Regulation of purified hepatic PC-1 (phosphodiesterase-I/nucleotide pyrophosphatase) by threonine auto(de)phosphorylation and by binding of acidic fibroblast growth factor

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The plasma cell differentiation antigen PC-1 was purified to homogeneity from rat liver membranes. Denaturing electrophoresis revealed polypeptides of 118 and 128 kDa, which were both recognized by antibodies against recombinant murine PC-1. During gel filtration PC-1 migrated as a protein of about 500 kDa, suggesting a tetrameric structure. Purified PC-1 displayed a phosphodiesterase-I/nucleotide pyrophosphatase activity that could be completely blocked by EDTA, dithiothreitol and acidic fibroblast growth factor (extrapolated $K_i = 1.3$ nM). Purified PC-1 was also capable of threonine autophosphorylation and of phosphorylation of histone IIa. The autophosphorylation of PC-1 was inhibited by addition of histone IIa, and it was blocked by phosphodiesterase-I inhibitors (acidic fibroblast growth factor, dithiothreitol), by nucleotides (ATP, ADP, AMP),

and by vanadate. When added to autophosphorylated PC-1, these compounds caused a prompt dephosphorylation. However, the same agents did not affect the (de)phosphorylation of histone IIa, which is not a substrate for the PC-1 phosphatase. These data indicate that phosphodiesterase-I inhibitors, nucleotides and vanadate affect the (de)phosphorylation of PC-1 by stimulating the PC-1 phosphatase and/or by shielding the autophosphorylation site from the PC-1 kinase. The rate of dephosphorylation of PC-1 was independent of the dilution, suggesting an autocatalytic intramolecular process. We propose that the autophosphorylation of PC-1 serves to block its nucleotide pyrophosphatase activity when extracellular ATP becomes scarce.

INTRODUCTION

The plasma-cell differentiation antigen-1 PC-1 was originally described as a cell-surface marker of differentiated immunoglobulin-secreting B-lymphocytes [1]. The protein is, however, also expressed in some non-lymphoid tissues, such as liver and kidney [1–3]. Apart from being an integral plasma-membrane protein [1,4], PC-1 may also be associated with the endoplasmic reticulum [5]. In addition, there is preliminary evidence that a soluble form of PC-1 is generated by intracellular proteolysis before insertion in the membrane and subsequently released extracellularly [6].

PC-1 has been identified as an enzyme with a phosphodiesterase-I/nucleotide pyrophosphatase activity capable of hydrolysing various nucleotides and nucleotide sugars [5–9]. The active site of the plasma-membrane-associated PC-1 appears to be located extracellularly [4,6], whereas the active domain of intracellular PC-1 is believed to protrude into the lumen of the endoplasmic reticulum [5]. The results of a detailed comparison of cell types that express widely different levels of PC-1 suggest that intracellular PC-1 may regulate the availability of nucleotide-linked sugars and sulphate which serve as precursors for the glycosylation of proteins and for the sulphation of oligosaccharides, respectively [7,10–12]. Plasma-membrane-associated enzyme(s) with nucleotide pyrophosphatase/phosphodiesterase I activity are probably involved in the clearance of extracellular nucleotides such as ATP and diadenosine polyphosphates [13,14].

Remarkably, PC-1 is also a protein threonine kinase, capable

of autophosphorylation as well as phosphorylation of exogenous substrates such as histones and myelin basic protein [3]. In accordance with PC-1 being an ecto-protein kinase, a putative ATP-binding site has been identified in the extracellular domain [3]. We have characterized a protein in rat liver membranes with properties similar to those of PC-1, including its capacity for threonine autophosphorylation [15]. A novel feature was that the autophosphorylation was strongly inhibited by adenine nucleotides. Thus, autophosphorylation only occurred below a threshold concentration of total nucleotides (ATP, ADP and AMP), whereas the addition of nucleotides to phosphorylated PC-1 resulted in rapid dephosphorylation. In this paper we report the complete purification of PC-1 from rat liver and show that it is actually a trifunctional enzyme, displaying a phosphodiesterase-I/nucleotide pyrophosphatase activity, a protein threonine kinase activity, and a nucleotide-stimulated protein threonine phosphatase activity. In addition, it is shown that the activity of PC-1 is inhibited by nanomolar concentrations of acidic fibroblast growth factor (aFGF).

