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Protein Phosphatase $2A_1$ Is the Major Enzyme in Vertebrate Cell Extracts that Dephosphorylates Several Physiological Substrates for Cyclin-dependent Protein Kinases

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Okadaic acid (2 nM) inhibited by 80–90% the protein phosphatase activities in diluted extracts of rat liver, human fibroblasts, and *Xenopus* eggs acting on three substrates (high mobility group protein-I(Y), caldesmon and histone H1) phosphorylated by a cyclin-dependent protein kinase (CDK) suggesting that a type-2A phosphatase was responsible for dephosphorylating each protein. This result was confirmed by anion exchange chromatography of rat liver and *Xenopus* extracts, which demonstrated that the phosphatases acting on these substrates coeluted with the two major species of protein phosphatase 2A, termed $PP2A_1$ and $PP2A_2$. When matched for activity toward glycogen phosphorylase, $PP2A_1$ was five- to sevenfold more active than $PP2A_2$ and 35-fold to 70-fold more active than the free catalytic subunit ($PP2A_C$) toward the three CDK-labeled substrates. Protein phosphatases 1, 2B, and 2C accounted for a negligible proportion of the activity toward each substrate under the assay conditions examined. The results suggest that $PP2A_1$ is the phosphatase that dephosphorylates a number of CDK substrates in vivo and indicate that the A and B subunits that are associated with $PP2A_C$ in $PP2A_1$ accelerate the dephosphorylation of CDK substrates, while suppressing the dephosphorylation of most other proteins. The possibility that $PP2A_1$ activity is regulated during the cell cycle is discussed.

INTRODUCTION

Two classes of protein kinase that are currently the focus of intense research interest are the cyclin-dependent protein kinases (CDKs) that regulate cell cycle progression (Pines and Hunter, 1991) and the mitogen-activated protein kinases (MAPKs) that are stimulated by signals that cause cells to proliferate or differentiate (Sturgill and Wu, 1991). Although these two types of protein kinase play distinct physiological roles and are activated at different times in vivo, they resemble one another in phosphorylating proteins at serine and threonine residues that are followed by a proline residue. The preferred consensus sequence for phosphorylation by CDKs is (Lys/Arg)-Ser/Thr-Pro-Xaa-Lys/Arg (Chambers and Langan, 1990), whereas the sequences phosphorylated most frequently by MAPKs are Pro/Leu-Xaa-Ser/Thr-Pro- (Stokoe *et al.*, 1992; Sutherland *et al.*, 1993).

The identification of protein phosphatases that oppose the actions of CDKs and MAPKs is clearly of considerable interest, because they must play a major role in determining the level of phosphorylation of CDK and MAPK substrates in vivo. We recently reported that protein phosphatase 2A (PP2A) accounts for about 90% of the phosphatase activity in rat liver cytosol toward histone H1 phosphorylated by a CDK (Sola *et al.*, 1991) and for 96% of the activity in rat brain extracts toward the microtubule-associated protein tau phosphorylated by MAPK (Goedert *et al.*, 1992a). Furthermore, the trimeric species of PP2A, termed $PP2A_1$ (consisting of the catalytic [C] subunit complexed to A and B subunits), was more effective in dephosphorylating both substrates than the dimeric species $PP2A_2$ (consisting of the C and A subunits only) that, in turn, was more effective than the free C subunit, termed $PP2A_C$. When all three forms of PP2A were matched for activity toward glycogen

phosphorylase, $PP2A_1$ was six- to eightfold more active than $PP2A_2$ and 30- to 50-fold more active than $PP2A_C$ toward either histone H1 or tau (Sola *et al.*, 1991; Goedert *et al.*, 1992a). The results suggested that the A and B subunits enhance activity toward histone H1 and tau, while suppressing activity toward glycogen phosphorylase. Other investigators (Agostinis *et al.*, 1992) also found that purified trimeric forms of PP2A were 10- to 20-fold better histone H1 phosphatases than dimeric forms of PP2A and 100-fold better histone H1 phosphatases than $PP2A_C$ and that PP1 dephosphorylates histone H1 rather poorly. Similar results were obtained with several synthetic peptide substrates phosphorylated at Ser/Thr-Pro motifs by a CDK (Agostinis *et al.*, 1992).

Histone H1 is an atypical substrate for PP2A because, in common with certain other highly basic proteins, it is a potent activator of $PP2A_1$ and $PP2A_2$ (reviewed by Cohen, 1989), whereas tau is not normally phosphorylated by MAPK in vivo and only becomes highly phosphorylated at Ser-Pro and Thr-Pro sequences in the brains of patients with Alzheimer's disease (Goedert *et al.*, 1992b). It was therefore important to extend these studies to additional physiological substrates and different cells. In this paper we demonstrate that $PP2A_1$ is the major phosphatase acting on three different CDK substrates in extracts prepared from several vertebrate tissues.

