Conversion of a phosphoseryl/threonyl phosphatase into a phosphotyrosyl phosphatase

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By use of the autophosphorylated epidermal-growth-factor receptor and the synthetic peptide RRLIEDAEY(P)AARG, representing an autophosphorylation site of the transforming protein of Rous-sarcoma virus, it is demonstrated that the phosphotyrosyl phosphatase activity of the polycation-stimulated phosphatases is substantially increased by an enzyme-directed effect of ATP or PP_i . Concomitant with this increase in phosphotyrosyl phosphatase activity, the phosphorylase phosphatase activity is decreased, thus dramatically changing the substrate specificity of these enzymes. The dephosphorylation of four different phosphotyrosyl sites of the epidermal-growth-factor receptor is neither consecutive nor at random, but a preferred dephosphorylation of the P_1 site over the $P_3 > P_2 > P_4$ sites is observed. This phosphatase activity represents a substantial fraction of the total phosphotyrosyl phosphatase activity in the post-mitochondrial supernatant of *Xenopus laevis* oocytes.

INTRODUCTION

A role for the phosphorylation of proteins on tyrosine residues in the control of cell proliferation is implicated by the observations that protein tyrosine kinase activities are intrinsic to a number of growth-factor receptors [1–5] and that they are also subverted in the generation of a number of oncogenes [6–8]. The importance of these activities in the signalling role played by these individual proteins is evident from studies demonstrating that abolition of kinase activity through site-directed mutagenesis abolishes the signalling/transforming capabilities [9–11]. In the model systems that have been used to elucidate the control of cellular functions by phosphorylation, it has become increasingly clear that the role played by protein phosphatases is far from a passive one, and that complex regulatory mechanisms exist to affect steady-state protein phosphorylation through alterations in protein phosphatase activity [12–14]. Although a number of phosphotyrosyl phosphatase activities have been described (see [15] for references), there has been as yet no evidence that these activities can be regulated in an acute fashion.

Recently the PCS_L (subunits 35 and 62 kDa) phosphoseryl/threonyl phosphatase from *Xenopus laevis* oocytes was isolated in high yield through a procedure involving tyrosine-agarose chromatography at an early point in the purification scheme. It was observed that a major *p*-nitrophenyl phosphatase activity co-purified with this phosphorylase phosphatase activity [16]. It has been known for some time that *p*-nitrophenyl phosphatase [17,18] and low phosphotyrosyl phosphatase activities [19,20] are associated with this type of enzyme, and highly variable but so far unexplained *p*-nitrophenyl

phosphate/phosphorylase phosphatase activity ratios have been reported [21,22]. The *p*-nitrophenyl phosphatase activity is specifically inactivated during purification, but can be restored by incubation with ATP, its analogues or PP_i [16].

During incubation with these effectors the enzyme loses its phosphorylase phosphatase activity [16–18], thus dramatically changing the specificity of the preparation by converting the phosphoseryl phosphatase activity into a phosphophenyl phosphatase activity [16]. We have utilized two phosphotyrosyl substrates to investigate whether this specificity change is also accompanied by an increase in phosphotyrosyl phosphatase activity. We report here that the EGF receptor autophosphorylated on tyrosine residues and phosphorylated RR-*src* [RRLIEDAEY(P)AARG; a synthetic peptide representing an autophosphorylation site of the *src* kinase] are substrates for the PCS phosphatases and that this activity is increased by preincubation with ATP and PP_i . Dephosphorylation by the activated PCS_L phosphatase of the four different tyrosine residues in the EGF receptor is not strictly consecutive, but a preferred order of $P_1 > P_3 > P_2 > P_4$ is observed.

MATERIALS AND METHODS

The PCS_H, PCS_M, PCS_L and PCS_C phosphatases were purified from rabbit skeletal muscle [23,24], and the PCS_L phosphatase was also purified from *Xenopus laevis* oocytes [16]. The active catalytic subunit of the ATP Mg^{2+} -dependent phosphatase was purified from dog liver [25]. Calcineurin purified from bovine brain [26] was

Abbreviations used: PCS phosphatases, polycation-stimulated (PCS_H, PCS_M and PCS_L: high-, medium- and low- M_r) phosphatases; PCS_C, catalytic subunit of the polycation-stimulated protein phosphatases; EGF, epidermal growth factor; *src* kinase, transforming protein of Rous-sarcoma virus; *p*NPP, *p*-nitrophenyl phosphate.

