Phosphorylation and nucleotide-dependent dephosphorylation of hepatic polypeptides related to the plasma cell differentiation antigen PC-1

Matxalen URIARTE,* Willy STALMANS,*† Scot HICKMAN† and Mathieu BOLLEN*

*Afdeling Biochemie, Fakulteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium,

and †Washington University Medical Service, Veterans Affairs Medical Center, John Cochran Division, St. Louis, MO 63106, U.S.A.

A glycoprotein fraction was isolated from rat liver membranes by affinity chromatography on immobilized wheat-germ lectin. Incubation of this fraction with MgATP or MgGTP resulted in a sequential phosphorylation and dephosphorylation of a complex of three polypeptides (118, 128 and 197 kDa on SDS/PAGE) with N-linked sialyloligosaccharides. Each polypeptide was recognized by polyclonal antibodies against recombinant plasma cell differentiation antigen PC-1. The relationship of the 118 kDa and 128 kDa polypeptides with PC-1 was confirmed by observations that they are linked by disulphide bonds into a larger protein, and that they are exclusively phosphorylated on Thr residues. Phosphorylation of p118, p128 and p197 only occurred after a lag period (up to 90 min at 30 °C), which lasted until most of the ATP had been converted to adenosine and P_i, with ADP and AMP as intermediate products. The length of the latency period increased with the concentration of initially added ATP (5–1000 µM) and could be prolonged by a second addition of similar concentrations of ATP, ADP, AMP and various nucle-

otide analogues. Most potent were the αβ-methylene derivatives of ADP and ATP. Adenosine was poorly effective. AMP, ADP, and perhaps ATP, emerge as the direct determinants of the latency. After further purification of the lectin-purified membrane fraction on anion-exchange and molecular-sieve columns, the complex of p118, p128 and p197 was still capable of auto-phosphorylation and dephosphorylation. The dephosphorylation was not affected by classical inhibitors (NaF, okadaic acid, EDTA, EGTA, phenylalanine). It was stimulated about 20-fold by various adenine nucleotides and analogues, with the same order of efficiency as noted for the induction of the latency. A similar stimulation of dephosphorylation was caused by 0.5 mM Na₃VO₄, which also prevented the phosphorylation of the three polypeptides. The likely explanation for the latency that precedes the phosphorylation of the membrane proteins is that the action of a protein kinase is initially offset by nucleotide-stimulated dephosphorylation.

INTRODUCTION

It is now well established that many membrane-associated processes (e.g. signal transduction and transport) are tightly controlled by the reversible phosphorylation of proteins on specific serine, threonine and tyrosine residues. Several integral membrane proteins are themselves protein kinases or protein phosphatases. Well-known examples are the receptor-linked tyrosine kinases and tyrosine phosphatases [1,2]. The plasma membrane also contains ecto-protein kinases and ecto-protein phosphatases, which can only (de)phosphorylate proteins on the cell surface or in the extracellular space [3,4].

The plasma cell differentiation antigen PC-1 was originally described as a cell surface marker present on differentiated, antibody-secreting B-lymphocytes (plasma cells) but lacking from thymocytes [5]. However, the protein is also expressed very selectively in non-lymphoid tissues; it is present on liver cells [5,6], some kidney cells and gland ducts, but not for instance on striated and smooth muscle cells nor in the gastrointestinal tract [7]. Murine and human PC-1s have been cloned; the cDNA-derived primary structure (871 amino acids) indicates that 95 % of the amino acids are located extracellularly, with a short (24-amino-acid) N-terminal cytoplasmic tail [8]. Recently it has been demonstrated that PC-1 is actually a bifunctional enzyme; it possesses both a Thr-specific ecto-protein kinase activity capable of autophosphorylation [6] and a nucleotide pyrophosphatase/

alkaline phosphodiesterase I activity [9] that had previously been purified from liver plasma membranes [10].

In the course of a study on the identity of protein kinases and protein phosphatases that phosphorylate/dephosphorylate the insulin receptor, we noted that such preparations of insulin receptor, partially purified from rat liver membranes by affinity chromatography on wheat-germ lectin–Sephacrose, also contained three polypeptides that became phosphorylated on threonine residues upon incubation with MgATP. Interestingly, the phosphorylations only started after a lag period that critically depended upon the exact conditions of incubation. We report here on the nature of the latency before phosphorylation and show that the phosphopolypeptides are structurally related to PC-1.

EXPERIMENTAL

Materials

[α-³²P]ATP, [γ-³²P]ATP, [2,8-³H]ATP and [γ-³²P]GTP were obtained from Amersham. ATP, adenosine 5′-[αβ-methylene]-diphosphate (p[CH₂]pA), adenosine 5′-[αβ-methylene]triphosphate (pp[CH₂]pA), adenosine 5′-[βγ-methylene]triphosphate (p[CH₂]ppA), adenosine 5′-[βγ-imido]triphosphate (p[NH₂]ppA), AMP and lectin from *Triticum vulgaris*, coupled to Sepharose-6MB, were purchased from Sigma. Adenosine 5′-[β-thio]diphosphate (ADP[S]), N-glycosidase F from *Flavo-*

Abbreviations used: p[NH₂]ppA, adenosine 5′-[βγ-imido]triphosphate; pp[CH₂]pA, adenosine 5′-[αβ-methylene]triphosphate; p[CH₂]pA, adenosine 5′-[αβ-methylene]diphosphate; p[CH₂]ppA, adenosine 5′-[βγ-methylene]triphosphate; ADP[S], adenosine 5′-[β-thio]diphosphate; PC-1, plasma cell differentiation antigen-1.