EXPERIMENTAL

Materials

aFGF, also known as the endothelial cell-growth factor, from bovine brain was obtained from Boehringer. 5'-Nucleotidase from *Crotalus atrox* venom and phosphodiesterase-I from bovine intestinal mucosa were purchased from Sigma. The origin of other materials has been reported previously [15].

Purification of PC-1

Membranes were prepared from five rat livers as detailed previously [15]. The detergent-solubilized membrane fraction (50–100 ml) was diluted 2-fold in standard buffer [50 mM Tris/HCl, pH 7.5, 0.1% (v/v) Triton X-100 and 50 mM NaCl] and stirred for 60 min at 4 °C with 80 ml of DEAE-Sephadex A-50, equilibrated in the same buffer. After washing with 5 vol. of the buffer on a sintered-glass funnel, the gel was poured into a column. The retained proteins were eluted with a linear 800 ml gradient of 50–600 mM NaCl in the same buffer. The peak fractions containing PC-1, as detected by Western blotting (see Figure 1), were pooled and diluted with NaCl-free standard buffer to a final NaCl concentration of 50 mM. The pool was stirred for 60 min at 4 °C with 50 ml of heparin–Sephacrose, equilibrated in standard buffer. Subsequently, the gel was rinsed with 5 vol. of buffer on a sintered-glass funnel, poured into a column and eluted with a linear 150 ml gradient of 50–1000 mM NaCl in standard buffer. Fractions containing PC-1, as detected by the assay of phosphodiesterase-I, were pooled, diluted with standard buffer to a final NaCl concentration of about 150 mM, and mixed for 60 min at 4 °C with 10 ml of wheat-germ lectin–Sephacrose-6MB, equilibrated with standard buffer containing 150 mM NaCl. The gel was washed and transferred into a column as above, and the retained proteins were eluted with 45 ml of standard buffer containing 0.3 M *N*-acetyl-D-glucosamine. The pooled fractions were concentrated by ultrafiltration (Amicon Centriprep 30) to 1–2 ml, dialysed against standard buffer and applied to a Mono Q column (5 cm × 0.5 cm), equilibrated in standard buffer. PC-1 was eluted with a linear 20 ml gradient of 50–500 mM NaCl. Finally, the fractions containing phosphodiesterase-I activity were pooled, concentrated by ultrafiltration (Amicon Centriprep 30 and Centricon 30) down to 250 µl, and applied to a Superdex-200 gel-filtration column, equilibrated in standard buffer containing 150 mM NaCl. The peak fractions containing phosphodiesterase-I activity were pooled and stored at –20 °C.

Assays

The phosphodiesterase-I activity was measured at 37 °C in the presence of 100 mM glycine at pH 9.0, with 1.7 mM *p*-nitrophenyl thymidine phosphate (PNTP) as substrate. The hydrolysis of PNTP was stopped by a 10-fold dilution in 3% (w/v) trichloroacetic acid. Subsequently, 0.1 vol. of 5 M NaOH was added and *p*-nitrophenol was quantified colorimetrically at 410 nm. One unit of phosphodiesterase-I was defined as the amount of enzyme that hydrolyses 1 µmol of substrate/min under the adopted assay conditions.

The oligonucleotide pyrophosphatase activity was detected at 37 °C and at pH 9.0 (100 mM glycine) with 1 mM UDP-[U-¹⁴C]-glucose as substrate. The reaction was arrested by addition of 10 vol. of 30 mM ammonium formate (pH 4.35). The non-hydrolysed substrate was separated from the generated glucose 1-phosphate by the selective binding of the UDP-glucose to added DEAE-cellulose, equilibrated in 30 mM ammonium formate at pH 4.35 [16]. Glucose 1-phosphate was quantified after sedimentation of the resin (1 min at 10000 *g*) by counting the radioactivity in the supernatant.

The hydrolysis of [2,8-³H]ATP (50 µM) was monitored by counting the radioactivity associated with ATP, ADP, AMP and adenosine after their separation by t.l.c. [15].

Western blotting was done with polyclonal antibodies raised against recombinant PC-1 [15]. The total protein concentration was measured by the method of Bradford [17].

Statistics are given as means ± S.E.M. for the indicated number (*n*) of observations.

