^{32}P] ATP (32 μCi/nmol), and 1 mg/ml histone H1 (Boehringer Mannheim) in a reaction volume of 100 μl. After incubations at 25°C, the reaction was terminated by spotting 50-μl aliquots on

Whatmann P81 paper squares (2.5 × 2.5 cm), which were immersed in tap water and air dried; radioactivity was determined using Universol scintillation solution (ICN Biomedicals, Costa Mesa, CA). The reaction was linear for up to 15 min. Activity was expressed as pmol of phosphate transferred/10 min/10^6 cells.

Phosphatase Assays

Enzymes. DH5 *Escherichia coli* transformed with PGEX-25-BD (a generous gift of Dr P. Russell, The Scripps Institute, La Jolla, Ca) or pGEX-KGcdc25M2, a murine homologue of cdc25B (a generous gift of Dr. T. Hunter, The Salk Institute, San Diego, CA), were grown in L-Broth at 37°C for an optical density of 0.4 and induced at 25°C for 3 h in the presence of 0.3 mM IPTG. Fusion proteins were purified from the bacterial lysate using a glutathione-Sepharose (Pharmacia, Piscataway, NJ) column as described in detail elsewhere [Millar et al., 1991a; Kakizuka et al., 1992]. GST-cdc25 and GST-cdc25B were stored at −20°C (0.1 mg/ml, 25 mM Hepes pH 7.4, 1 mM DTT, 50% glycerol) pending assays.

Assays. We then added 10 μl of enzymes to 180 μl of 25 mM Hepes, pH 7.4, buffer containing the inhibitors and preincubated for 30 min at 25°C. Reactions were initiated by adding 10 μl of p-nitrophenyl phosphate (p-NPP, 10 mM final). The reactions were stopped by adding 800 μl 0.2 M NaOH and absorbance measured at 410 nm against blanks (no enzyme) in a Beckman DU 650 [Tonks et al., 1988].

Immunofluorescence Microscopy

Cells cultured on glass coverslips were fixed in methanol (−20°C, 20 min), rinsed in PBS, incubated for 1 h with antivimentin monoclonal antibody 2E9 produced by a mouse immunized with chick erythrocyte vimentin [Granger and Lazarides, 1982], and positively identified by immunoblotting and immunofluorescence labelling of chick embryo fibroblasts and several mammalian cell lines. The cells were then rinsed abundantly in PBS, incubated for 1 h with a goat anti-mouse IgG antibody coupled with rhodamine, washed three times in PBS, and mounted in with p-phenylene diamine-PBS in glycerol [Platt and Michael, 1983]. The cells were observed and photographed under a Zeiss Axiophot microscope equipped with epifluorescence optics.

RESULTS

Proliferation and Viability of Cells Cultivated in the Presence of Peroxovanadium Derivatives

The effects of bpV[phen] and bpV[pic] on the proliferation and viability of NB and C6 cells were assessed. In preliminary experiments, the addition of 20 μM of bpV[phen] or bpV[pic] to NB cells resulted in cell rounding after 2 h of culture. No apparent changes were observed thereafter for the cells cultivated in the presence of bpV[pic] compared with control cells. However, after 24 h in the presence of 20 μM bpV[phen], 31% of the cells were detached; the viability of attached cells was 86%. After 48 h of culture, 70% of the cells were detached and viability was low (8%). Therefore, we determined the doses that would allow the cells to grow in the presence of bpV[phen]. Concentrations of 0.5 to 2 μM of this compound were not toxic for NB cells, as detachment and decreased viability occurred only at higher doses (5–10 μM) (Table I). In contrast to NB cells, C6 cells were less sensitive to this drug, with detachment occurring at the 10-μM concentration (Table I).

TABLE I. Percentages of Detached Cells With Increased Doses of bpV[phen]*

Concentration of bpV[phen](μM)	% Detached cells (h)					
	NB (%)			C6 (%)		
	24	48	72	24	48	72
0.0	5	6	8	2	2	2
0.5	5	6	4	ND	ND	ND
1.0	6	5	7	1	1	1
2.0	5 (97)	9 (99)	15 (95)	1	1	1
5.0	7 (95)	64 (66)	ND	2 (96)	2 (99)	2 (98)
10	28 (60)	73 (20)	ND	2 (99)	51 (62)	ND
20	ND	ND	ND	2 (89)	73 (15)	ND

*Cells were cultivated in 75 cm^2 flask at an initial plating of 3 × 10^5 for NB and 2 × 10^5 for C6 cells. After 24 h, the culture medium was replaced with fresh medium and the cells were cultivated for an additional 24, 48, or 72 h in the absence or presence of the indicated concentration of peroxovanadium. Cell viability (numbers in parentheses) and number were measured immediately as described in Materials and Methods, directly on the culture medium (detached cells) or after trypsinization (attached cells). Values are the mean of three experiments with S.E ranging from 2 to 8%. ND, not determined. Viability for concentrations 0, 0.5, and 1 μM bpV[phen] was >95%.