MATERIALS AND METHODS

Materials

The catalytic subunit of protein phosphatase-1($PP1_C$) and $PP2A_C$ were purified from rabbit skeletal muscle by Dr. D. Schelling at Dundee (Cohen *et al.*, 1988) and caldesmon from chicken gizzard smooth muscle (Bretscher, 1984). Histone H1 was isolated from calf thymus (Langan, 1982). Human High Mobility Group protein-I(Y) (HMG-I(Y))[1] and inhibitor-2, both expressed in bacteria, were generous gifts from Dr. R. Reeves (Department of Biochemistry, Washington State University) and Dr. A. DePaoli-Roach (Department of Biochemistry, University of Indiana), respectively. Okadaic acid was provided by Dr. Y. Tsukitani (Fujisawa Pharmaceutical, Tokyo).

Preparation of ^{32}P-labeled Substrates

^{32}P-labeled glycogen phosphorylase (10^6 cpm/nmol) containing 1.0 mol phosphate per mol subunit was prepared by phosphorylation with phosphorylase kinase (Cohen *et al.*, 1988). ^{32}P-labeled histone H1 (2.1–2.4 mol phosphate per mol protein), ^{32}P-labeled HMG-I(Y) (0.75–0.9 mol phosphate per mol protein), and ^{32}P-labeled caldesmon (0.3–0.9 mol phosphate per mol protein) were prepared by phosphorylation for 6–16 h at 37°C in the presence of Mg-γ^{32}P[ATP] (4 × 10^6 cpm/nmol) and growth-associated histone H1 kinase (a CDK homologue) from randomly growing Novikoff rat hepatoma cells (Chambers and Langan, 1990). The ^{32}P-labeled histone H1 was precipitated with 25% (by weight) trichloroacetic acid, and after standing on ice for 10 min, the protein was collected by centrifugation for 3 min at 13 000 × *g*. The histone H1 pellet was washed twice with 25% (by weight) trichloroacetic acid, once with acetone:HCl (200:1), and once with acetone. After evaporation of the acetone at ambient temperature, histone H1 was redissolved in 10 mM tris(hydroxymethyl)-aminomethane (Tris)/HCl pH 8.0 to a final concentration of 4 mg/ml. ^{32}P-labeled HMG-I(Y) and ^{32}P-labeled caldesmon were freed from ATP by gel-filtration on Sephadex G50 Superfine (Pharmacia, Hilton Keynes, United Kingdom) equilibrated in 50 mM Tris/HCl pH 7.0, 0.1 mM Na-ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate-EGTA) and 0.1% (by volume) 2-mercaptoethanol.

Previous work has established that our CDK preparation phosphorylates the same tryptic peptides in vitro as cyclin B-p34cdc2 from *Xenopus* eggs (Langan *et al.*, 1989) and that the serine and threonine residues phosphorylated in histone H1 (Langan *et al.*, 1980) and HMG-I(Y) (Reeves *et al.*, 1991) all lie in CDK consensus sequences that are phosphorylated in vivo during mitosis (Langan *et al.*, 1981; Nissen *et al.*, 1991).

Protein Phosphatase Assays

The dephosphorylation of glycogen phosphorylase was carried out in duplicate 30 μl incubations at 30°C in the presence of 5 mM caffeine as described previously (Cohen *et al.*, 1988; Cohen *et al.*, 1989; Cohen, 1991). The dephosphorylation of HMG-I(Y), caldesmon, and histone H1 was carried out in an identical manner except that caffeine was omitted. After terminating the reactions with 0.1 ml of 25% (by weight) trichloroacetic acid, 0.2 ml of 1.25 mM KH_2PO_4 in 0.5 M H_2SO_4 was added, followed by 0.4 ml of isobutanol:heptane (1:1 by volume) and 0.1 ml of 5% (by weight) ammonium molybdate. The mixture was vortexed and after allowing the phases to separate, 0.2 ml of the upper layer was removed, mixed with 1.0 ml of xylene-based scintillant, and counted. This procedure, in which an inorganic phosphate/molydate complex is extracted into isobutanol/heptane, was adopted because histone H1 and HMG-I(Y) are difficult to precipitate quantitatively with trichloroacetic acid leading to high reaction blanks. The final substrate concentrations in the assay are given in the legends of tables and figures presented under RESULTS.