† To whom correspondence should be addressed: Afdeling Biochemie, KULeuven Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

bacterium meningosepticum, neuraminidase from *Arthrobacterium ureafaciens* and acidic fibroblast growth factor were obtained from Boehringer. The catalytic subunit of protein phosphatase-1 [11] and different species of protein phosphatase-2A [12] were purified from rabbit skeletal muscle. A kit for the development of Western blots by enhanced chemiluminescence was obtained from Amersham.

Preparation of lectin-purified membrane fraction

Rat liver membranes were prepared according to Havrankova et al. [13], with slight modifications. The livers of five Wistar rats (250–500 g) were homogenized in a Potter–Elvehjem tube in 4 vol. of an ice-cold solution at pH 7.6, containing 1 mM NaHCO₃, 1 mM phenylmethanesulphonyl fluoride and 10 mM benzamidine (buffer A). After a further 10-fold dilution in the same buffer, the homogenate was centrifuged for 30 min at 600 g. The supernatant was filtered through glass wool and centrifuged for 60 min at 15000 g. The pellet was washed once in 2–3 vol. of buffer A and resedimented for 30 min at 20000 g. Subsequently, the pellet was dissolved in 3–4 vol. of a buffer containing 50 mM Tris/HCl, pH 7.5, 140 mM NaCl, 2.5 mM KCl, 4 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 25 mM benzamidine. The resuspended membranes were solubilized by addition of 1–2 vol. of a buffer containing 50 mM Tris/HCl, pH 7.5, 3% (v/v) Triton X-100, 4 mM phenylmethanesulphonyl fluoride and 51 mM benzamidine. This suspension was incubated for 2 h at 4 °C and subsequently subjected to centrifugation for 120 min at 120000 g. The supernatant (about 50–100 ml) was mixed with 1 vol. of a buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% (v/v) Triton X-100 (buffer B), and applied to 10 ml of wheat-germ lectin–Sepharose-6MB, equilibrated in buffer B. The flow-through was re-applied twice to the same column. Subsequently, the column was washed with 60 ml of buffer B and the retained proteins were eluted with 0.3 M *N*-acetyl- α -D-glucosamine in buffer B. The fractions containing proteins were pooled, concentrated by ultrafiltration (Amicon Centriprep 30) to about 1 ml (1–2 mg of protein/ml) and dialysed against buffer B. This lectin-purified membrane fraction was used in the phosphorylation/dephosphorylation experiments unless otherwise stated.

Phosphorylation and analysis of membrane proteins

The lectin-purified membrane fraction was diluted 3–50-fold with 50 mM Hepes, pH 7.6, and incubated at 30 °C with the indicated concentrations of MgCl₂ and [γ -³²P]ATP. At different time points an aliquot was boiled in SDS-electrophoresis buffer [14] and subjected to SDS/PAGE (7.5% gels). Subsequently the gels were dried and exposed to autoradiography films (Hyperfilm-Mp; Amersham). The relative extent of phosphorylation of p118, p128 and p197 (proteins of 118, 128 and 197 kDa respectively) was quantified by laser densitometry. The apparent molecular mass was calculated by comparison with myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa) and BSA (66 kDa).

The glycoprotein nature of the phosphorylated polypeptides was investigated as follows. After maximal phosphorylation, the preparation was incubated for 30 min at 37 °C in the absence or presence of neuraminidase (1.3 units/ml). An aliquot of either mixture was prepared for SDS/PAGE. The remainder was boiled in 0.2% SDS and incubated for 16 h at 37 °C with N-glycosidase F (6.8 units/ml) plus 0.5% Triton X-100. Subsequently the latter mixtures were also subjected to SDS/PAGE.

The nature of the phosphoamino acid(s) was determined after maximal phosphorylation of the 118 kDa and 128 kDa polypeptides. The latter were located by autoradiography after SDS/PAGE, cut out separately and subjected to hydrolysis for 90 min at 110 °C in either 5 M KOH or 6 M HCl. The digests were supplemented with phosphoamino acid standards and analysed by two-dimensional thin-layer electrophoresis at pH 1.9 and then at pH 3.5 [15]. Ser-*P*, Thr-*P* and Tyr-*P* were located by development of the plate with ninhydrin and the radioactive phosphoamino acid was identified by comparison with an autoradiogram.

Partial purification of an autophosphorylating holoenzyme

In a few experiments, 0.6 ml of the concentrated lectin-purified membrane fraction was diluted with 2 vol. of buffer containing 50 mM Tris/HCl, pH 7.5, and 0.1% (v/v) Triton X-100, so as to lower the NaCl concentration to 50 mM. The sample was then applied to an anion-exchange column (Mono Q; 5 cm $\times$ 0.5 cm) connected to an f.p.l.c. system (Pharmacia LKB). The column was eluted with a linear NaCl gradient (50–500 mM) in the same buffer. The fractions were dialysed against buffer B. An aliquot was then incubated for 20–30 min at 30 °C with 50 μ M [γ -³²P]ATP and analysed for labelled polypeptides as described above. The polypeptides p118, p128 and p197 co-eluted (results not shown) with a peak at 320 $\pm$ 14 mM NaCl (mean $\pm$ S.E.M.; *n* = 5). The (unphosphorylated) peak fractions were pooled, concentrated (Amicon Centriprep 30) and applied to a column of Superose 6 (30 cm $\times$ 1 cm; Pharmacia LKB) equilibrated in buffer B, and eluted with the same buffer. An aliquot of each fraction was incubated with 50 μ M [γ -³²P]ATP and analysed for labelled polypeptides as described above (see Figure 2b).