RESULTS

Purification of PC-1

The complete purification of PC-1 from the detergent-solubilized fraction of rat liver membranes required five chromatographic steps. In the first step, which involved chromatography on DEAE-Sephadex A-50, the phosphodiesterase-I activity was eluted as a much broader peak than PC-1 (Figure 1), that was composed of the typical monomers of 118 and 128 kDa (p118 and p128) [15]. Moreover, Western blotting with antibodies against recombinant PC-1 also revealed two polypeptides of 165 ± 1 kDa (*n* = 9) and 54 ± 1 kDa (*n* = 9) that migrated independently of the p118–p128 complex. The structural and functional relationship between these polypeptides and PC-1 is not clear. However, the 165- and 54-kDa polypeptides were not phosphorylated under conditions (see below) where PC-1 was phosphorylated and, unlike PC-1, they were not retained by wheat-germ lectin–Sephacrose (results not shown). Using the same antibodies, Rebbe et al. [5] also noted cross-reaction with a polypeptide of 45 kDa in Cos-7 cell lysates.

Figure 1 Elution of phosphodiesterase-I activity and of PC-1 from DEAE-Sephadex A-50

Panel (a) shows the elution pattern of the phosphodiesterase-I activity (●). In the same fractions PC-1 was detected by Western blotting and quantified by gel scanning of the signals corresponding to polypeptides of 118 and 128 kDa (○). Panel (b) illustrates the Western blots of the relevant column fractions.

Figure 2 Subunit composition of PC-1

A sample of the pool of the peak fractions from the Superdex-200 gel-filtration column containing phosphodiesterase-I activity was subjected to SDS/PAGE (7.5% gel) and either silver staining (lane **a**) or Western blotting with antibodies against recombinant murine PC-1 (lane **b**). Lane (**c**) shows an autoradiogram after SDS/PAGE of a sample previously incubated with 5'-nucleotidase (3 units/ml) and 5 μ M [32 P]ATP for 10 min.

The above data strongly indicate that several phosphodiesterase-I species are present in the eluate of the DEAE-Sephadex column. It was not until chromatography on wheat-germ lectin-Sephadex (step 3) that the phosphodiesterase-I activity coincided exactly with the immunologically detected polypeptides of 118 and 128 kDa. Because of the initial contamination with other type-I phosphodiesterases, a correct estimate of the recovery and total purification of PC-1 could not be obtained. However, a minimal recovery of the purification, taking the phosphodiesterase-I activity of the pooled fractions from DEAE-Sephadex as 100%, amounted to 3%. Combined with observations that about 1 μ g of homogeneous PC-1 was obtained per g of liver, this indicates that the membranes contain maximally 30 μ g of PC-1/g of liver.

In agreement with previous estimates [15,18], purified PC-1 migrated on Superdex 200 as a protein of about 500 kDa (results not shown). After SDS/PAGE, silver staining revealed only two major bands, of 118 and 128 kDa (Figure 2, lane **a**). The positions of these bands corresponded exactly to the polypeptides detected by Western blotting with antibodies against recombinant murine PC-1 (Figure 2, lane **b**) and by autoradiography after incubation with [32 P]ATP (Figure 2, lane **c**). Sometimes, a minor band of 197 kDa was also visible (see, e.g., Figure 2c). As noted previously [7,15], this band probably represents a PC-1 dimer linked by non-disulphide bonds and resistant to dissociation by denaturing electrophoresis.

Phosphodiesterase-I and nucleotide pyrophosphatase activities

With PNTP as substrate, homogeneously purified PC-1 displayed a phosphodiesterase-I activity of 411 ± 84 units/mg of protein ($n = 6$), which is similar to the specific activity of other membrane-associated type-I phosphodiesterases [18,19]. PC-1 also released glucose 1-phosphate from UDP-glucose (results not shown) and converted ATP into AMP with little accumulation of ADP (Figure 3). The latter data contrast with earlier observations on the partially purified enzyme suggesting that PC-1 could convert ATP quantitatively into adenosine [15]. This discrepancy is likely to be explained by the removal of contaminating 5'-

Figure 3 Hydrolysis of ATP by purified PC-1

PC-1 was incubated with 30 μ M [2,8- 3 H]ATP. At the indicated time points, samples were analysed for the presence of ATP, ADP, AMP, IMP and adenosine (Ado) by t.l.c. No IMP was detected (results not shown).

nucleotidase(s) during further purification of PC-1 (see also below).