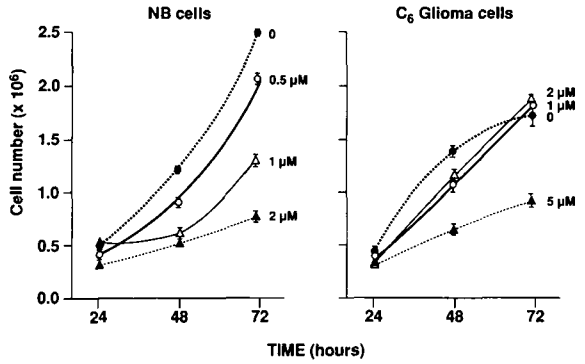

Fig. 1. Proliferation of NB41 and C6 cells treated with bpV-[phen]. NB41 and C6 cells were cultivated in 75-cm^2 flasks at an initial plating of 3×10^5 and 2×10^5, respectively. After 24 h of culture, the medium was changed and the cultures were continued for 24, 48, or 72 h in the presence or absence of the indicated concentrations of bpV[phen]. Cells were trypsinized and counted. Results are the mean ± SD of five to eight experiments.

In the presence of 2 μM bpV[phen], the number of NB cells decreased progressively to 55, 40, and 30% of control values after 24, 48, and 72 h of culture, respectively (Fig. 1). A similar effect, at higher doses, was observed for C6 cells. Hence, at a 5 μM dose of bpV[phen], 45 and 52% of the control values were observed after 48 and 72 h of culture, respectively (Fig. 1).

Similar observations were made at doses ranging from 20 to 100 μM when bpV[pic] was used instead of bpV[phen]. Orthovanadate, used at 1 to 5 μM concentrations, had no apparent effects. At a concentration of 20 μM a small accumulation at G2/M was inconsistently observed. Marked detachment of both cell lines and decreased viability were observed at concentrations of vanadate of 20 to 100 μM and for the ligand 1,10-phenanthroline at a concentration of 2 μM. This latter observation suggests degradation of the compound bpV[phen] would be toxic to the cultured cells.

Cell-Cycle Analysis of Asynchronous NB and C6 Cells Cultures

The relative number of cells in the different phases of the mitotic cycle was estimated by measuring their intracellular DNA contents by fuorescence-activated cell sorting (Tables II and III, Fig. 2). After 24, 48, and 72 h of culture, control NB cells in active proliferation displayed similar cell-cycle transition profiles: about 50% of the cells in G1, 30% in S, and 20% in the G2/M phases.

No changes occurred when the cells were treated with 0.1 and 0.25 μM bpV[phen] (not shown). At 0.5 μM and after 48 h of culture, a significant ($P < 0.005$) 30% decrease of the percentage of cells in G1 was observed, together with a concomitant increase in G2/M. This latter percentage was further significantly increased as a function of dose and time (Table II). Thus, after 48 and 72 h of culture and at the 2 μM dose, 60 to 70% of the cells were in G2/M. The same effect was observed with bpV[pic] but at 10-fold greater doses (Fig. 2). At higher doses of this latter compound (30, 40 μM), the effect was more pronounced, but viability was markedly decreased as assessed by the exclusion of PI.

Control C6 cells presented a rather different cell cycle profile with 70% of the cells in the G1 transition after 48 h of culture. Augmented accumulation at the G2/M transition was observed in the presence of 5 μM bpV [phen] (Table III). No effect was observed with bpV[pic], as elevated doses of this compound (30–100 μM) resulted in low cell viability.

Intracellular Levels of RNA and Protein and Expression of Vimentin in NB Cells Treated With bpV[phen]

The ratios of RNA to DNA and RNA to protein levels were used as an index of unbalanced growth [Crissman et al., 1985a,b). Table IV shows that for doses of 0.5 to 5 μM bpV[phen], the ratios were unaltered at each transition of the cycle. A general, pronounced decrease was observed thereafter with increasing doses of bpV[phen].

To evaluate possible oxidative damage to DNA, we measured DNA breakages using a very sensitive ^{32}P-3′ end-labeling technique [Mirault et al., 1991; Tremblay and Mirault, 1992]. No labeled DNA smears or fragments were observed after the cultures were exposed for 48 and 72 h to pV derivatives at 50 μM (not shown).

To evaluate further the integrity of the treated cells at the time of the maximal effect of pV on the cycle, we investigated the expression and intracellular organization of vimentin in NB cells, a component of the intermediate filament cytoskeleton in most mammalian cultured cells [Traub, 1985], by using indirect immunofluorescence microscopy. In control NB cells, we observed a characteristic perinuclear pattern of staining that was expanding in the cytoplasm [Traub, 1985] and dense in the neuritic net-

TABLE II. Effect of bpV[phen] on the Mitotic Cycle of NB Cells*

bpV[phen] concentration (μM)	24 h (% cells)			48 h (% cells)			72 h (% cells)		
	G1	S	G2	G1	S	G2	G1	S	G2
0	44.1 ± 6.8 (9)	30.0 ± 3.9 (9)	24.4 ± 5.8 (9)	51.4 ± 6.6 (11)	27.9 ± 4.8 (10)	20.7 ± 6.3 (10)	48.7 ± 6.7 (4)	30.2 ± 8.4 (4)	21.0 ± 5.6 (4)
0.5	41.0 ± 2.8 (2)	29.0 ± 1.4 (2)	30.0 ± 4.2 (2)	37.5 ± 8.4[b] (4)	30.5 ± 6.8 (4)	29.5 ± 3.9 (4)	ND	ND	ND
1	33.5 ± 4.9 (2)	34.0 ± 5.6 (2)	32.5 ± 10.6 (2)	24.0 ± 7.8[c] (5)	26.4 ± 4.4 (5)	49.8 ± 7.1[d] (5)	16 (1)	25 (1)	59 (1)
2	33.0 ± 6.9[a] (4)	36.7 ± 6.8 (4)	30.2 ± 6.0 (4)	12.8 ± 4.4 (5)	29.6 ± 3.7 (5)	57.6 ± 7.6[e] (5)	15.2 ± 7.6 (5)	19.8 ± 1.9 (5)	67 ± 5.9 (5)