Immunological procedures

Polyclonal antisera against recombinant PC-1 (fusion proteins derived from clones 3-1 and 5) were produced in rabbits [9]. Western blots were performed as described [16], except that the incubation with primary antiserum was done for 8 h at a final dilution of 1:5000 and that the peroxidase-labelled secondary antibodies were detected by enhanced chemiluminescence.

Assay of ATP and breakdown products

A trace amount of [2,8-³H]ATP was added to a phosphorylation mixture containing 50 μ M unlabelled ATP, in order to investigate the fate of the adenine moiety of ATP during incubation with the lectin-purified membrane fraction. Proteins were precipitated in aliquots with ice-cold 10% (w/v) trichloroacetic acid, which was immediately removed from the supernatant by multiple ether extractions. Subsequently ATP, ADP, AMP, IMP and adenosine in the supernatant were separated by t.l.c. [17] and quantified by comparison of the distribution of the radioactivity with the migration of adenine compounds during standard runs.

The fate of the [γ -³²P]phosphoryl group during phosphorylation of the three membrane polypeptides was investigated at various times after addition of 50 μ M [γ -³²P]ATP to the pooled peak fractions of the autophosphorylating holoenzyme (cf. Figure 2b). Deproteinization and subsequent removal of trichloroacetic acid were performed as described above. After addition of unlabelled P_i and ATP (1 mM each), a sample was applied to a column of Mono Q (5 cm $\times$ 0.5 cm) equilibrated with 50 mM Tris/HCl at pH 7.5. A non-linear NaCl gradient (0–1 M) was applied to elute separately P_i and ATP, and fractions were collected for determination of radioactivity.

Figure 1 Phosphorylation of three polypeptides in a glycoprotein fraction from rat liver membranes

The lectin-purified membrane fraction was incubated at 30 °C in the presence of 50 μ M [γ - 32 P]ATP. At the indicated times aliquots were boiled in electrophoresis sample buffer. After SDS/PAGE the radioactively labelled polypeptides were visualized by autoradiography for 2 h (a) or 16 h (b). Identical results were obtained when the incubation was performed in the presence of 100 nM insulin.

RESULTS

Phosphorylation of three polypeptides

The reversible phosphorylation of the insulin receptor is often studied in a lectin-purified membrane fraction [18]. However, we noted that the mere incubation of this fraction with 50 μ M [γ - 32 P]ATP, in the presence or absence of insulin, in itself resulted in the labelling of three polypeptides of 118, 128 and 197 kDa (Figure 1). Optimal visualization of these polypeptides was achieved by autoradiography for periods that were too short to reveal any autophosphorylated insulin receptor (95 kDa). Intriguingly, the labelling of p118, p128 and p197 only occurred after a lag period (Figure 1) of 10–90 min at 30 °C, the actual length depending on the precise incubation conditions (see below). Subsequently the labelling proceeded rapidly for 5–10 min, after which the amount of label decreased progressively. The relative distribution of the label between p118, p128 and p197 amounted to 1.0, 1.53 ± 0.04 and 0.16 ± 0.01 respectively (means $\pm$ S.E.M.; $n = 51$). The ratio was not affected by the incubation conditions.

The labelling of the three polypeptides could be observed at concentrations of [γ - 32 P]ATP as low as 0.1 μ M, and was equally pronounced when [γ - 32 P]ATP was replaced by [γ - 32 P]GTP (results not shown). The presence of a metal was essential, as indicated by the absence of any labelling in the presence of [γ - 32 P]ATP plus EDTA (results not shown). However, the metal

concentration required was very low, since an optimal labelling was obtained in the absence of added Mg^{2+} . At low concentrations of [γ - 32 P]ATP (5 μ M), increasing concentrations of Mg^{2+} (0.05–10 mM) were even found to inhibit the phosphorylation (50% inhibition at about 0.5 mM $MgCl_2$), but this effect was much less pronounced at 50 μ M labelled ATP.

Nature of the labelling

A covalently linked phosphate was strongly suggested by the persistence of the label after SDS/PAGE (Figure 1) and by the absence of label with [α - 32 P]ATP (results not shown). The covalent link was acid-stable (see below), which precludes a Tyr-P [15], an N-linked phosphate [19] and an acyl-phosphate [3]. However, the label was promptly and completely released by incubation with Ser/Thr-specific protein phosphatases (results not shown), i.e. either the catalytic subunit of protein phosphatase-1 or a mixture of different species of protein phosphatase-2A (PCS_{H1}, PCS_L and PCS_M [12]). In acid hydrolysates of p118 and of p128, Thr-P was identified as the single labelled amino acid (results not shown). An alkaline digest of either polypeptide did not contain any labelled phosphoamino acid, which confirms the absence of Tyr-P.

When phosphorylated p118 and p128 were incubated with trypsin (10 μ g/ml) for 1–3 min, their apparent molecular masses on SDS/PAGE decreased by about 8 kDa (results not shown). A longer trypsinolysis period (5–30 min) converted the label progressively to fragment(s) which were no longer retained by the gel. Exhaustive trypsinolysis of either polypeptide allowed us to recover the label in a single peptide with identical behaviour on reverse-phase h.p.l.c. (results not shown).