Preparation of Tissue Extracts

Xenopus eggs and finely chopped rat livers were homogenized in 3 vol of ice-cold 2 mM Na-EDTA, 2 mM Na-EGTA, 250 mM sucrose, 0.1% (by vol) 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM benzamidine using three strokes of a Potter-Elvehjem homogenizer (Fisons, Loughburough, United Kingdom). Human foreskin fibroblasts (1–2 × 10^6 cells) lysed in 50 mM Hepes pH 7.0, 78 mM KCl, 10 mM Na-EGTA, 8.37 mM $CaCl_2$, 4 mM $MgCl_2$, 0.5 mM spermine, 0.15 mM spermidine, 1 mM dithiothreitol, 0.3 mM PMSF, 40 kallikrein inhibiting units/ml aprotinin, 6 μg/ml leupeptin, 10 μg/ml cytochalasin D, and 0.2% (by weight) NP40 were kindly provided by Dr. C. Hutchison (Department of Biological Sciences, University of Dundee).

RESULTS

The dephosphorylation of three CDK substrates (HMG-I(Y), caldesmon, and histone H1) was examined initially in a previously defined system, namely the rat liver homogenate, together with glycogen phosphorylase, a well characterized substrate for PP1 and PP2A. When liver homogenates are assayed at final dilutions of 600-fold, PP2A is inhibited >90% by 2 nM okadaic acid, at which concentration the activity of PP1 is only inhibited about 5% (Cohen *et al.*, 1989). In the absence of divalent cations, the dephosphorylation of glycogen phosphorylase was inhibited 41% by 2 nM okadaic acid (Table 1), in-

[1] The designation HMG-I(Y), rather than HMG-I, has been adopted (Lund *et al.*, 1983; Bussemakers *et al.*, 1991) to avoid confusion with the unrelated nonhistone chromosomal protein HMG-1.

Table 1. Inhibition of the dephosphorylation of phosphorylase and CDK substrates in vertebrate cell lysates by okadaic acid

Substrate	Rat liver	*Xenopus* eggs	Human fibroblasts
10 μM phosphorylase a	41 ± 1 (n = 23)	56 ± 4 (n = 4)	30 ± 2 (n = 15)
2 μM caldesmon	91 ± 3 (n = 9)	79 (n = 1)	76 ± 4 (n = 6)
2 μM HMG-1 (Y)	85 ± 2 (n = 8)	89 ± 5 (n = 2)	80 ± 5 (n = 7)
2 μM histone H1	79 ± 2 (n = 7)	72 ± 7 (n = 3)	80 ± 2 (n = 8)
10 μM histone H1	85 ± 4 (n = 2)	81 ± 3 (n = 4)	89 ± 1 (n = 2)

Percentage inhibition of the dephosphorylation of each substrate by 2 nM okadaic acid is given as ±SE.

Values in parentheses are number of lysates.

Rat liver, *Xenopus* eggs, and human foreskin fibroblast extracts were prepared and assayed as described under MATERIALS AND METHODS at 600-, 1200-, and 150-fold final dilutions, respectively. Protein concentrations of the undiluted extracts determined by the method of Bradford (1976) were 20 mg/ml rat liver, 50 mg/ml *Xenopus* eggs, and 8 mg/ml human fibroblasts.

dicating that PP2A accounted for ~40% and PP1 for ~60% of the phosphorylase phosphatase activity in the homogenates. This was confirmed by the finding that 200 nM inhibitor-2 (a specific inhibitor of PP1 [Cohen, 1989]) inhibited the phosphorylase phosphatase activity of the homogenates by 60%. These values are in good agreement with those reported previously (Cohen *et al.*, 1989; Sola *et al.*, 1991). In contrast, the dephosphorylation of HMG-I(Y), caldesmon, and histone H1 was inhibited 85, 91, and 79–85%, respectively, by 2 nM okadaic acid (Table 1) suggesting that a phosphatase(s) with a sensitivity to okadaic acid similar to that of PP2A was the dominant phosphatase acting on these substrates in the liver homogenates.

In the presence of 0.1 μM okadaic acid (to inhibit PP2A), the inclusion of either 0.3 mM $CaCl_2$ (to activate protein phosphatase 2B) or 10 mM $MgCl_2$ (to activate protein phosphatase 2C) did not increase the rate of dephosphorylation of either HMG-I(Y) or caldesmon in the rat liver homogenates, indicating that protein phosphatases 2B and 2C make a negligible contribution to the dephosphorylation of these CDK substrates.