*The percentage of NB cells in each phase of the cell cycle was estimated by measuring the intracellular DNA content. Cells were cultivated in 75 cm^2 flasks with an initial plating of 300,000. After 24 h of culture, the medium was changed and the cultures were continued for 24, 48, or 72 h in the presence or the absence of bpV[phen]. Cells were trypsinized, fixed and DNA was measured as described in Materials and Methods. Numbers in parentheses represent the number of independent cultures. Results are the mean ± SD. ND, not determined. Versus 0 dose control: [a]$P < 0.02$; [b]$P < 0.005$; [c]$P < 0.0001$; [d]$P < 0.0001$; [e]$P < 0.0001$.

TABLE III. Effect of bpV[phen] on the Mitotic Cycle of C6 Cells*

bpV[phen] concentration (μM)	24 h (% cells)			48 h (% cells)			72 h (% cells)		
	G1	S	G2	G1	S	G2	G1	S	G2
0	54.7 ± 5.0 (12)	27.2 ± 6.4 (12)	17.9 ± 4.0 (12)	67.1 ± 10.6 (9)	18.2 ± 8.5 (9)	14.7 ± 3.8 (9)	79.0 ± 2.8 (2)	13.0 ± 4.2 (2)	8.0 ± 1.4 (2)
2.5	48.5 ± 0.7[a] (2)	31.5 ± 0.7 (2)	20.0 ± 0.0 (2)	51.2 ± 1.9[d] (4)	22.2 ± 4.7 (4)	26.5 ± 1.5[e] (4)	48 (1)	28 (1)	24 (1)
5	38.0 ± 9.1[b] (8)	48.9 ± 7.6[c] (8)	21.2 ± 8.5 (8)	22.0 ± 2.8 (2)	36.0 ± 0.0 (2)	41.5 ± 2.1 (2)	19 ± 2.0 (3)	20 ± 1.1 (3)	61 ± 2.0 (3)
10	68.4 ± 5.7 (5)	19.6 ± 2.5 (5)	11.8 ± 6.6 (5)	43 (1)	35 (1)	10 (1)	ND	ND	ND
20	62.7 ± 2.4 (4)	21.7 ± 0.5 (4)	15.2 ± 2.7 (4)	ND	ND	ND	ND	ND	ND

*The percentage of C6 cells in each phase of the mitotic cycle was estimated by measuring the intracellular DNA content. C6 cells were cultivated in 75 cm^2 flasks with an initial plating of 2×10^5 cells. After 24 h of culture, the medium was changed and the cultures were continued for 24, 48, or 72 h in the presence or the absence of bpV[phen]. Cells were trypsinized, fixed, and DNA content measured as described in Materials and Methods. Numbers in parentheses represent the number of independent cultures. Results are the mean ± SD; ND, not determined. Versus 0 dose control: [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$; [d]$P < 0.025$; [e]$P < 0.05$.

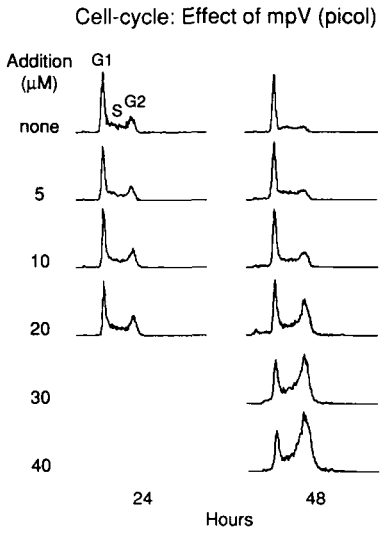

Fig. 2. Accumulation of asynchronous cultures treated with bpV[pic] at the G2/M transition of the cell cycle. NB41 cells were cultivated in 75-cm^2 flasks with an initial plating of 3 × 10^5. After 24 h, the medium was changed and the culture was continued for 24 and 48 h in the presence or absence of the indicated concentration of bpV[pic]. Cells were trypsinized, fixed, and DNA measured using a cell sorter as described in Materials and Methods.

work. Cells treated with 2 μM bpV[phen] for 48 and 72 h displayed the same intracellular pattern. Thus, no morphological changes or disorganization of the cytoskeleton network were apparent in treated cells (Fig. 3).

Reversibility of the G2/M Block

In preliminary experiments, replacing the media of cultures treated with 2 μM bpV[phen] for 48 and 72 h with fresh media reversed the G2/M block. After a release of 24 h, proliferation and cell-cycle profiles were similar to control cultures. These release experiments were continued by measuring BrdU incorporation into newly synthesized DNA to assess whether cells in G2/M were able to reenter the cycle. The results shown in Figure 4 confirmed that control NB cells could complete one cycle in 24 h, as BrdU was incorporated in most cells during this period (Fig. 4A: 82.6% incorporation). In cultures maintained for 48 h in the presence of 2 μM bpV-[phen] and exposed with BrdU and 2 μM bpV-[phen] for the next 24 h, 48.3% of the cells incorporated BrdU (total number of cells in G2/M: 61.0%). Of these, 16.2% had undergone at least one cycle, as they were found in G1 (Fig. 4D). In a flask where, after 48 h of treatment with bpV[phen], the medium was replaced by fresh medium containing 10 μM BrdU for a further 24 h of culture, the cytogram showed that most cells (75.4%) had incorporated the dye into their DNA (Fig. 4E). Taken together, these data demonstrate that treated cells were not definitively arrested but still participated, albeit at a much slower rate in the cycle.