Characteristics of the glycan moiety

The reversible adsorption of the phosphorylatable polypeptides to immobilized wheat-germ lectin indicates the presence of either clustered O-glycosidically linked sialyloligosaccharides or an N-linked oligosaccharide with a mannose branch point [20]. The latter alternative applies to p118, p128 and p197, since treatment with N-glycosidase F decreased their apparent mass by 14–19 kDa (Table 1).

Asn-linked oligosaccharides with affinity for wheat-germ lectin

Table 1 Effects of neuraminidase and of N-glycosidase F on the apparent molecular mass of the Thr-phosphorylated proteins

The phosphorylated membrane proteins were treated as described in the Experimental section. Results are the means $\pm$ S.E.M. ($n = 8$). The values in parentheses indicate the mean decrease in the apparent molecular mass (in kDa).

Addition	Apparent molecular mass on SDS/PAGE (kDa)		
None	118 $\pm$ 2	128 $\pm$ 2	197 $\pm$ 2
Neuraminidase	112 $\pm$ 2 (–6)	123 $\pm$ 2 (–5)	193 $\pm$ 3 (–4)
Glycosidase F	99 $\pm$ 2 (–19)	112 $\pm$ 2 (–16)	183 $\pm$ 4 (–14)
Neuraminidase + glycosidase F	99 $\pm$ 2 (–19)	111 $\pm$ 2 (–17)	183 $\pm$ 4 (–14)

Figure 2 Molecular mass of the native protein containing p118, p128 and p197

The membrane fraction was incubated for 60 min at 30 °C in the presence of 0.25 mM [γ - 32 P]ATP and 3 mM MgCl₂. An aliquot of this fraction was then supplemented with SDS electrophoresis buffer in the absence or presence of reducing agents, and boiled. Panel (a) shows an autoradiogram after SDS/PAGE of these samples; panel (b) shows the migration of p118, p128 and p197 on Superose 6, after purification of the holoenzyme on lectin-Sepharose and Mono Q as described in the Experimental section. The polypeptides were identified by scanning of autoradiograms after SDS/PAGE of samples that had been preincubated for 30 min at 30 °C with 50 μ M [γ - 32 P]ATP. The arrow indicates the elution position of the marker protein ferritin (450 kDa). The inset shows an autoradiogram of the peak fractions of the phosphopolypeptides.

may have a terminal neuraminic acid residue attached to galactose [20]. This was indeed established for all three phosphopolypeptides, whose apparent masses on SDS/PAGE were decreased by 4–6 kDa after incubation with neuraminidase (Table 1). As expected, the effect of neuraminidase was not additive to that of N-glycosidase F.

Quaternary structure

SDS/PAGE in the absence of reducing agents yielded only one major radioactive band, of about 230 kDa (Figure 2a). This implies that the 118 kDa and 128 kDa polypeptides are linked into a larger protein by disulphide bonds, for example as homo- or hetero-dimer(s). It is not clear whether the 230 kDa band also encompasses the poorly phosphorylated 197 kDa polypeptide, which was not detected when reducing agents were omitted.

The apparent molecular mass of the native protein containing p118, p128 and p197, as deduced from gel filtration on Superose 6, amounted to about 500 kDa (Figure 2b). The co-migration of the three polypeptides during successive anion-exchange chromatography (results not shown) and molecular sieving (Figure 2b) indicates strongly that they comprise a single protein.

Immunological characterization

The specific phosphorylation of the hepatic polypeptides on Thr residues, and their migration during SDS/PAGE before and after treatment with reducing agents (Figure 2a) or N-glycanase (Table 1), is reminiscent of what has previously been described for the plasma cell differentiation antigen PC-1 [6,9,21]. These similarities prompted us to investigate in more detail the structural relationship between the hepatic phosphopolypeptides and PC-1. Western blotting with polyclonal antibodies raised against recombinant PC-1, expressed as a fusion protein in bacteria [9],

Figure 3 Western blots with PC-1-specific antibodies

The hepatic phosphopolypeptides were purified until after the Superose 6 step, as described in the Experimental section. An aliquot of the partially purified polypeptides (0.6 μ g of protein; lane 1) and of a lysate of variant MOPC 315 plasmacytoma cells expressing high levels of PC-1 (lanes 2 and 3) were used for Western blotting with polyclonal antibodies against recombinant PC-1. The blots were developed by autoradiography for 1 min (lanes 1 and 2) or 5 min (lane 3). The antibodies used in this experiment were raised against a fusion protein, termed clone 3-1 in [9], but the same results were obtained with antibodies against clone 5 (results not shown). No cross-reaction was observed with similarly diluted control serum.

revealed a cross-reaction with all three hepatic phosphopolypeptides (Figure 3). Interestingly, p197 was less intensively immunostained than were p118 and p128. Together with the lesser 32 P labelling of p197, this indicates that the concentration of p197 is lower than that of the other phosphopolypeptides. The antibodies used here have previously been shown to recognize, under reducing conditions in MOPC 315 plasmacytoma cell extracts, a major polypeptide of 115–120 kDa and a minor polypeptide of 220 kDa [9]. With the SDS/PAGE system adopted in the present study, the migration of the two cross-reacting

Figure 4 Effect of dilution and of the ATP concentration on the phosphorylation of p118–p128

The membrane fraction was incubated at 30 °C at the indicated final protein concentrations (a) in the presence of 0.25 mM [γ - 32 P]ATP and 3 mM MgCl₂. At the indicated times, samples were taken for SDS/PAGE. All samples were diluted to the same final concentration prior to electrophoresis. The extent of phosphorylation of the p118–p128 doublet was quantified by laser densitometry of autoradiograms. Panel (b) shows the effect of the indicated concentrations of labelled ATP (plus 3 mM MgCl₂) on the phosphorylation of the doublet, at a final protein concentration of 0.3 mg/ml. The specific radioactivity of ATP was kept constant.