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a gift from Dr. J. H. Wang, University of Calgary, Calgary, Alberta, Canada. Alkaline phosphatase from *Escherichia coli* (type III-R) was purchased from Sigma Chemical Co. Phosphorylase *b* was purified from rabbit skeletal muscle [27], and A431 cell membranes were prepared in accordance with ref. [28] but omitting Ca^{2+} and Mg^{2+} . RR-*src* peptide was prepared by using an Applied Biosystems model 430A peptide synthesizer.

Radiolabelled phosphorylase *a* was prepared as previously described [29]. Autophosphorylated EGF receptor was prepared by incubating 375 μg of A431 cell membranes (preincubated at room temperature for 20 min with 1 μM -EGF) with 20 mM-Hepes/NaOH buffer, pH 7.5, containing 10 mM- Mg^{2+} , 200 μM - Na_3VO_4 , 0.3% (v/v) 2-mercaptoethanol and 100 μM - ^{32}P ATP (2500 c.p.m./pmol) at 30°C for 15 min. The membranes were then pelleted by centrifugation at 10000 *g* and resuspended in solubilization buffer [20 mM-Hepes/NaOH buffer, pH 7.5, containing 2 mM-EDTA, 1% (v/v) Triton X-100 and 0.3% (v/v) 2-mercaptoethanol]. Solubilized membranes were applied to a 1.0 ml column of wheat-germ lectin-Sepharose 6MB (Sigma Chemical Co.) that had been equilibrated in solubilization buffer. The column was washed with several volumes of 20 mM-Hepes/NaOH buffer, pH 7.5, containing 0.3% (v/v) 2-mercaptoethanol, to remove any free ATP from the column, and then the autophosphorylated receptor was eluted from the column with washing buffer containing 0.3 M-*N*-acetylglucosamine. Fractions (200 μl) were collected and their radioactivities determined by Čerenkov scintillation counting. Fractions containing peak amounts of radioactivity were pooled and chromatographed on a Sephadex G-50 column (1 cm $\times$ 10 cm) to remove *N*-acetylglucosamine. The column was equilibrated and run in 20 mM-Hepes/NaOH buffer, pH 7.5, containing 0.1% (v/v) Triton X-100 and 0.3% (v/v) 2-mercaptoethanol. Fractions containing peak amounts of radioactivity were pooled and a sample was analysed by SDS/10%-(w/v)-polyacrylamide-gel electrophoresis to determine the purity of the phosphorylated receptor. The synthetic RR-*src* peptide was phosphorylated by EGF receptor kinase in a 1.0 ml reaction mixture containing 120 μg of A431 cell membranes, 0.2 μM -EGF, 50 mM-Hepes/NaOH buffer, pH 7.4, 150 mM-NaCl, 0.02% (v/v) Triton X-100, 5% (v/v) glycerol, 2 mM- MnCl_2 , 100 μM - Na_3VO_4 , 1.4 mM-RR-*src* and 250 μM - ^{32}P ATP (2000–3000 c.p.m./pmol). After incubation of the mixture at room temperature for 1 h in the absence of ATP, the phosphorylation was initiated by the addition of ATP and continued for 16 h at 30°C. The reaction was stopped with an equal volume of 1 M-acetic acid, centrifuged to remove any denatured protein, and the supernatant applied to an anion-exchange column (Bio-Rad AG-1X2; 1.5 cm $\times$ 47 cm) that had been equilibrated in 0.5 M-acetic acid. The phosphopeptide was eluted with 1 M-acetic acid while unutilized ATP remained bound to the column. Peak fractions of phosphopeptide were dried by vacuum centrifugation, resuspended in water and pooled. To separate phospho- and dephospho-peptides, the pool was applied to a 1.0 ml column of anti-phosphotyrosine monoclonal antibody 1G2 (Oncogene Science, Long Island, NY, U.S.A.) conjugated to Sepharose 4B that had been equilibrated in 50 mM-ammonium acetate buffer, pH 6.8. The column was washed in equilibration buffer and the phosphopeptide was eluted with 0.5 M-ammonium acetate buffer, pH 6.8.