p34^{cdc2} Kinase Tyrosine Phosphorylation Levels and Kinase Activity Towards Histone H1

The p34^{cdc2} kinase is an important regulator of the onset of mitosis [Norbury and Nurse, 1992]. To determine whether hyperphosphorylation of p34^{cdc2}, and hence reduced kinase activity, is a consequence of the treatment of cells by bpV[phen], we measured the p34^{cdc2} activity levels toward histone H1 in treated cells. Figure 5 shows that the steady-state levels of enzyme in both cell lines were comparable in control and treated cultures as assessed by immunoblotting on whole cell extracts (Fig. 5A,C). However, p34^{cdc2} was detected only in antiphosphotyrosine immunoprecipitates of cells treated with bpV[phen]. This signal disappeared after a further 24 h of culture in the absence of bpV[phen].

The catalytic activity measured in NB cells of specifically immunoprecipitated p34^{cdc2} toward histone H1 (Fig. 6A) showed that after 24 h of culture with 2 μM bpV[phen], p34^{cdc2} activity

TABLE IV. Mean Values of the RNA to DNA and RNA to Protein Ratios*

bpV[phen] (μM)	RNA to DNA			RNA to protein		
	G1	S	G2/M	G1	S	G2/M
0	1.00 ± 0.01	0.95 ± 0.01	0.94 ± 0.02	1.00 ± 0.01	0.96 ± 0.03	1.02 ± 0.03
0.5	0.96 ± 0.05	0.91 ± 0.01	0.99 ± 0.01	0.99 ± 0.04	0.89 ± 0.05	0.96 ± 0.05
2	0.93 ± 0.04	0.91 ± 0.02	0.89 ± 0.05	0.91 ± 0.09	1.07 ± 0.13	0.97 ± 0.08
5	0.91 ± 0.05	0.89 ± 0.05	0.91 ± 0.06	0.90 ± 0.05	0.87 ± 0.05	0.95 ± 0.06
10	0.83 ± 0.06	0.82 ± 0.08	0.78 ± 0.03	0.82 ± 0.06	0.88 ± 0.09	0.82 ± 0.03
20	0.46 ± 0.1	0.43 ± 0.11	0.47 ± 0.11	0.53 ± 0.12	0.54 ± 0.14	0.57 ± 0.13

*Mean values of ratios ± SD (n = 3) were obtained from NB41 cells cultures treated for 48 h. Fluorescence units were standardized in individual experiments using C6 cells as an internal control.