Figure 5 Effect of a second addition of labelled ATP on the phosphorylation state of p118–p128

The membrane glycoprotein fraction was incubated with 0.25 mM [γ - 32 P]ATP and 3 mM MgCl₂. After 2 h the mixture was divided in two parts, which were either again supplemented with 0.25 mM [γ - 32 P]ATP and 3 mM MgCl₂ (●) or not (○). At the indicated times samples were taken for SDS/PAGE and the extent of phosphorylation of the p118–p128 doublet was quantified by laser densitometry of an autoradiogram.

plasmacytoma cell polypeptides corresponded to molecular masses slightly lower than those of p128 and p197 (Figure 3).

Role of adenine nucleotides in the latency

Figures 4–8 illustrate laser-quantified changes in the phosphorylation of the p118–p128 doublet; however, within the limits of detection, the same changes applied to p197. The latency was markedly prolonged by decreasing the temperature in the range 0–37 °C (results not shown). The lag period also increased from less than 30 min to 90 min when the membrane fraction was

diluted over an 8-fold range before incubation with [γ - 32 P]ATP (Figure 4a). Conversely, this dilution increased the maximal phosphorylation at least 20-fold. In searching for an explanation for these dilution effects, we obtained a similar pattern at a constant protein concentration by increasing stepwise the concentration of labelled ATP between 0.125 and 1 mM (Figure 4b). Additional evidence for a role of ATP in the generation of the lag period came from observations that the latency could be prolonged by an extra addition of [γ - 32 P]ATP before the phosphorylation had started (results not shown). Furthermore, an extra addition of [γ - 32 P]ATP after the peak of phosphorylation caused a prompt and near-complete dephosphorylation of the labelled doublet, followed by a new lag period and then a swift rephosphorylation (Figure 5). The complete rephosphorylation seems to exclude the possibility that the preceding ATP-induced loss of label was caused by ATP-dependent protease(s) (such enzymes are known to be present in particulate fractions from mammalian cells [22]).

[2,8- 3 H]ATP was rapidly degraded during the phosphorylation assay (Figure 6). There was a temporary accumulation of ADP and AMP, but eventually all ATP was quantitatively converted to adenosine. IMP did not accumulate at any time (results not shown). The phosphorylation of p118 and p128 (Figure 6) and p197 (results not shown) only started when nearly all of the ATP had been hydrolysed. The fate of the terminal phosphoryl group of [γ - 32 P]ATP was monitored during incubation with the partially purified autophosphorylating holoenzyme (peak fractions from Superose 6; cf. Figure 2b). Ultimately, all of the non-protein-associated radioactivity was recovered as P_i. No evidence was found for an intermediate phosphoryl acceptor (results not shown).

We subsequently investigated whether ATP itself or one of its degradation products causes the lag period. Because of the extremely short life of the adenine nucleotides (Figure 6), we also used analogues that are more resistant to enzymic cleavage [23]. When added at the end of the lag period, various adenine nucleotides and analogues decreased the rate of phosphorylation of p118 and p128 (Figure 7). AMP, ADP[S], p[NH]ppA and

Figure 6 Degradation of ATP during incubation with the membrane fraction

The membrane fraction was incubated with $50 \mu\text{M}$ [γ - ^{32}P]ATP. At the indicated times samples were taken for assay of the extent of phosphorylation of p118–p128 by scanning of autoradiograms after SDS/PAGE (broken line). In a parallel incubation the membrane fraction was incubated with $50 \mu\text{M}$ ATP labelled in the adenine moiety. The degradation products of ATP were identified after separation by t.l.c. as described in the Experimental section.

Figure 7 Inhibition of the phosphorylation of p118–p128 by adenine compounds

The lectin-purified membrane fraction was incubated at 30°C with $50 \mu\text{M}$ [γ - ^{32}P]ATP. The dilution of the preparation was adjusted so that the phosphorylation of p118–p128 started after a lag period of about 10 min. The indicated concentrations of the adenine compounds shown were added at 11 min. Samples were taken at 15, 17 and 20 min for determination of the phosphorylation rate of the doublet on autoradiograms after SDS/PAGE.

$\text{p}[\text{CH}_2]\text{ppA}$ were approximately equipotent: they prevented the phosphorylation completely at 50 – $250 \mu\text{M}$, and caused half-maximal inhibition at 10 – $30 \mu\text{M}$. In contrast, α,β -methylene-substituted ATP and ADP were about 50 times more efficient at decreasing phosphorylation. Adenosine had no effect on the phosphorylation rate at $50 \mu\text{M}$, and decreased it by only 25% at $250 \mu\text{M}$.