Similar results were obtained when highly diluted lysates of dividing cells (human fibroblasts) or cells that are primed to divide (*Xenopus* eggs) were used instead of liver homogenates. The dephosphorylation of HMG-I(Y), caldesmon, and histone H1 were largely inhibited by 2 nM okadaic acid, this toxin having a much less marked effect on the dephosphorylation of glycogen phosphorylase (Table 1).

To determine whether PP2A itself, or a distinct PP2A-like enzyme, was responsible for dephosphorylating the CDK substrates, rat liver cytosol was subjected to anion exchange chromatography and assayed for phosphorylase phosphatase, HMG-I(Y) phosphatase, and caldesmon phosphatase activities (Figure 1). As reported previously (Sola *et al.*, 1991), three peaks of phosphorylase phosphatase activity were detected (Figure 1). The first to elute was unaffected by 2 nM okadaic acid, inhibited completely by 200 nM inhibitor-2, and corresponded to PP1, whereas the second and third peaks are inhibited completely by 2 nM okadaic acid, are unaffected by inhibitor-2, and correspond to two major forms of PP2A (Sola *et al.*, 1991). It is well established that these two forms of PP2A correspond to the trimeric and dimeric species termed $PP2A_1$ and $PP2A_2$, respectively (see Ingebritsen *et al.*, 1983a). $PP2A_1$ and $PP2A_2$ each contain the 36-kDa PP2A catalytic subunit complexed to a 60-kDa A subunit, whereas $PP2A_1$ contains an additional 55-kDa B subunit, which is complexed to subunit A (reviewed in Cohen, 1989). When the same fractions were assayed with either HMG-I(Y) (Figure 1, A) or caldesmon (Figure 1, B), two peaks of activity were observed; the major one coeluting with $PP2A_1$ and the minor one with $PP2A_2$. Little activity was associated with PP1 (Figure 1), consistent with the results of the experiments in liver homogenates (Table 1).

The relative activities of different forms of PP2A and PP1 toward glycogen phosphorylase, HMG-I(Y), caldesmon, and histone H1 are summarized in Table 2. Relative to glycogen phosphorylase, $PP2A_1$ had a much higher activity toward CDK substrates than $PP2A_2$, which in turn had much higher activity than $PP2A_C$. Both the purified catalytic subunit of PP1 from skeletal muscle and the form of PP1 isolated by anion exchange chromatography of liver cytosol (Figure 1) dephosphorylated each CDK substrate poorly in comparison to glycogen phosphorylase (Table 2).

It was recently reported that the dephosphorylation of CDK-labeled histone H1 by concentrated *Xenopus* egg extracts was inhibited ~30% at very high (10 μM) concentrations of inhibitor-2 (Walker *et al.*, 1992) in apparent conflict with the results reported in this paper. We therefore chromatographed *Xenopus* egg cytosol on Q Sepharose (Pharmacia) and assayed the fractions for phosphorylase phosphatase and histone H1 phosphatase activities. The elution profile (Figure 2) was similar to that obtained with rat liver cytosol (Figure 1), (Sola *et al.*, 1991). The first peak of phosphorylase phosphatase activity was blocked by inhibitor-2 (Figure 2, A) and therefore corresponds to a form(s) of *Xenopus* PP1, whereas the second and third peaks were inhibited by