The phosphodiesterase-I activity of PC-1 was completely blocked by the addition of 1 mM EDTA, but was not affected by 1 mM $MgCl_2$ and/or $CaCl_2$ (results not shown). Since PC-1 was dissolved in a buffer without added metals, this suggests that the inhibitory action of EDTA is caused by the removal of tightly bound metal(s). The phosphodiesterase-I activity of PC-1 was also blocked by the reducing agent dithiothreitol ($IC_{50} = 2.3 \pm 0.4$ mM, $n = 3$).

Since aFGF was previously reported to enhance the autophosphorylation of PC-1 [3], we have investigated whether this growth factor perhaps also affects the phosphodiesterase-I activity of PC-1. Indeed, as is shown in Figure 4, aFGF turned out to be a very potent inhibitor of the phosphodiesterase-I activity of PC-1. Actually, aFGF was inhibitory at nearly stoichiometric concentrations, which explains why the inhibitory concentrations depended on the dilution of PC-1. For such tightly bound inhibitors Michaelis-Menten kinetics do not apply, since the enzyme-bound fraction of inhibitor is not negligible [20]. An estimate of the K_i is then given, however, by $IC_{50} - E/2$, where IC_{50} is the inhibitor concentration that causes 50% inhibition and E is the enzyme concentration [21]. When applied to the inhibition of PC-1 by aFGF (Figure 4) the extrapolated K_i value was 1.3 ± 0.3 nM ($n = 4$).

PC-1 as a protein kinase

In contrast with the partially purified enzyme [15], homogeneously purified PC-1 did not become phosphorylated upon incubation with ATP, unless exogenous 5'-nucleotidase was added to convert the accumulating AMP into adenosine (Figure 5). These data can be rationalized by our observations that adenine nucleotides (ATP, ADP, AMP), but not adenosine, prevent a net phosphorylation of PC-1 ([15], see also below). As seen with the partially purified enzyme [15], the maximal extent of phosphorylation of PC-1 varied with the added concentration of ATP (Figure 5). Moreover, at higher ATP concentrations (100 μ M in Figure 5), the phosphorylation only started after a latency. The length of this latency increased with the con-

Figure 4 Effect of aFGF on the phosphodiesterase-I activity of PC-1

The indicated concentrations of PC-1-monomer (taking 100 kDa as carbohydrate-free molecular mass) were incubated with various concentrations of aFGF (indicated on a log scale). After a 2 min preincubation, the phosphodiesterase-I activity was measured.

Figure 5 Influence of 5'-nucleotidase and of the nucleotide concentration on the autophosphorylation of PC-1

PC-1 was incubated with 10 μ M (circles) or 100 μ M (squares) [32 P]ATP in the absence (open symbols) or presence (filled symbols) of 5'-nucleotidase (3 units/ml). At the indicated time points, samples were taken for SDS/PAGE (7.5% gel). The extent of phosphorylation of the p118–p128 doublet was quantified by laser densitometry of autoradiograms.

centration of added ATP and corresponded to the time required to convert most of the ATP into adenosine and P_i (see also Figure 6 in [15]). We did not observe any phosphorylation of commercial intestinal alkaline phosphodiesterase-I (results not shown), indicating that phosphorylation is not a property common to all enzymes with a phosphodiesterase-I/nucleotide pyrophosphatase activity.

The metal requirement for autophosphorylation of purified PC-1 was very low, as indicated by observations that the rate and extent of phosphorylation were the same, whether or not ATP was supplemented with 1 mM $MgCl_2$ (results not illustrated). Nevertheless, the autophosphorylation of PC-1 was completely blocked by 1 mM of the metal chelator EDTA (results not shown). The autophosphorylation of PC-1 in the presence of 100 μ M ATP was not affected by the protein kinase inhibitors [22,23] staurosporine (30 μ M), genistein (30 μ M) and heparin (10 μ g/ml), but was inhibited by the adenosine analogue and protein kinase inhibitor 5-iodotubercidin [24] at concentrations

Figure 6 Effect of aFGF on the PC-1 autophosphorylation and on the phosphorylation of histone IIa

PC-1 (2 nM) was incubated with 100 μ M [32 P]ATP, 50 μ M histone IIa and 5'-nucleotidase (3 units/ml) in the absence (open symbols) or presence (filled symbols) of 5 nM aFGF. At the indicated time points, samples were taken for Tricine/SDS/PAGE (12% gel). The extent of phosphorylation of the PC-1 doublet (circles) and of histone IIa (triangles) was quantified by laser densitometry of autoradiograms.