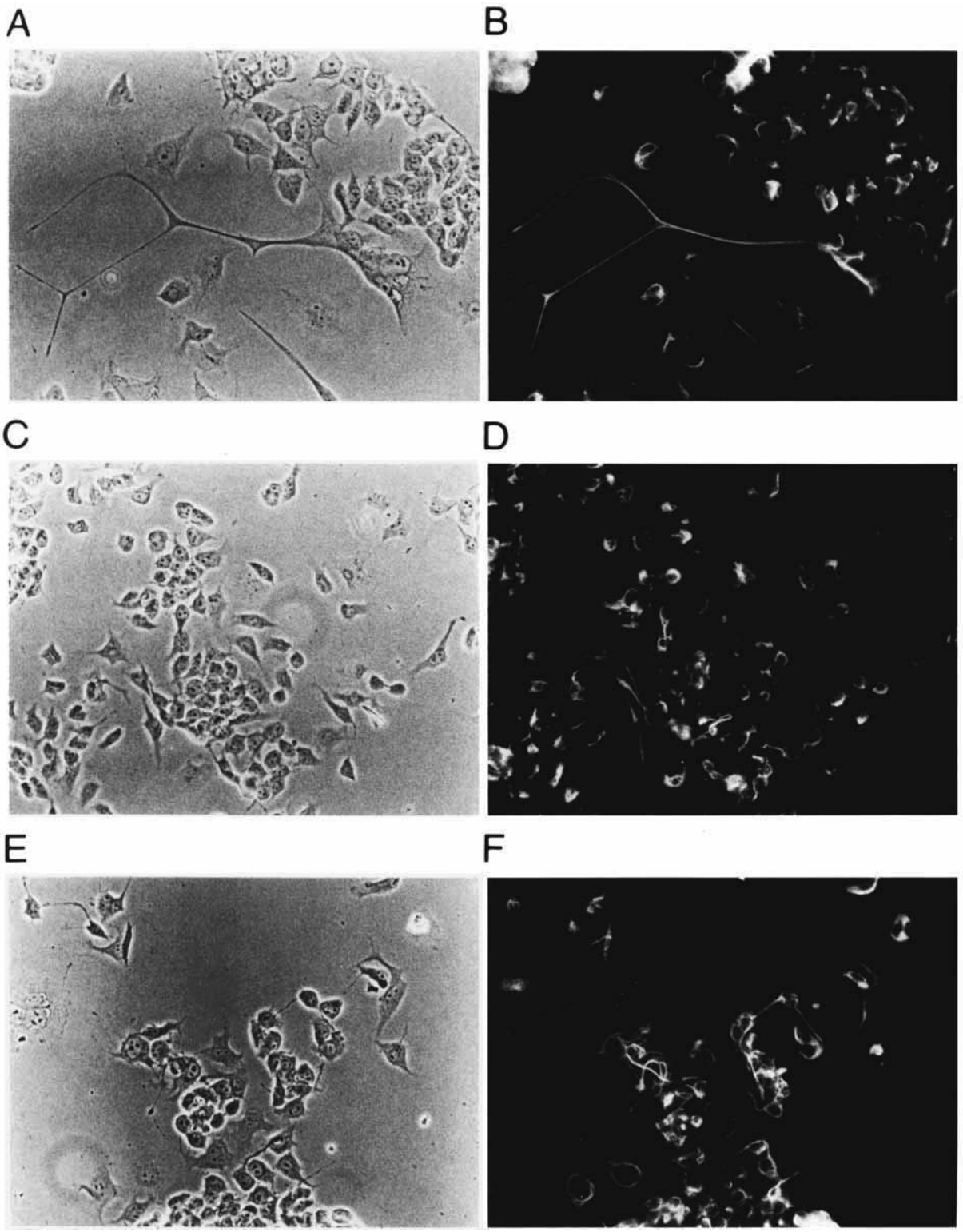

Fig. 3. Localization of vimentin by indirect immunofluorescence in NB 41 cells treated or not with 2 μM bpV[phen]. Cells were cultivated for 48 h (A, D) or 72 h (E, F). **A:** Phase contrast, untreated cells; **B:** fluorescence, untreated cells; **C:** phase contrast, treated cells; **D:** fluorescence, treated cells; **E:** phase contrast, treated cells; **F:** fluorescence, treated cells.

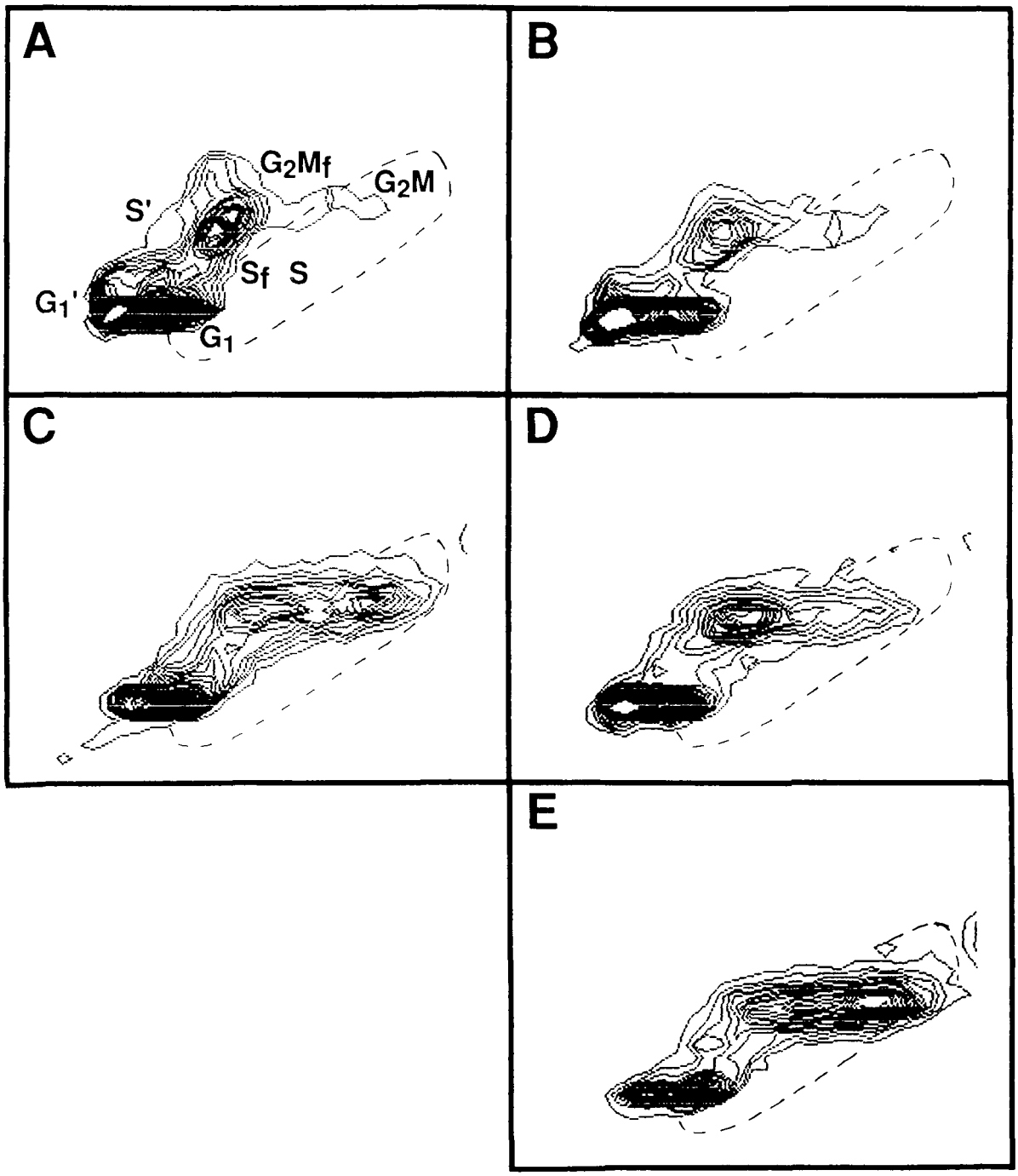

Fig. 4. Incorporation of BrdU into newly synthesized DNA of NB41 cells treated or not with bpV[phen]. NB41 cells were cultivated in the presence or absence of 2 μM bpV[phen]; 10 μM BrdU was added during the last 24 h before harvesting. The dotted line represents the target for cells that did not incorporate the dye. Abscissa: red PI; ordinate: blue-Hoechst.