Eluted fractions containing peak amounts of radioactivity and low (0.002 maximum) to undetectable absorbance at 280 nm were dried by vacuum centrifugation, redissolved in 50 μl of water and combined, and bovine serum albumin was added to a concentration of 1 mg/ml. Phosphopeptide concentration was calculated on the basis of the specific radioactivity of the ATP in the phosphorylation reaction (only one tyrosine residue is present in the peptide substrate).

Phosphorylase phosphatase was assayed [23] with 1 mg of phosphorylase/ml. *p*NPP phosphatase was measured, after a preincubation with or without 2 mM-ATP, in an incubation mixture containing 20 mM-*p*NPP and 20 mM- MgCl_2 [16]. Dephosphorylation of the *src* peptide was measured after a 10 min preincubation at 30°C of the phosphatase in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml in the presence or in the absence of 1 mM-ATP or 50 μM - PP_i (as indicated in the Results and discussion section). The preincubation was stopped by diluting the mixture 5-fold with 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM- MgCl_2 , 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml. The diluted enzyme (5 μl) was included in a 50 μl reaction volume that routinely contained 0.3–0.4 μM -phospho-*src*, 0.5 mM-dithiothreitol, 1 mg of bovine serum albumin/ml and 50 mM- MgCl_2 in 50 mM-Tris/HCl buffer, pH 8.6, or 2 mM- MnCl_2 in 50 mM-Tris/HCl buffer, pH 7.5. The enzyme concentration in each reaction was adjusted (3–6 $\mu\text{g}/\text{ml}$) so that reaction rates remained linear for 5–10 min at 30°C and substrate depletion never exceeded 30%. Different parameters in the standard assay were varied as indicated in the Results and discussion section. The phosphatase reaction was stopped by the addition of 0.5 ml of 5% (w/v) trichloroacetic acid in 0.1 M-potassium phosphate. Released ^{32}P P_i was separated as described previously [30] by passing each stopped reaction mixture over a 0.5 ml column of cation-exchange resin (Bio-Rad AG-50W X8), which was then rinsed twice with 1.0 ml of water. Eluent was collected in a scintillation vial for counting of radioactivity. Dephosphorylation of the EGF receptor was carried out at 30°C in a reaction mixture containing 12 μg of preincubated and diluted PCL_T phosphatase/ml (as described above), 8–12 nm-phosphorylated receptor, 20 mM- MgCl_2 , 50 mM-Tris/HCl buffer, pH 8.6, 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml. Samples of the reaction were removed at various times and added to a 5-fold excess of 10% (w/v) trichloroacetic acid. Stopped reactions were placed on ice for 15 min and centrifuged at 10000 *g*. Free ^{32}P P_i in the supernatant was quantified by liquid-scintillation counting. For analysis of the dephosphorylation of individual residues in the receptor, the pellets were redissolved in 1 M-NaOH, neutralized with 1 M-Tris/HCl buffer, pH 8.0, and 1 M-HCl, and diluted to 0.5 ml with water. Then 100 μg of trypsin [L-tosyl-phenylalanylchloromethane- ('TPCK') treated; Cooper Biomedical) was added and the mixtures were incubated for 24 h at 37°C. Non-trypsin-digested protein was precipitated with trichloroacetic acid and pelleted at 10000 *g*, and the supernatant was applied to a Brownlee RP 300 column (2.1 mm $\times$ 100 mm) connected to a Hewlett-Packard 1090 h.p.l.c. system. Bound material was eluted with a 0–50% (v/v) acetonitrile gradient in 0.08% (v/v) trifluoroacetic acid [31], and the fractions containing phosphopeptide (identified by Čerenkov scin-

tillation counting) were subjected to phospho amino acid analysis.