($IC_{50} = 78 \pm 15 \mu$ M; $n = 3$) that did not measurably affect the phosphodiesterase-I activity of PC-1. The enzyme did not become autophosphorylated in the presence of 0.1 mM vanadate, which also caused a 20% inhibition of the phosphodiesterase-I activity. Furthermore, the autophosphorylation of PC-1 was inhibited by the same concentrations of dithiothreitol (results not shown) and aFGF (Figure 6) that also blocked the phosphodiesterase-I activity of PC-1. Since many protein kinases are themselves substrates for phosphorylation by other kinases, we have investigated whether this also applies to PC-1. However, neither cyclic-AMP-dependent protein kinase nor casein kinase 2 was able to phosphorylate PC-1 (results not shown).

PC-1 also phosphorylated histone IIa (Figure 6), but the affinity for this exogenous substrate was extremely low ($K_m > 100 \mu$ M). We did not obtain any phosphorylation by PC-1 of other model substrates for protein kinases such as myelin basic protein (at variance with another report [3]) or casein or phosvitin. Addition of histone IIa inhibited the autophosphorylation of PC-1 ($IC_{50} = 20$ –100 μ M), which may be explained by competition for the same kinase catalytic site. In contrast with the autophosphorylation of PC-1, the phosphorylation of histone IIa occurred without a latency (Figure 6), did not require the presence of 5'-nucleotidase (results not shown), and was not inhibited by the addition of nucleotides (e.g. 100 μ M ATP) or 0.1 mM vanadate or 10 mM dithiothreitol (results not shown) or 5 nM aFGF (Figure 6). These data suggest that these compounds do not prevent the autophosphorylation of PC-1 by blocking the kinase catalytic site.

Autodephosphorylation of PC-1

After an initial period of autophosphorylation, PC-1 became gradually dephosphorylated again (Figures 5 and 7). This dephosphorylation may be explained by depletion of the ATP pool by the nucleotide pyrophosphatase activity of PC-1. The dephosphorylation of PC-1 could be enhanced by the addition of general protein kinase inhibitors such as EDTA or 5-iodotubercidin at the time of maximal phosphorylation (results not shown), or by addition of aFGF, or nucleotides (ATP, ADP,

Figure 7 Stimulation of the autodephosphorylation of PC-1 by aFGF, ADP and vanadate

PC-1 was preincubated with 100 μ M [32 P]ATP and 5'-nucleotidase (3 units/ml). After 30 min, when the phosphorylation of the p118–p128 doublet was maximal, the mixture was supplemented with the additions shown, and samples were taken for determination of the extent of autophosphorylation of PC-1 by scanning of autoradiograms after SDS/PAGE.

AMP, but not adenosine), or vanadate (Figure 7). Interestingly, the nucleotide- and vanadate-induced dephosphorylation of PC-1 always occurred initially about 2-fold faster than the dephosphorylation observed in the presence of aFGF (Figure 7) or EDTA or dithiothreitol (results not shown). When 100 μ M [32 P]ATP was added to autophosphorylated PC-1, a prompt and nearly complete dephosphorylation of the enzyme occurred, followed by a latency and a subsequent rephosphorylation of PC-1 (results not shown), as previously described for the partially purified enzyme (Figure 5 in [15]). Remarkably, none of the above compounds (EDTA, 5-iodotubercidin, adenine nucleotides, vanadate, dithiothreitol) caused a dephosphorylation of PC-1-phosphorylated histone IIa (results not shown), suggesting

Figure 8 The dephosphorylation of PC-1 is independent of the dilution

PC-1 was incubated with 100 μ M [32 P]ATP in the presence of 5'-nucleotidase (3 units/ml), resulting in maximal phosphorylation after 30 min. At that time, the enzyme was further incubated with 1.1-fold dilution, caused by the addition of buffer (○) or by the addition of 1 mM EDTA plus 0.1 mM ADP (●), or after a 16-fold dilution in buffer containing 1 mM EDTA plus 0.1 mM ADP (▲). Samples were withdrawn at the indicated times for measurement of the radiolabel in PC-1 by scanning of autoradiograms after SDS/PAGE.

that histone IIa is not a substrate for the PC-1-associated phosphatase.