Stimulation of dephosphorylation by nucleotides

We next addressed the question of whether adenine nucleotides create the latency by inhibiting the protein kinase reaction or by

Figure 8 Effects of nucleotides and of vanadate on the dephosphorylation of p118–p128

The dilution of the membrane fraction was adjusted so as to obtain a maximal phosphorylation of p118–p128 after incubation at 30°C with $50 \mu\text{M}$ [γ - ^{32}P]ATP for 10 min. At this time, the mixture was supplemented with the additions shown, and samples were taken at the indicated times for SDS/PAGE and determination of the extent of phosphorylation of the doublet. The results are means $\pm$ S.E.M. ($n = 3$) or the mean of data from two experiments (ADP[S]).

stimulating dephosphorylation. Figure 8 shows that the rate of dephosphorylation of the p118–p128 doublet was increased when the kinase activity was blocked by EDTA. This indicates that, even when maximal phosphorylation had been obtained, there was still a continuous phosphorylation/dephosphorylation cycle. Remarkably, the addition of nucleotides resulted in a much faster dephosphorylation (Figure 8). During the first 1 min after the addition of a maximally effective nucleotide concentration, the p118–p128 doublet was dephosphorylated at a 15–25-fold higher rate than that observed in the presence of EDTA. The slower dephosphorylation in the presence of EDTA cannot be attributed to an inhibition of a protein phosphatase, since the combined addition of EDTA (1 mM) plus a nucleotide ($4 \mu\text{M}$ $\text{pp}[\text{CH}_2]\text{pA}$) elicited essentially the same rate of dephosphorylation of the doublet as seen with the nucleotide alone (results not shown).

We have also observed that the relative potencies of different adenine compounds in blocking the phosphorylation of p118–p128 (Figure 7) were essentially the same as their potencies in stimulating dephosphorylation of the membrane proteins. For example, $\text{pp}[\text{CH}_2]\text{pA}$ was maximally effective at just $2 \mu\text{M}$ (Figure 8), whereas a concentration of at least $50 \mu\text{M}$ was required for the maximal effect of ADP[S] to be observed (results not shown). In addition, adenosine did not show any stimulatory effect at concentrations below $100 \mu\text{M}$.

Other characteristics of the dephosphorylation

The dephosphorylation of p118–p128 was not affected by general inhibitors of Ser/Thr-specific protein phosphatases [24,25], such as 10 mM NaF or 5 mM pyrophosphate (results not shown). Neither was there any effect of more specific inhibitors of protein phosphatases-1 and -2A ($1 \mu\text{M}$ okadaic acid or $1 \mu\text{M}$ microcystin), of protein phosphatase-2B (1 mM EGTA) and of protein phosphatase-2C (1 mM EDTA; see above). Finally, the de-

phosphorylation of p118–p128 was not affected by inhibitors of alkaline phosphatases [26], i.e. phenylalanine, homoarginine or levamisole (each at 10 mM). Paradoxically, vanadate, a tyrosine phosphatase inhibitor [24], was a powerful stimulator of the dephosphorylation; at 0.5 mM, vanadate was equipotent with optimal concentrations of nucleotides (Figure 8). Therefore 0.5 mM vanadate was also added to a fresh membrane fraction together with MgATP, and found to prevent any phosphorylation of p118, p128 and p197 (results not shown).

We have also observed that the phosphatase activity against p118–p128 in the lectin-purified membrane fraction could not be separated from the autophosphorylating holoenzyme by successive chromatographies on Mono Q and on Superose 6 (results not shown).

DISCUSSION

Structure of the autophosphorylating protein kinase and its relationship to PC-1

Because of the limited amount of material available, we have not yet been successful in obtaining a partial amino acid sequence of p118, p128 or p197. However, in several respects our data provide strong evidence for a close structural relationship between the hepatic phosphopolypeptides and PC-1. (1) The three phosphopolypeptides were recognized by antibodies against recombinant segments of PC-1 (Figure 3). (2) The rat liver phosphopolypeptides (Table 1) and plasmacytoma PC-1 [9] have a similar N-linked oligosaccharide content. (3) The rat liver polypeptides p118 and p128 (the present work) and bovine liver PC-1 [6] are phosphorylated on Thr and not on Ser residues. Analysis of alkaline hydrolysates allowed us, furthermore, to establish the absence of Tyr phosphorylation, which could not be concluded for PC-1 [6]. (4) Like PC-1 from bovine liver [6], the enzyme from rat liver (purified until after the Superose 6 step) could phosphorylate histone IIa but not caseine, and the autophosphorylation was nearly completely inhibited by 1 mM dithiothreitol (results not shown). (5) The apparent molecular mass of the native liver enzyme (500 kDa; Figure 2b) corresponds to the previously reported molecular mass (about 520 kDa) of placental nucleotide pyrophosphatase [27], which is likely to be PC-1 [9]. This suggests that the native hepatic holoenzyme is a p128–p118 tetramer. (6) There are evident similarities in the apparent molecular masses as determined by SDS/PAGE, both in the absence and in the presence of reducing agents. The plasma cell antigen PC-1 has been described as a disulphide-linked homodimer (220–230 kDa) of a 115 kDa polypeptide [9,21]. However, PC-1 from bovine liver appeared on SDS/PAGE under reducing conditions as a doublet with a major band (130 kDa) and a minor one (110 kDa), and under non-reducing conditions as a single band of 260 kDa [6]. These values correspond reasonably well to our data on rat liver, with a phosphorylated 128–118 kDa doublet under reducing conditions and a single 230 kDa band without reduction. The presence of a monomeric doublet may be explained by partial proteolysis or by distinct post-translational modification. In addition to the doublet, we detected under reducing conditions a small but fairly constant proportion of a 197 kDa phosphopolypeptide. In our SDS/PAGE system this polypeptide migrated similarly to the 220 kDa band described for plasmacytoma PC-1 [9]. The presence of this 197/220 kDa polypeptide even after extensive reduction has previously been taken as evidence that some PC-1 dimer could be linked by non-disulphide bonds [9].