	G_1'	G_1	S	S_f	G_2M	G_2/M_f	T
A	46.6	10.3	2.3	20.3	4.8	15.7	82.6
B	21.9	11.6	17.3	9.9	29.6	9.7	41.5
C	45.2	3.2	7.3	20.5	13.2	10.6	76.3
D	16.2	10.3	2.4	9.5	39.3	22.3	48.3
E	45.1	6.0	2.5	9.4	13.3	23.7	78.2

Nomenclature as in: Ormerod and Kubbies [1992]. G_1', cells in G_1 which have completed one cycle; S, cells incorporating the BrdU; S_f, slower cells incorporating lesser amounts of BrdU; G_2/M_f cells in G_2M that have incorporated the BrdU. **A:** Control cells cultivated for 24 h in the presence of 10 μM BrdU. **B:** Cells treated with 2 μM bpV[phen] for 24 h in the presence of 10 μM BrdU. **C:** Cells treated for 24 h with 2 μM bpV[phen] and released for an additional 24 h with fresh medium containing 10 μM BrdU. **D:** Cells treated for 48 h with 2 μM bpV[phen] and in the presence of 10 μM BrdU for the last 24 h. **E:** Cells treated for 48 h with 2 μM bpV[phen] and released for an additional 24 h in the presence of 10 μM BrdU.

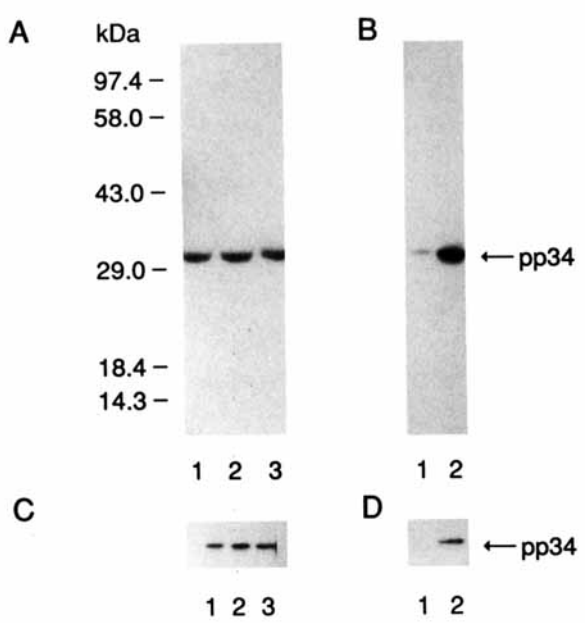

Fig. 5. Phosphorylation of p34^{cdc2} in NB and C6 cells treated with bpV[phen]. Cells were cultivated for 48 h in the absence or presence of bpV[phen]. Total homogenates were submitted directly to immunoblotting with antibody to p34^{cdc2} (A,C). In parallel, the extracts were immunoprecipitated with antiphosphotyrosine antibody, and the immunoprecipitates were subjected to immunoblotting with antibody to p34^{cdc2} (B,D). The following were the concentrations of bpV[phen] in the cultures. **A** (NB cells): *lane 1,* 0 μM; *lane 2,* 1 μM; *lane 3,* 2 μM; **B** (NB cells): *lane 1,* 0 μM; *lane 2,* 2 μM; *lane 3,* 5 μM; **D** (C6 cells): *lane 1,* 0 μM; *lane 2,* 5 μM.

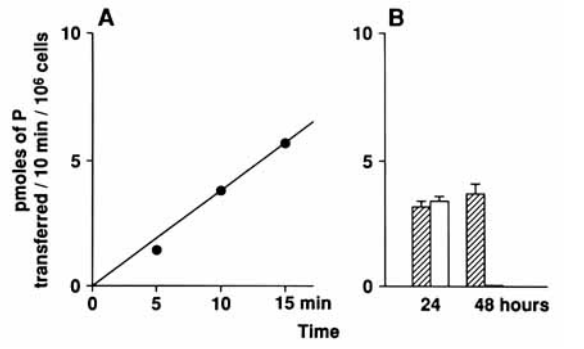

Fig. 6. Kinase activity of p34^{cdc2} toward histone H1 in NB 41 cells treated with bpV[phen]. NB 41 cells were cultivated for 24 or 48 h in the presence of 2 μM bpV[phen]. Cells were solubilized, p34^{cdc2} immunoprecipitated, and assayed for kinase activity as described in Materials and Methods. **A:** Time course of histone H1 phosphorylation at 25°C by p34^{cdc2} immunoprecipitated from untreated NB cells. **B:** Phosphorylation (±1/2 the range of n = 2, 10 min at 25°C) of histone H1 by p34^{cdc2} immunoprecipitated from cells cultivated for 24 and 48 h in the presence (open bars) or absence (dashed bars) of bpV[phen].