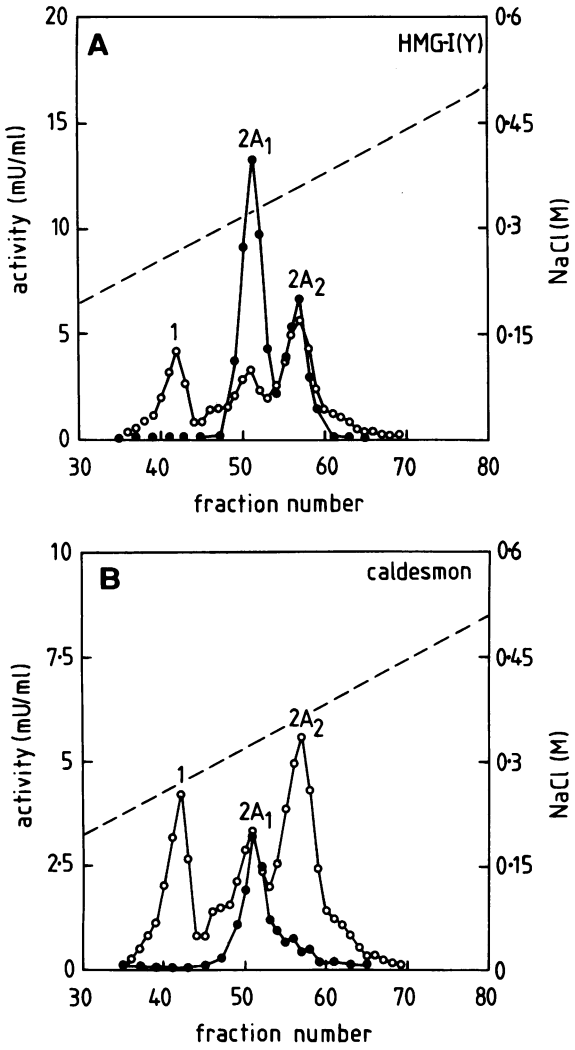

Figure 1. Separation of PP1 and two forms of PP2A by chromatography of rat liver cytosol on Q-Sepharose. Rat liver homogenates were centrifuged for 1 h at 100 000 × *g*, and 18 ml of the supernatant (350 mg) was applied to a 10 × 1.6 cm column of Q-Sepharose equilibrated in 20 mM triethanolamine/HCl pH 7.5, 0.1 mM Na-EGTA, 5% (by volume) glycerol, and 0.1% (by volume) 2-mercaptoethanol (buffer A). After washing with buffer A until the absorbance at 280 nm was <0.02, the column was developed at fraction 1 with a 400 ml linear salt gradient to 0.5 M NaCl. The flow rate was 3.0 ml/min and fractions of 5 ml were collected and assayed in A for HMG-I(Y) phosphatase activity (●) and in B for caldesmon phosphatase activity (●). The open circles show the phosphorylase phosphatase activity. The substrate concentrations were: 2 μM HMG-I(Y), 2 μM caldesmon, and 10 μM glycogen phosphorylase. The salt gradient is shown by the broken line and the elution positions of hepatic PP1, $PP2A_1$, and $PP2A_2$ are marked. No protein phosphatase activity was found in either the flowthrough fractions or in any other part of the gradient other than the region shown in the figure.

low concentrations of okadaic acid (Figure 2, A) and therefore correspond to forms of PP2A. Nearly all the histone H1 phosphatase activity was associated with the two peaks of *Xenopus* PP2A and inhibited by low concentrations of okadaic acid (Figure 2, B). On the basis of their elution positions and histone H1 phosphatase: phosphorylase phosphatase activity ratios, these two peaks correspond to $PP2A_1$ and $PP2A_2$, respectively. A small amount of histone H1 phosphatase activity insensitive to low concentrations of okadaic acid was associated with the peak of PP1 (Figure 2, B) but only accounted for ~5% of the total histone H1 phosphatase activity. Histone H1 is an unusual substrate for PP1 (purified from mammalian and avian tissues), because its dephosphorylation is unaffected by inhibitor-2 at concentrations that prevent the dephosphorylation of glycogen phosphorylase (Agostinis *et al.*, 1992; Alessi *et al.*, 1993). This is not explained by contamination with PP2A, because the dephosphorylation of histone H1 is prevented by inhibitor-1, another specific inhibitor of PP1 (Alessi *et al.*, 1993). The *Xenopus* PP1 that had been partially purified by chromatography on Q Sepharose (Figure 2) behaved in a similar manner. Its histone H1 phosphatase activity was only inhibited ~10% by 10 nM inhibitor-2, at which concentration the phosphorylase phosphatase activity was inhibited by 80%. Interestingly, the histone H1 phosphatase activity of PP1 is also more resistant to okadaic acid and is not inhibited at 10 nM (Figure 2), at which concentration the phosphorylase phosphatase activity of PP1 is inhibited by ~40%.

To investigate whether PP2A activity is inhibited as p34cdc2 becomes activated, we measured phosphatase activity toward HMG-I(Y) in *Xenopus* egg extracts blocked in interphase or mitosis after the addition of a glutathione S-transferase-cyclin B fusion protein to activate p34cdc2 (Pfaller *et al.*, 1991). The HMG-I(Y) phosphatase activity was assayed at a 1500-fold final dilution 45 min after the addition of cyclin B, at which time the histone H1 kinase activity was maximal. In two experiments, the HMG-I(Y) phosphatase activity after addition of cyclin B was 91 ± 10% of the activity of control incubations in which cyclin B was replaced by buffer.