At variance with a previous report on PC-1 from bovine liver [6], we did not find any stimulatory effect of acidic fibroblast

growth factor, nor of equimolar concentration of Mn^{2+} and pyrophosphate, on the phosphorylation of p118–p128 (results not shown). At concentrations above 0.5 mM each, Mn^{2+} plus pyrophosphate were even found to inhibit the phosphorylation of p118–p128. On the other hand, pyrophosphate alone (up to 5 mM) did not affect the dephosphorylation.

Nature of the latency before phosphorylation

The lectin-purified membrane fraction has been widely used for the initial characterization of the insulin receptor [18]. It is therefore somewhat surprising that the phosphorylation of p118, p128 and p197 has not been described previously in this preparation. A likely explanation is that the phosphorylation of the three polypeptides is preceded by a lag (which depends critically on factors such as dilution, initial ATP concentration and temperature). Furthermore, at low ATP concentrations the phosphorylation of the three polypeptides is inhibited by Mg^{2+} concentrations that are routinely included in phosphorylation mixtures.

Several lines of evidence lead us to conclude that the presence of adenine nucleotides accounts for the lag period preceding the phosphorylation of p118, p128 and p197. Most importantly, phosphorylation only occurred when nearly all the nucleotides had been hydrolysed (Figure 6), and a new latency could be induced by an extra addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Figure 5). Our observation that both the duration of the latency and the maximal extent of phosphorylation depended on the dilution of the membrane fraction (Figure 4a) is also in agreement with this view. Indeed, at higher dilutions of the membrane fraction (lower ATPase concentration) it takes longer to reach the threshold concentration of ATP at which phosphorylation starts (i.e. longer latency), but the ATP concentrations in the useful substrate range for the autophosphorylating kinase will conceivably also be maintained for a longer time (thus ensuring a higher maximal phosphorylation).

Out of a series of nucleotides and nucleotide analogues, $p[\text{CH}_2]\text{pA}$ and $pp[\text{CH}_2]\text{pA}$ emerged as superior inhibitors of phosphorylation (Figure 7). These are also the two analogues with a non-metabolizable $\alpha\beta$ -linkage. When added to isolated hepatocytes, $p[\text{CH}_2]\text{ppA}$ and ATP were indeed converted to adenosine at the same rate, whereas $pp[\text{CH}_2]\text{pA}$ did not yield adenosine [28]. $p[\text{NH}]\text{ppA}$ can be hydrolysed by alkaline phosphatases [23]. Taken as a whole, our results indicate that at least AMP and ADP, and perhaps ATP, inhibit the labelling of p118–p128, and that conversion of AMP to adenosine terminates the latency.

We have shown that the rate of dephosphorylation of p118, p128 and p197 increased some 20-fold upon addition of nucleotides (Figure 8). Therefore a likely explanation for the lag period after addition of ATP is that the action of a protein kinase is initially nullified by nucleotide-stimulated dephosphorylation. As a result, no net phosphorylation is observed as long as the nucleotide concentration remains sufficiently high. This view is supported by the fact that various adenine nucleotides and adenosine displayed the same relative potencies in eliciting a latency and in stimulating the dephosphorylation. Although nucleotides evidently stimulate the dephosphorylation of the membrane proteins, we cannot rule out the possibility that the nucleotides may also inhibit the phosphorylation of p118, p128 and p197. However, our failure to identify an inhibitor of the dephosphorylation (see below) has hampered efforts to test the latter possibility.

Oda et al. [6] have reported that the autophosphorylation of PC-1 from bovine liver is inhibited by guanine and uridine

nucleotides. Inasmuch as our work deals with the equivalent protein from rat liver, it seems that the effects described by Oda et al. [6] would rather be explained by nucleotide-stimulated dephosphorylation of PC-1. However, the picture could even be more complex; since at least GTP is an alternative substrate for the autophosphorylating kinase (present data), the reported inhibition by unlabelled GTP [6] may well have been due to non-radioactive autophosphorylation. Although rare, such a dual nucleotide specificity has also been noted for some cytoplasmic protein kinases, such as casein kinase II [29].

A holoenzyme capable of reversible autophosphorylation?

The primary structure of PC-1 reveals a putative ATP-binding site in the extracellular domain, which has been taken as evidence that it is an ecto-protein kinase [6]. Inasmuch as the protein containing p118, p128 and p197 indeed represents rat hepatic PC-1, our data predict that this protein should then become Thr-autophosphorylated in the extracellular domain by the simple addition of MgATP or MgGTP.