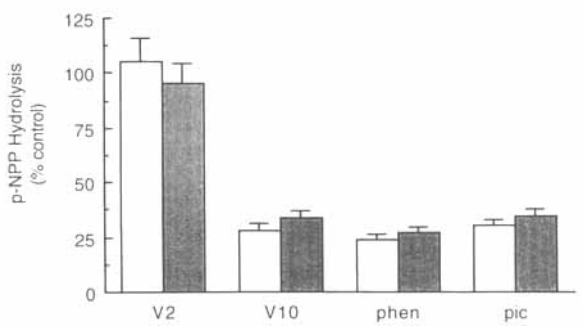

Fig. 7. Inhibition of GST-cdc25 and GST-cdc25M2 activities. The ability of bpV[phen] and bpV[pic] to inhibit the catalytic activity of GST-cdc25 and GST-cdc25M2 was assessed. Values represent a percentage of activity obtained with the enzymes in the absence of inhibitors. Assays were done in triplicate as described in Materials and Methods (1 μg GST-cdc25, range: 0.195–0.260; 1 μg GST-cdc25M2, range: 0.155–0.215 O.D. at 410 nm, 2 h at 25°C). V2: 2 μM orthovanadate; V10: 10 μM orthovanadate; phen: 2 μM bpV[phen]; pic: 2 μM bpV[pic] (±1/2 range of two experiments). White bars, GST-cdc25; Grey bars, GST-cdc25M2.

was unchanged. After 48 h of culture in the presence of bpV[phen], no kinase activity was detectable (Fig. 6B).

Inhibition of cdc25 and cdc25MB In Vitro

We verified whether bpV[phen] and bpV[pic] could inhibit cdc25 and cdc25B, two distant cdc25s. The catalytic activity of both enzymes was shown by the ability to dephosphorylate p-nitrophenyl phosphate (p-NPP) to p-nitrophenolate [Millar et al., 1991a; Kakizuka et al., 1992]. Low but consistent activity was measured. This phosphatase activity was inhibited by vanadate at a concentration of 10 μM. A preincubation step was necessary to obtain a marked inhibition, which is consistent with the possibility that vanadate slows but does not completely block turnover of the phosphoryl group. Similarly, the compounds bpV[phen] and bpV[pic] inhibited GST-cdc25 and GST-cdc25B activities. These compounds were substantially more potent than vanadate, as a similar inhibition was observed at a 2 μM concentration. However, no differences in the potency of these two compounds were observed under these conditions (Fig. 7).

DISCUSSION

In cultures treated continuously with bpV[phen] or bpV[pic], two highly potent PTP inhibitors, cellular proliferation markedly decreased, and there was progressive accumulation of cells at G2/M as assessed by fluorescence-activated cell-sorting analysis of DNA content. Treatment

with the bpVs was not toxic, as judged by the ability of treated cells to exclude propidium iodide (Table I), the absence of detectable DNA damage, and the reversibility of the inhibitory effect of these compounds after being removed from the incubation medium (Fig. 4). At maximal effective doses, total cellular protein and RNA were normally synthesized, and the RNA/protein ratio, a sensitive index of unbalanced growth [Crissman et al., 1985b], was unchanged (Table IV). Phase microscopy and indirect immunofluorescence showed that the expression and localization of vimentin were unaltered and that the morphology and cytoskeleton pattern of the treated cells were undisturbed (Fig. 3). Thus, at the dosage employed, prolonged incubations with the bpV compounds did not induce gross cellular changes.

The simplest hypothesis to explain restriction at the G2/M transition is that a PTP(s) controlling entry of the cell into mitosis was a target for the drugs. This was supported by a previous study showing that vanadate inhibited $p34^{cdc2}$ dephosphorylation [Morla et al., 1989]. Several phosphatases acting on the cell cycle are potential targets for the pV compounds. Thus, it was documented that the overexpression of the dual phosphatase cdi delayed the G1/S transition [Gyuris et al., 1993] and overexpression of pyp1 and pyp2 delayed entry into mitosis by acting on the inhibitory wee1 pathway [Millar et al., 1992]. In fission yeast, M-phase entry is governed by the mitotic inducer genes *cdc25*$^+$ and *cdrl*$^+$/*niml*$^+$ [Feilotter et al., 1991; Norbury and Nurse, 1992] and two mitotic inhibitor genes, *weel*$^+$ and *mikl*$^+$ [Lundgren et al., 1991; Norbury and Nurse, 1992]. The products of these genes regulate the function of $p34^{cdc2}$/cyclin B in initiation of the M-phase. The *weel*$^+$, *cdrl*$^+$/*niml*$^+$, and *mikl*$^+$ genes encode protein kinases, whereas the *cdc25* gene product promotes dephosphorylation of $p34^{cdc2}$ [Charbonneau and Tonks, 1992; Norbury and Nurse, 1992]. The mitotic activity of fission yeast cdc25 is highly conserved in Drosophila [Edgar and O'Farrel, 1990], mice [Kakizuka et al., 1992], and humans [Galactionov and Beach, 1992; Nagata et al., 1991; Sadhu et al., 1990]. Mouse and human *cdc25* genes functionally complemented for cdc25 mutations in yeast [Sadhu et al., 1990; Millar et al., 1991b; Nagata et al., 1991; Galactionov and Beach, 1992; Kakizuka et al., 1992] showing that despite their divergence in sequence the vertebrate enzymes have the capacity to perform the same function as the yeast cdc25 in vivo. These observations suggested that cdc25s are important regulators of growth in mammalian cells. Our studies provide strong support for this. Thus, treatment with bpVs resulted in hyperphosphorylation of $p34^{cdc2}$, as depicted in Figures 5 and 6, along with the loss of immunoprecipitated kinase activity. This is the predicted consequence of the inhibition of PTPs of the cdc25 subfamily, which should result in the hyperphosphorylation of $p34^{cdc2}$ and a concomitant reduction in its catalytic activity.