RESULTS AND DISCUSSION

Preliminary experiments indicated that the phosphotyrosyl peptide *RR-src* was indeed a substrate for the PCS_L phosphatase. Since this peptide substrate is readily available, it was used to characterize the phosphotyrosyl phosphatase activity in preference to the auto-phosphorylated EGF receptor, which is also a substrate for the PCS_L phosphatase (see below). The low stoichiometry of peptide phosphorylation (less than 5%) and an observed product inhibition of phosphatase activity by the unphosphorylated peptide (see below) necessitated separation of the phosphorylated and non-phosphorylated peptides before experimental use of the substrate. This was achieved by using an anti-phosphotyrosine monoclonal antibody covalently linked to Sepharose. Non-phosphorylated peptide was not retained by the column whereas phosphorylated peptide was retained and then eluted from the column (see the Materials and methods section).

The purified PCS_L phosphatase from rabbit muscle or *Xenopus* oocytes displays a basal activity towards the purified phospho-*src* peptide. As previously observed for the *p*-nitrophenyl phosphatase activity [16], this phosphotyrosyl phosphatase activity could be stimulated 3–5-fold (Fig. 1) by incubation with ATP ($A_{50} = 220 \mu\text{M}$) or PP_i ($A_{50} = 34 \mu\text{M}$). Both basal and stimulated activities are variable among different enzyme preparations and seem to be related to a previous inactivation during the purification. The ATP- and PP_i -mediated phosphotyrosyl phosphatase activation is dependent upon the time of preincubation of these effectors with the enzyme (Fig. 2), indicating that their effect is at the enzyme level and not substrate-directed. Maximum activation can be observed following incubation in the presence of 2 mM-ATP or 0.1 mM- PP_i . After activation (optimal at 20 min), the preincubation mixture is diluted 50-fold in MgCl_2 to decrease free ATP/ PP_i (see below) before assay. The ability of the enzyme to remain activated after dilution of the effector to $\ll K_a$ (40 μM -ATP or 2 μM - PP_i) in the presence of a large excess of Mg^{2+} or Mn^{2+} suggests that these effectors have induced a relatively stable change in the enzyme. The activation of the phosphatase by PP_i as well as by ATP indicates that phosphorylation does not play a role in the activation. Furthermore, the ATP-mediated enzyme activation does not occur if excess Mg^{2+} is present in the preincubation mixture (Fig. 1).

In contrast with the activation process, phosphotyrosyl phosphatase activity is itself completely dependent on the presence of Mg^{2+} or Mn^{2+} in the assay mixture. The basal and the ATP/ PP_i -stimulated phosphotyrosyl phosphatase activities exhibit different pH optima in the presence of Mn^{2+} (pH 7.5) or Mg^{2+} (pH 8.5). At these pH optima, the phosphotyrosyl phosphatase activity displays apparent A_{50} values of 30 μM and 10 mM for Mn^{2+} and Mg^{2+} respectively.

Examination of the kinetic parameters of phosphotyrosyl-*src* phosphatase activity shows that preincubation of the enzyme with ATP results in an increased V_{max} without affecting the affinity for the substrate (apparent K_m unchanged). When assayed in the presence of Mg^{2+} and at pH 8.5, activation of the enzyme by ATP results in an increase in V_{max} from 7.1 to 34.8 nmol/min per

Fig. 1. Activation of the PCS_L phosphatase by different concentrations of ATP or PP_i with *RR-src* as the substrate

The PCS_L phosphatase was preincubated for 10 min at 30 °C with the indicated concentrations of ATP (●), PP_i (▲) or ATP + Mg^{2+} (✱), MgCl_2 being present in 5-fold excess over ATP, diluted and assayed for *RR-src* phosphatase activity in 50 mM-Tris/HCl buffer, pH 8.5, in the presence of 20 mM- MgCl_2 as described in the Materials and methods section.