DISCUSSION

In the present study we extended our analysis of the protein phosphatases acting on CDK substrates to HMG-I(Y) and caldesmon. These results, together with earlier studies (Sola *et al.*, 1991; Agostinis *et al.*, 1992; Goedert *et al.*, 1992a), indicate that trimeric forms of PP2A, such as $PP2A_1$, account for nearly all the phosphatase activity toward a variety of CDK and MAPK substrates in both proliferating and nonproliferating cells. Furthermore, the major enzyme in *Xenopus* egg extracts that dephosphorylates and inactivates cdc25 is inhibited by okadaic acid (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Clarke *et al.*, 1993) but not by inhibitor-2 (Clarke *et al.*, 1993), and therefore it appears to be a type-2A phosphatase. cdc25 is the protein phos-

Table 2. Rates of dephosphorylation of CDK substrates relative to phosphorylase (100%) by different forms of PP2A and PP1

	Substrate					
				Histone H1		
Phosphatase	Phosphorylase (10 μM)	HMG-I(Y) (2 μM)	Caldesmon (0.5 μM)	(0.5 μM)	(2 μM)	(10 μM)
$PP2A_1$	100	530	140	144	365	750
$PP2A_2$	100	106	22	26	55	134
$PP2A_C$	100	10	2	2	7	22
$PP1_C$	100	2	0.1	2	2	2
$PP1_L$	100	2	0.3	2	4	7

Assays were carried out using partially purified hepatic $PP2A_1$, $PP2A_2$, and PP1 ($PP1_L$) from Figure 1 and the purified catalytic subunits of PP1 ($PP1_C$) and PP2A ($PP2A_C$) from rabbit skeletal muscle. Similar results were obtained in several different experiments.

phatase responsible for activating p34cdc2 and is itself phosphorylated and activated by p34cdc2, creating an autoamplification mechanism for rapidly driving the activation of p34cdc2 to completion after the first traces of active CDK have been formed (Hoffman *et al.*, 1993).

Two forms of the 36-kDa PP2A catalytic subunit (PP2Aα, PP2Aβ) (Cruz e Silva and Cohen, 1987; Stone *et al.*, 1987) and two forms of its associated 65-kDa A-subunit (Aα, Aβ) (Hemmings *et al.*, 1990) have been identified in mammalian cells. PP2Aα and PP2Aβ show 97% amino acid sequence identity and Aα and Aβ 87% amino acid sequence identity. The relative proportions of these species in the $PP2A_1$ and $PP2A_2$ species we have studied are unknown but in view of their high degree of homology significant differences in specificity seem unlikely. The mRNA encoding PP2Aα is 10-fold higher than that of PP2Aβ in most mammalian cells (Khew-Goodall *et al.*, 1991), suggesting that the latter may only be a minor component of most PP2A preparations.

The HMG-I(Y)* proteins are a family of nonhistone chromosomal proteins, of which HMG-I and HMG-Y are the best studied members (Lund *et al.*, 1983; Johnson *et al.*, 1988; Johnson *et al.*, 1989). These proteins bind preferentially in the minor groove of adenine-thymine (AT)-rich sequences of DNA (Solomon *et al.*, 1986; Elton *et al.*, 1987; Disney *et al.*, 1989) via a conserved 11–amino acid binding domain present in one or two copies in murine and human HMG-I(Y) proteins (Reeves and Nissen, 1990). HMG-I(Y) proteins are expressed at elevated levels in proliferating, neoplastically transformed, and metastatic cells (Giancotti *et al.*, 1987; Giancotti *et al.*, 1989; Johnson *et al.*, 1990; Bussemakers *et al.*, 1991), suggesting a function in growth control. Although a number of specific functions have been proposed for these proteins, including nucleosome phasing (Strauss and Varshavsky, 1984) and 3′-end processing of mRNA transcripts (Russnak *et al.*, 1988), the most direct evidence suggests a role in transcriptional regulation of genes associated with AT-rich sequences. Transcriptional activation of rRNA genes by HMG-I(Y) proteins has been demonstrated directly in vitro (Yang-Yen and Rothblum, 1988), and recently HMG-I(Y) has been shown to bind specifically to AT-rich regulatory regions of the tumor necrosis factor-β gene, which may enhance or repress transcription depending on the presence of other factors (Fashena *et al.*, 1992). Phosphorylation of HMG-I(Y) proteins takes place in vivo in a cell cycle regulated manner with maximum phosphorylation occurring at metaphase (Lund and Laland, 1990; Reeves *et al.*, 1991), and in vitro these proteins are substrates for CDKs (Lund and Laland, 1990; Meijer *et al.*, 1991; Reeves *et al.*, 1991). Phosphorylation in vivo and in vitro occurs within the conserved DNA binding domain of HMG-I(Y), which contains a CDK phosphorylation site consensus sequence, and causes a ~20-fold reduction in DNA binding affinity (Meijer *et al.*, 1991; Nissen *et al.*, 1991). This suggests that CDK phosphorylation could have important consequences for HMG-I(Y) function during the cell cycle. It follows from this that PP2A may be an important regulator of transcriptional reinitiation after mitosis.