Interestingly, the maximal rate of dephosphorylation of PC-1, in the presence of 1 mM EDTA plus 0.1 mM ADP, was independent of the dilution of the enzyme (Figure 8). This suggests that the PC-1 phosphatase is not a minor contaminant of the preparation, but an intrinsic activity of PC-1.

DISCUSSION

Multiple membrane-associated species of phosphodiesterase-I

Although the substrate specificity of PC-1 was similar to that of previously purified enzymes with phosphodiesterase-I and nucleotide pyrophosphatase activities [3,18,19], it seems unlikely that they are all the same enzyme. Thus, PC-1 could be solubilized with Triton X-100 (the present work; [3,18]), whereas the major fraction of the membrane-associated phosphodiesterase-I from rat liver was reported not to be detergent-extractable, but could be released by proteolytic cleavage at trypsin concentrations [19] that completely destroy PC-1 [15]. Moreover, in contrast with what can be expected for a transmembrane protein like PC-1, it has been shown that the plasma-membrane-bound phosphodiesterase-I is partially released by incubation with a phosphatidylinositol-specific phospholipase C [14,25,26], indicating an anchorage by a glycosylphosphatidylinositol. Even the detergent-extracted membrane fraction may contain several species of phosphodiesterase-I, since the activity was eluted during fractionation on DEAE-Sephadex A-50 as a much broader peak than the immunologically detected PC-1 (Figure 1). Further, we found that at least the intestinal enzyme did not autophosphorylate.

Enzymic activities of PC-1

PC-1 emerges from this study as a trifunctional enzyme displaying a phosphodiesterase-I/nucleotide pyrophosphatase activity, a protein threonine kinase activity and a protein threonine phosphatase activity. The most important argument suggesting that the three activities belong to the same enzyme is their copurification. It is indeed unlikely that the (de)phosphorylation of PC-1 is catalysed by trace amounts of contaminating kinases and phosphatases, since the rate of (de)phosphorylation of PC-1 did not change during purification (compare [15] with the present work). Moreover, the rate of dephosphorylation of purified PC-1 did not decrease with dilution (Figure 8), providing additional evidence that the phosphatase (besides the kinase, [3]) is an intrinsic activity of PC-1. The results (Figure 8) also allow us to conclude that the dephosphorylation is an intramolecular, rather than an intermolecular, process.

The primary structure of PC-1 also shows some features that agree with its proposed multifunctional role. Thus, PC-1 contains a domain that is homologous to the active site of intestinal phosphodiesterase-I [8,9]. PC-1 does not show similarities to known protein kinases [3] and with Ser/Thr protein phosphatases-1, -2A and -2B (our analysis); however, PC-1 contains a consensus sequence (GXGXXG---A/GXK) for the binding of the phosphates of ATP [3], and we identified two consensus sequences (H/R/KX₍₅₋₈₎ϕXϕϕD/E, where ϕ represents a hydrophobic residue) for binding of the adenine moiety [27]. The latter two domains (residues 343–353 and 736–748 in human PC-1) are conserved in mouse PC-1 [28]. Although the physiological role of these putative adenine-binding sites remains to be established, it is tempting to speculate that they mediate the nucleotide-stimulated dephosphorylation of PC-1.

An important question is whether or not the three activities of

PC-1 are catalysed by different catalytic sites. Mechanistic studies of the intestinal phosphodiesterase-I have revealed a catalytic intermediate whereby a nucleoside phosphate is covalently bound to a phylogenetically conserved Thr residue [29,30] that corresponds to Thr-204 in PC-1 [3,28]. This reaction mechanism differs completely from the phosphorylation and dephosphorylation of PC-1, which involves the transfer of the γ -phosphate group of ATP to threonine and the hydrolysis of a threonine-linked phosphomonoester bond, respectively. A different catalytic mechanism is also indicated by findings that some potent effectors of the auto(de)phosphorylation of PC-1 (e.g. 0.1 mM vanadate, 0.1 mM 5-iodotubercidin) had little or no effect on the phosphodiesterase-I activity of PC-1. Along the same lines, the complete inhibition of the phosphodiesterase-I activity by aFGF (Figure 4) or EDTA still allowed a rapid dephosphorylation of PC-1 (Figures 7 and 8).