Fig. 2. Time course of the phosphotyrosyl phosphatase activation by ATP

The PCS_L phosphatase was preincubated at 30 °C with 1 mM-ATP (■), 0.25 mM-ATP (●) or without ATP (▲). At the indicated time points, samples were diluted and assayed at pH 8.5 in the presence of 50 mM- MgCl_2 as described in the Materials and methods section.

Fig. 3. Lineweaver-Burk plot of the activated and non-activated PCS_L phosphatase with RR-*src* as the substrate

PCS_L phosphatase was preincubated with (●) and without (▲) 1 mM-ATP, diluted and assayed as described in the Materials and methods section with various concentrations of the substrate (0.5–1.7 μM) at pH 8.5 in the presence of 50 mM-MgCl₂.

mg without significantly modifying the K_m of 6.9 μM (Fig. 3). The different effectors influence the phosphotyrosyl phosphatase and *p*-nitrophenyl phosphatase activities of the PCS_L phosphatase in a remarkably similar way. This is probably related to the mechanism of catalysis and the similarity of the substrates. However, the kinetic parameters differ largely (K_m 4 mM for *p*NPP [16] compared with 6.9 μM for the phosphopeptide; V_{max} 53 μmol/min per mg for *p*NPP [16] compared with 35 nmol/min per mg for the phosphopeptide), indicating that the integration of the phenyl phosphate into a peptide can strongly influence the affinity for the substrate and the efficiency of catalysis.

After purification, the PCS_L phosphatase displays phosphorylase phosphatase activity and a basal *p*-nitrophenyl phosphatase or *src* peptide phosphatase activity. Phosphorylase phosphatase activity is competitively inhibited by *p*NPP, indicating that the same catalytic site may be involved in the dephosphorylation of these two substrates [16]. Non-phosphorylated *src* peptide also inhibits phosphorylase phosphatase activity (I_{50} = 0.5 mM). The ATP/PP_i-induced activation of the phosphotyrosyl phosphatase activity of the PCS_L phosphatase results in a concomitant loss of phosphorylase phosphatase activity, so that phosphorylase is no longer a substrate after preincubation with these effectors. Apparently, following the ATP/PP_i-induced activation of the phosphatase the catalytic site is no longer accessible to the alkyl phosphate of phosphorylase, whereas the phenyl phosphate moiety of phosphotyrosine or *p*NPP is now effectively hydrolysed. The dephosphorylation of the *src* peptide is inhibited by the alternative substrate *p*NPP (I_{50} = 1.5 mM, measured at 0.39 μM-phosphopeptide) and by non-phosphorylated peptide (I_{50} = 0.8 mM, measured at 0.39 μM-phosphopeptide). The latter product inhibition appears to be a common property of aryl phosphatases [32,33]. The rather low affinity for

product, as indicated by the high I_{50} value, does not suggest any physiological relevance. However, since all tyrosine-containing proteins could be considered potential inhibitors of phosphotyrosyl phosphatases and since there is some indication that phosphotyrosyl phosphatase inhibitors are present in the brain [34], the observed inhibition requires further investigation.

In order to determine whether the PCS_L phosphatase could dephosphorylate phosphotyrosine-containing proteins, we utilized partially purified autophosphorylated EGF receptor (3.1–3.5 mol of phosphate/mol of receptor) as a substrate. The ATP-activated PCS_L phosphatase shows a specific activity of 2.4 nmol/min per mg towards this protein (present at 10 nM). The conditions of enzyme activation and requirements for phosphatase activity (metal ion, pH) are identical with those determined for the *src* peptide dephosphorylation (results not shown). The activated phosphatase completely removes the phosphate present exclusively on tyrosine residues (results not shown). Analysis of the time course (Figs. 4a and 4b) of dephosphorylation of the individual tyrosine residues demonstrates that removal is not strictly consecutive; it appears that the enzyme preferentially hydrolyses phosphate from the tyrosine residue 1173 of site P₁ [31], followed by the sites P₃ (residue 1068) and P₂ (residue 1148), and that the recently identified site P₄ (J. J. Hsuan, personal communication) is the least preferred site. Although these findings show that the PCS_L phosphatase can indeed dephosphorylate a tyrosine-containing protein substrate, further studies are necessary to identify other such substrates and to determine whether or not the enzyme exhibits any degree of phosphotyrosyl phosphatase specificity akin to that evidenced by its phosphoserine/threonine phosphatase specificity [35,36].