Caldesmon is a protein present in smooth muscle and nonmuscle cells that interacts with actin, myosin, calmodulin, and microtubules. It contains a highly conserved N-terminal domain that interacts with myosin (Velaz *et al.*, 1990) and a highly conserved C-terminal domain that contains the binding sites for actin and calmodulin (Bryan *et al.*, 1989; Bartegi *et al.*, 1990). In fibroblasts, caldesmon is dissociated from microfilaments during mitosis, apparently as a consequence of mitosis-specific phosphorylation (Yamashiro *et al.*, 1990). A human CDK phosphorylates several Ser/Thr-Pro sequences in the C-terminal domain of both nonmuscle and smooth muscle caldesmon (Mak *et al.*, 1991), and peptide mapping indicates that the phosphorylation sites are the same as those phosphorylated in vivo during mitosis (Yamashiro *et al.*, 1991). Because caldesmon inhibits actomyosin ATPase activity, it has been suggested that phosphorylation of this protein by CDK may play a role in microfilament reorganization during mitosis. Phosphorylation of caldesmon by CDK in vitro

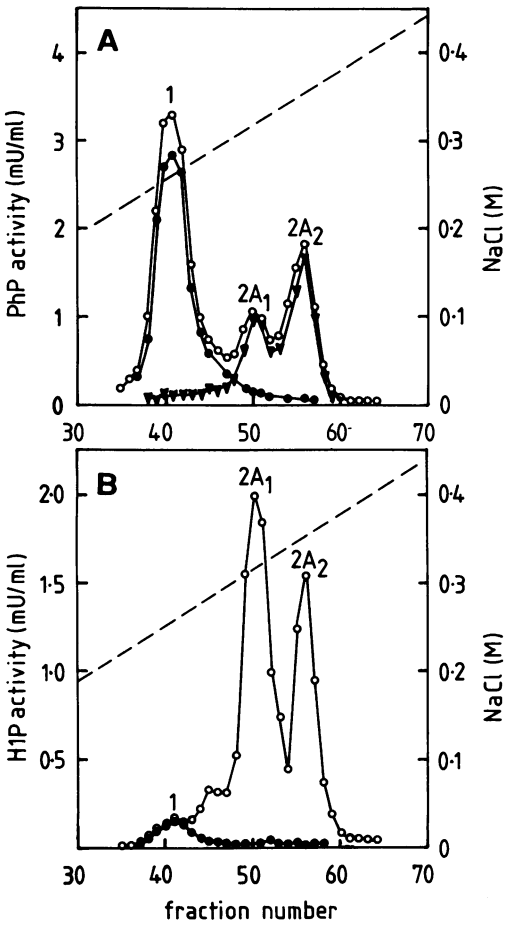

Figure 2. Separation of PP1 and two forms of PP2A by chromatography of *Xenopus* egg cytosol on Q-Sepharose. High speed supernatants were prepared from *Xenopus* egg interphase extracts as in Smythe and Newport, (1991). An aliquot of the supernatant (0.6 ml, 40 mg) was diluted to 2 ml with buffer A and chromatographed on Q-Sepharose as described in the legend to Figure 1. (A) The fractions were assayed for phosphorylase phosphatase activity (PhP) with glycogen phosphorylase (10 μM) as substrate in the presence of 200 nM inhibitor-2 (▲), 2 nM okadaic acid (●), and absence of either inhibitor (○). (B) The fractions were assayed for histone H1 phosphatase activity (H1P) in the presence (●) and absence (○) of 10 nM okadaic acid. The histone H1 substrate was phosphorylated to 0.6 mol phosphate per mol protein and used in the assays at a concentration of 2.5 μM. The salt gradient is shown by the broken line. No protein phosphatase activity was found in the flowthrough or any other fraction apart from the region shown in the figure.

also eliminates its ability to bind to microtubules (Ishikawa *et al.*, 1992). It follows from this that PP2A may play an important role in restoring the cytoskeleton to an interphase state.