In human cells, the cdc25 phosphatases constitute a family of at least three distinct isoforms encoded by separated genes named A, B, and C [Galactionov and Beach, 1991; Sahdu et al., 1990] which appear to have distinct roles [Hoffman et al., 1994; Jinno et al., 1994]. Two were cloned in mouse, the cdc25B homologue [Kakizuka et al., 1992; Sebastian et al., 1993] and cdc25M1 which is closer in sequence to cdc25 and the human cdc25C [Nargi and Woodford-Thomas, 1994]. Figure 7 shows that both bpVs have comparable inhibitory potency in respect to the catalytic activity of the yeast cdc25 and cdc25B. Thus there is no specificity for these compounds as cdc25 inhibitors in this in vitro assay. However, given the low activity measured in this and previous studies with a number of artificial substrates [Millar et al., 1991a; Kakizuka et al., 1992], and the fact that cdc25, cdc25C and cdc25A are strongly activated in vivo by phosphorylation at activating site(s) [Charbonneau et al., 1994; Strausfeld et al., 1994; Hoffman et al., 1994], which may well influence the substrate specificity, the indirect assay using $p34^{cdc2}$ hyperphosphorylation in living cells may actually be a better index reflecting the action of bpVs on a dephosphorylation event at G2/M. The 24-h lag before a decrease in histone H1 activity was observed probably resulted from the presence of nonphosphorylated $p34^{cdc2}$ molecules and the time necessary to shift the population to the phosphorylated form(s). Furthermore, cells were not totally arrested as assessed by the continuous growth and incorporation of BrdU into cellular DNA, which may indicate that a subset of kinase escapes the inhibitory phosphorylation. The presence of newly synthesized, nonphosphorylated $p34^{cdc2}$ or the overlapping action of other kinases, may compensate in part for the loss of $p34^{cdc2}$ activity [Pelech and Sanghera, 1992]. This escape is unlikely to be the result of the degradation of bpV[phen], as its

effect becomes apparent after 48 h of treatment and is also observed at higher doses of the compound. In addition, the ligand 1, 10 phenanthroline, which would be released following the degradation of bpV[phen], was toxic to NB41 or C6 cells at a concentration of 2 μM, suggesting that it is not released in significant quantities during the incubation.

Marked differences were observed from one cell type to another and according to the nature of the synthetic pV compound used; however, final cellular responses were identical. The compounds bpV[phen] and bpV[pic] were comparably potent as inhibitors of GST-cdc25s in vitro (Fig. 7). However, bpV[pic] was less potent than bpV[phen] in promoting arrest at G2 in NB cells. In addition, C6 cells were less sensitive to bpV[phen] than NB cells, and bpV[pic] had no effect in these cells at nontoxic doses. Thus, other yet undetermined factors influence the potency of these compounds, such as the efficiency which they are delivered to their site(s) of intracellular action, their metabolism within particular cells, and their relative specificities as PTP inhibitors in vivo. Similarly, we were unable to observe such a marked effect with vanadate in either cell line while a 50 μM dose was employed on mouse 3T3 cells [Morla et al., 1991]. This may reflects its 5-fold lower potency to inhibit cdc25 in vitro and the attainment of toxic activity in these cell cultures at the PTP inhibitory doses. In addition the aqueous chemistry of vanadate is complex [Posner et al., 1990] and it is probable that vanadate is easily reduced to vanadyl intracellularly [Cantley and Aisen, 1979], which has been shown to stimulates yeast cells in culture [Willsky et al., 1984]. Vanadate is also easily chelated by EDTA, peptides, and proteins [Crans et al., 1989]. Thus, it inhibits PTPs in the millimolar range in complex protein mixtures [Faure et al., 1992], whereas it inhibits purified enzymes in the micromolar range in this and other studies [Tonks et al., 1988].

The present study demonstrates that prolonged incubation of two highly proliferative cell lines with potent and structurally defined PTP inhibitors markedly decreased cell proliferation by acting selectively at the G2/M transition of the mitotic cycle. We did not formally demonstrate that this was due to selective inhibition of a mouse cdc25C in vivo. A selective block at G2/M may be due to a higher dependency of $p34^{cdc2}$/cyclinB than other cdks/cyclin complexes on dephosphorylation events. In this regard, an efficient positive feedback loop of activation was reported for p34cdc2/cyclin B/cdc25C [Clarke et al., 1993; Strausfeld et al., 1994; and cdc25 phosphorylation appeared to occur prior to p34cdc2/cyclinB activation [Charbonneau et al., 1994]. The molecular mechanism(s) underlying the apparent potency and selectivity of these pV compounds remains to be further studied.

ACKNOWLEDGMENTS

This work was supported by Fonds de la Recherche en Santé du Québec (R.F.) and Nordic Merrel Dow Research, Laval, Québec, and the MRC (B.I.P.). We thank Dr. Marc Edouard Mirault, who performed the analysis of DNA nicks in his laboratory, Dr. Jesse B. Ng for preparing the pV compounds, Dr. P. Russell for the gift of the GST-cdc25 construct, and Dr. T. Hunter for the gift of the GST-cdc25M2 (cdc25B) construct.

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