The experiments described above were carried out with the low- M_r form of the PCS phosphatase (PCS_L). Other forms of PCS phosphatases, which differ in M_r , subunit composition, phosphoserine/threonine substrate specificity and activity requirements [14,15,35,36], were also tested for ATP/PP_i-stimulated RR-*src* peptide phosphatase activity. The PCS_{H1}, PCS_{H2}, PCS_M and PCS_C phosphatases from rabbit skeletal muscle all show such activity (results not shown). The degree of stimulation by ATP or PP_i is variable, as found with the PCS_L phosphatase, and does not appear to be related to a particular form of PCS phosphatase. The fact that the ATP/PP_i activation is also observed with the catalytic subunit (PCS_C) suggests that this effect is not mediated by the regulatory subunits present in the holoenzymes. No phosphotyrosyl phosphatase activity could be measured with the catalytic unit of the ATP Mg²⁺-dependent phosphatase (500 units of phosphorylase phosphatase/ml), even after incubation with PP_i. No ATP or PP_i stimulation of the phosphotyrosyl phosphatase activity of *E. coli* alkaline phosphatase (specific activity 28 nmol/min per mg) or of calcineurin isolated from bovine brain (specific activity 11 nmol/min per mg) was observed, indicating that this phenomenon is a unique property of the PCS phosphatases.

The ATP/PP_i-mediated stimulation of phosphotyrosyl phosphatase activity and the ensuing loss of phosphorylase phosphatase activity of the PCS enzymes represents a novel mechanism of phosphatase regulation. This process appears to involve a change in the catalytic subunit of the phosphatase by the direct interaction of the effector molecules with this subunit. Whether this

Fig. 4. Dephosphorylation of the individual tyrosine residues of the EGF receptor by the PCS_L phosphatase

(a) Tryptic phosphopeptide map of EGF receptor from A431 cell membranes. The EGF receptor was phosphorylated and tryptic phosphopeptides were prepared and separated as described in the Materials and methods section. The map shown results from the receptor before dephosphorylation (100% phosphate label). (b) Time course of dephosphorylation of the EGF receptor. During dephosphorylation of the receptor (see the Materials and methods section) samples of the reaction mixture were

interaction results in a stable change or whether continued protein-effector interaction is required to maintain activity remains to be investigated. The ATP/PP_i-activated and non-activated forms of the enzyme appear to share a common active site that specifically hydrolyses either phosphotyrosine or phosphoserine/threonine respectively. So far it has only been possible to demonstrate the conversion of a phosphoserine phosphatase into a phosphotyrosyl phosphatase by ATP or PP_i, but future studies may elucidate conditions that lead to an inter-conversion, an extremely interesting prospect in terms of physiological enzyme regulation. To have an idea of the cellular importance of this phenomenon in terms of relative phosphotyrosyl phosphatase activity represented by the PCS phosphatases, we measured the RR-*src* phosphatase activity in the post-mitochondrial supernatant of oocytes from *Xenopus laevis*, after separation of the different phosphatases on a DEAE-Sephacel column eluted with a 0–0.5 M-NaCl gradient. Measured under the optimal conditions for the activated phosphotyrosyl phosphatase activity (pH 8.5, plus Mg²⁺), the activity associated with the PCS phosphatases represents about 30% of the total measurable phosphotyrosyl phosphatase. Therefore the conversion of serine/threonine specificity into tyrosine specificity described above suggests a viable mode of regulation for the potentially multifunctional PCS phosphatases.

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withdrawn at the times indicated and tryptic phosphopeptides were generated and mapped as in (a). The amount of phosphate label remaining in each peptide was quantified and expressed as a function of time. □, Site P₁; ■, site P₂; ○, site P₃; ●, site P₄. The points represent means of duplicate observations. The lines are derived from linear-regression analyses that yielded correlation coefficients > 0.94.

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