Our finding that forms of PP2A account for nearly all the histone H1 phosphatase activity in *Xenopus* egg extracts (under our assay conditions) is at variance with earlier results (Walker *et al.*, 1992) in which about 30% of the histone H1 phosphatase activity in concentrated *Xenopus* extracts could be blocked by inhibitor-2. This discrepancy is not explained by the use of different histone H1 and inhibitor-2 preparations, because ^{32}P-labeled histone H1 (phosphorylated by cyclin B/p34cdc2) generously provided to us by Dr. James Maller behaved similarly to our histone H1 substrate and the same inhibitor-2 preparation was used in each study. However, it is well known that PP1 and PP2A activities are both strongly suppressed in concentrated cell extracts (Ingebritsen *et al.*, 1983b). If PP2A activity were suppressed to a greater extent than PP1, this could account for the larger contribution of PP1 to histone H1 dephosphorylation in the experiments of Walker *et al.* (1992). On the other hand, rationalization of the data is complicated by the finding that the histone H1 phosphatase activity of PP1 is very resistant to inhibitor-2 in comparison to the phosphorylase phosphatase activity (Alessi *et al.*, 1993).

Studies on the specificities of protein phosphatases using synthetic peptide substrates have shown that the presence of a proline immediately C-terminal to a serine or threonine residue prevents the dephosphorylation of small peptides by the major types of protein serine/threonine phosphatases (Pinna *et al.*, 1989; Donella-Deana *et al.*, 1990) and presumably explains why the CDK and MAPK substrates we have studied are dephosphorylated slowly by PP1, PP2B, PP2C, and the catalytic subunit of PP2A. The intriguing finding is that the presence of the A and B subunits in PP2A$_1$ overcomes the negative effect of the C-terminal proline, allowing the catalytic subunit to dephosphorylate CDK and MAPK substrates extremely effectively. In contrast, the A and B subunits inhibit the dephosphorylation of most other substrates by PP2A (reviewed in Cohen, 1989). A mechanism may therefore exist in cells that would affect the rate of dephosphorylation of CDK and/or MAPK substrates by PP2A without affecting the dephosphorylation of other proteins. It will clearly be of interest to investigate whether a subunit(s) of PP2A$_1$ is phosphorylated in vivo under conditions where CDKs and MAPKs are activated and whether this is accompanied by inhibition of the dephosphorylation of CDK and MAPK substrates, because this would amplify the effects of these protein kinases on cellular processes. To begin to address this question, we investigated whether the dephosphorylation of HMG-I(Y) was inhibited in *Xenopus* egg extracts after addition of cyclin B to activate p34cdc2, but no significant decrease in phosphatase activity toward this substrate was observed. Clarke *et al.* (1993) also failed to detect any change in the rate of dephosphorylation of our ^{32}P-labeled histone H1 preparation by *Xenopus* egg extracts in similar experiments. These findings are not, however, definitive because of unique technical problems associated with studies of protein phosphatase regulation. If PP2A regulation is mediated by phosphorylation of one of the subunits of PP2A$_1$ or an as yet unidentified inhibitor protein, it will be necessary to add phosphatase inhibitors to prevent

reactivation during the assays. However, this would preclude the measurement of PP2A. For this reason we assayed the extracts after an extremely high (>1000-fold) dilution to try and prevent significant dephosphorylation of any putative regulatory site. The effectiveness of such a procedure is unknown and under these conditions the effect of an inhibitor protein may have been diluted out. Assays carried out by using undiluted extracts are even more hazardous because not only are rates of dephosphorylation higher, but there is a further technical problem. The activation of p34cdc2 increases the phosphorylation of a great many proteins in *Xenopus* eggs (Karsenti *et al.*, 1987) that in concentrated extracts will compete for phosphatases with any ^{32}P-labeled substrate added to the extract. This will lead to a decrease in the rate at which the ^{32}P-substrate is dephosphorylated and to the erroneous conclusion that a phosphatase has been inhibited. This technical problem might conceivably explain the observations of Walker *et al.* (1992) who reported that the dephosphorylation of ^{32}P-labeled histone H1 in concentrated *Xenopus* egg extracts decreased under conditions where p34cdc2 was activated and vice versa. It might also explain the observations of other investigators (Kumagai and Dunphy, 1992; Clarke *et al.*, 1993) who reported that the rate of dephosphorylation of exogenously added ^{32}P-labeled cdc25 decreased after p34cdc2 was activated in *Xenopus* egg extracts. However, other explanations are possible. For example, p34cdc2 could phosphorylate a protein that then binds to cdc25, selectively preventing its dephosphorylation by PP2A. Alternatively, cdc25 might not be dephosphorylated by PP2A but by another phosphatase with similar sensitivity to okadaic acid, such as PPX (Brewis *et al.*, 1993). The latter possibility can now be easily addressed using the methodology presented in this paper (Figure 2).

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