Although phosphorylation reactions are thermodynamically driven by the cleavage of a pyrophosphate bond, some of these reactions are reversible. Thus phosphorylation of the bacterial enzyme isocitrate dehydrogenase is reversible because the back reaction is tightly coupled to hydrolysis of the ATP formed to ADP and P_i [31]. The action of some other protein kinases is reversible, because protein-bound *O*-linked phosphates can be energy-rich [32–34]. It seems unlikely, however, that the dephosphorylation of PC-1 is explained by a reversal of the kinase reaction, since a rapid dephosphorylation could be obtained in the absence of precursors of ATP (e.g. vanadate in Figure 7). Moreover, the presence of a single kinase/phosphatase catalytic site is difficult to reconcile with our observations that histone-IIa is phosphorylated by PC-1, but is not a substrate for the PC-1-associated phosphatase.

Regulation of PC-1

An intriguing finding in this study is that the phosphodiesterase-I activity (Figure 4) as well as the autophosphorylation of PC-1 (Figure 6) was blocked by nearly stoichiometric concentrations of aFGF. These findings contrast with an earlier study [3] suggesting that aFGF stimulates the autophosphorylation of PC-1. This discrepancy can perhaps be explained by the extreme dependence of the autophosphorylation of PC-1 on the concentration of nucleotides. Indeed, Oda et al. [3] only measured the phosphorylation of PC-1 at a single time point after a fairly long incubation (30 min at 0 °C), when nearly all the added ATP (5 μ M) had possibly been hydrolysed and PC-1 was already being dephosphorylated. Under these conditions, sub-maximal concentrations of aFGF would be expected to stimulate the autophosphorylation of PC-1, since aFGF slows down the hydrolysis of ATP, thus leaving ATP available for a longer time for autophosphorylation of the fraction of non-inhibited PC-1.

The physiological significance of the inhibition of PC-1 by aFGF remains to be established. Interestingly, aFGF is one of the major growth factors controlling the differentiation and regeneration of liver parenchymal cells [35,36]. Since ATP has also been reported to be mitogenic in several cell types [37,38], it seems possible that some mitogenic effects of aFGF are mediated by inhibition of PC-1, allowing a longer action of ATP.

Besides aFGF nucleotides, dithiothreitol and vanadate also inhibited the autophosphorylation of PC-1. In addition, all these compounds promoted the dephosphorylation of PC-1 when added at the time of maximal phosphorylation. For all these agents, we can now exclude an inhibition at the PC-1 kinase site, since they failed to affect the phosphorylation of histone IIa by PC-1. Their effect should therefore be explained by stimulation of the PC-1 phosphatase or by making the autophosphorylation

site(s) unavailable for phosphoryl transfer. As for nucleotides and vanadate, we have been able to demonstrate that these agents act, at least in part, by stimulating the PC-1 phosphatase. However, at this stage, it is difficult to assess an additional shielding of the autophosphorylation site(s), since no inhibitors of PC-1 phosphatase are available. In this respect it is important to recall that the PC-1 phosphatase is not affected by a whole range of classical inhibitors of protein phosphatases and alkaline phosphatases [15].

One implication of a phosphatase-directed effect of the nucleotides is that the latency before the autophosphorylation of PC-1 should be viewed as a dynamic period, where the incorporated phosphate is rapidly hydrolysed by the PC-1 phosphatase. Net phosphorylation of PC-1 starts then when the concentration of nucleotides falls below the threshold concentration that is required for stimulation of the phosphatase.

We do not know the role of the autophosphorylation of PC-1. However, it is tempting to speculate that autophosphorylation occurs on the same threonine residue that participates in the formation of the catalytic intermediate of the phosphodiesterase-I/nucleotide pyrophosphatase reaction [29,30]. This would imply that PC-1 is inactivated by autophosphorylation. We have not yet been able to test this hypothesis. The underlying problem is the extreme nucleotide-dependence of the phosphorylation, which did not allow us to obtain a stoichiometry of autophosphorylation of more than 0.1 mol/mol of PC-1 monomer.

This work was supported financially by the Belgian Fund for Medical Scientific Research (3.0119.94), and by a Merit Review Grant from the U.S. Department of Veterans' Affairs. Ms. A. M. Hoogmartens provided expert technical assistance.

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Received 20 July 1994/5 October 1994; accepted 7 October 1994