The dephosphorylation of the p118–p128 doublet is a very unusual reaction in that it is not affected by general and specific inhibitors of the four classes of Ser/Thr-specific protein phosphatases, nor by inhibitors of alkaline phosphatases. Furthermore, dephosphorylation of the membrane proteins was stimulated some 20-fold by pp[CH₃]₃pA and by vanadate (Figure 8). However, the lectin-purified membrane fraction did not display any nucleotide-dependent or vanadate-dependent protein phosphatase activity towards phosphorylated glycogen phosphorylase, casein or histone IIA, i.e. Ser-phosphorylated model substrates of Ser/Thr protein phosphatases (results not shown). One possibility is that the dephosphorylation is catalysed by a novel 'nucleotide/vanadate-dependent' Thr protein phosphatase with a very narrow substrate specificity. However, our failure to separate a protein phosphatase from the autophosphorylating protein kinase during successive ion-exchange and molecular-sieve chromatographies indicates rather that the holoenzyme is also capable of autodephosphorylation. If so, then there is a striking resemblance to the bacterial isocitrate dehydrogenase kinase/phosphatase (reviewed by Laporte and Ikeda [30]). This protein kinase is also an ATP-dependent protein phosphatase; the two activities share a single ATP-binding site and occur probably at the same active site. The dephosphorylation is thought to occur by back-reaction of the kinase, tightly coupled to hydrolysis of ATP to ADP and P_i [30]. A regulatory protein in bacterial nitrogen metabolism has also been shown to undergo

ATP-stimulated dephosphorylation [31], but to our knowledge such a phenomenon has not been described in eukaryotes.

This work was supported by the Commission of the European Communities (SC1*.0223.C), by the Belgian Fund for Medical Scientific Research (3.0011.90), and by a Merit Review Grant from the U.S. Department of Veterans' Affairs. M.B. is 'Bevoegdverklaard navorser' of the National Fund for Scientific Research (Belgium). Ms. A. M. Hoogmartens provided expert technical assistance. Dr. J. Goris is acknowledged for generously providing PP-2A holoenzymes.

REFERENCES

- Ullrich, A. and Schlessinger, J. (1990) *Cell* **61**, 203–212
- Fischer, E. H., Charbonneau, H. and Tonks, N. K. (1991) *Science* **253**, 401–406
- Chen, X. Y. and Lo, T. C. Y. (1991) *Biochem. J.* **279**, 467–474
- Naik, U. P., Kornecki, E. and Ehrlich, Y. H. (1991) *Biochim. Biophys. Acta* **1092**, 256–264
- Takahashi, T., Old, L. J. and Boyse, E. A. (1970) *J. Exp. Med.* **131**, 1325–1341
- Oda, Y., Kuo, M.-D., Huang, S. S. and Huang, J. S. (1991) *J. Biol. Chem.* **266**, 16791–16795
- Harahap, A. R. and Goding, J. W. (1988) *J. Immunol.* **141**, 2317–2320
- Buckley, M. F., Loveland, K. A., McKinstry, W. J., Garson, O. M. and Goding, J. W. (1990) *J. Biol. Chem.* **265**, 17506–17511
- Rebbe, N. F., Tong, B. D., Finley, E. M. and Hickman, S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5192–5196
- Evans, W. H., Hood, D. O. and Gurd, J. W. (1973) *Biochem. J.* **135**, 819–826
- DeGuzman, A. and Lee, E. Y. C. (1988) *Methods Enzymol.* **159**, 356–368
- Waelkens, E., Goris, J. and Merlevede, W. (1987) *J. Biol. Chem.* **262**, 1049–1059
- Havrankova, J., Roth, J. and Brownstein, M. (1978) *Nature (London)* **272**, 827–829
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Cooper, J. A., Sefton, B. M. and Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402
- Vanden Abeele, C., Vandenheede, J. R. and Merlevede, W. (1988) *FEBS Lett.* **232**, 167–171
- Crabtree, G. W. and Henderson, J. F. (1971) *Cancer Res.* **31**, 985–991
- Zick, Y. (1989) *CRC Crit. Rev. Biochem. Mol. Biol.* **24**, 217–269
- Huang, J., Wei, Y., Kim, Y., Osterberg, L. and Matthews, H. R. (1991) *J. Biol. Chem.* **266**, 9023–9031
- Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G. and Strecker, G. (1986) in *Carbohydrate Analysis: A Practical Approach* (Chaplin, M. F. and Kennedy, J. F., eds.), pp. 143–204. IRL Press, Oxford
- Goding, J. W. and Shen, F.-W. (1982) *J. Immunol.* **129**, 2636–2640
- Fagan, J. M. and Waxman, L. (1989) *J. Biol. Chem.* **264**, 17868–17872
- Yount, R. G. (1975) *Adv. Enzymol.* **43**, 1–56
- Ballou, L. and Fischer, E. H. (1986) *Enzymes* 3rd Ed. **17**, 311–357
- Bollen, M. and Stalmans, W. (1992) *CRC Crit. Rev. Biochem. Mol. Biol.* **27**, 227–281
- Goldstein, D. J., Rogers, C. E. and Harris, H. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2857–2860
- Yano, T., Funakoshi, I. and Yamashina, I. (1985) *J. Biochem. (Tokyo)* **98**, 1097–1107
- Oetjen, E., Schweickhardt, C., Unthan-Fechner, K. and Probst, I. (1990) *Biochem. J.* **271**, 337–344
- Hathaway, G. M. and Traugh, J. A. (1982) *Curr. Top. Cell. Regul.* **21**, 101–127
- Laporte, D. C. and Ikeda, T. P. (1991) *Adv. Protein Phosphatases* **6**, 347–374
- Keener, J. and Kustu, S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4976–4980