Identification, characterization and purification to near-homogeneity of a novel 67 kDa phosphotyrosyl protein phosphatase associated with pig lung annexin extract

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During the purification of annexin VI from pig lung, we previously reported the isolation of another 67 kDa protein (protein 67E) differing from the former by immunological reactivity, amino acid composition, inability to interact with anionic phospholipids in the presence of Ca^{2+} and inability to inhibit phospholipase A_2 [Fauvel, Vicendo, Roques, Ragab-Thomas, Granier, Vilgrain, Chambaz, Rochat, Chap & Douste-Blazy (1987) FEBS Lett. 221, 397–402]. Attempts to phosphorylate protein 67E by the protein tyrosine kinase of epidermal-growth-factor receptor revealed a dramatic inhibition of receptor autophosphorylation, which was also observed with insulin receptor. This inhibitory effect was found to be supported by a phosphatase active towards *p*-nitrophenyl phosphate, phosphotyrosine, [^{32}P]phosphotyrosyl histones and [^{32}P]phosphotyrosyl poly(Glu,Tyr), but inactive towards phosphoserine, phosphothreonine and [^{32}P]phosphoserine histones. Although not purified to complete homogeneity, the enzyme was purified 273-fold over EGTA extracts from pig lung and corresponded to a monomeric protein displaying an apparent molecular mass of 67 kDa. With [^{32}P]phosphotyrosyl poly(Glu,Tyr) as substrate, the purified enzyme displayed K_m and V_{max} values of 10 μM and 1.93 $\mu\text{mol/min per mg}$ respectively, which compare reasonably well with other recently described phosphotyrosyl protein phosphatases. From these data and from its sensitivity to various inhibitors, it is concluded that protein fraction 67E contains a novel phosphotyrosyl protein phosphatase, the association of which with annexin extract might offer a clue to the understanding of its possible targeting to membrane substrates.

INTRODUCTION

Protein tyrosine phosphorylation is known to play an important role in the regulation of various cell functions such as proliferation, differentiation and transformation [1,2]. The state of cell protein tyrosine phosphorylation seems to be regulated by two opposite biochemical processes: one involves specific protein tyrosine kinases supported by growth-factor receptors or encoded by oncogenes [3–5]; the other is due to numerous phosphatases that are able to remove the phosphate group from protein phosphotyrosine residues. As recently reviewed by Lau *et al.* [6], various enzymes are able to perform such a task, but only some of them can be defined as true phosphotyrosyl protein phosphatases (PTPPs), on the basis of a strict specificity for phosphotyrosine residues. In addition, other phosphatases display similar activities towards protein phosphoserine and phosphotyrosine residues, as in the cases of alkaline phosphatase [7] and calcineurin [8].

The study of PTPP recently received a resurgence of interest after two key observations: (i) some somatostatin analogues were shown to act as growth inhibitors of pancreatic cancer cells through the activation of a PTPP [9]; (ii) a leucocyte antigen, CD 45, and a related protein, LAR, were identified as bearing a single transmembrane segment, an extracellular domain and an intracytoplasmic domain, the last displaying PTPP activity [10–14]. The number of known PTPPs is now rapidly expanding [15–17], which strongly argues in favour of a transmembrane signalling pathway involving a receptor-linked PTPP, in a way similar to but opposite to that previously described for numerous growth-factor receptors [18,19].

The latter observations were made on the basis of a strong

sequence similarity between the cytoplasmic domain of CD 45 antigen and a soluble 35 kDa PTPP purified from human placenta [10]. This protein belongs to a family of soluble PTPPs [14]; however, the soluble nature of these enzymes does not allow us at present to understand how their hydrolytic activity could be oriented towards specific targets such as membrane receptors to growth factors undergoing autophosphorylation on appropriate stimulation.

In a previous study dealing with the purification from pig lung of a 67 kDa Ca^{2+} - and phospholipid-binding protein, now known as annexin VI [20], we reported the isolation of a protein displaying a similar molecular mass to annexin VI but differing from it on the basis of amino acid composition, immunoreactivity, inability to interact with phospholipids and inability to inhibit phospholipase A_2 , as in the case of annexins [20]. Although later this protein fraction was revealed to be heterogeneous on the basis of *N*-terminal sequencing, it was referred to as protein 67E because of its exclusion from a column of polyacrylamide-immobilized phosphatidylserine loaded in the presence of Ca^{2+} , under which conditions annexins interact with the anionic phospholipid.

In the present study, protein 67E is shown to inhibit autophosphorylation of epidermal-growth-factor (EGF) receptor. Data are provided that this inhibition is supported by a minor protein displaying PTPP activity, the partial purification and characterization of which are reported.

MATERIALS AND METHODS

Materials

Poly(Glu,Tyr) (4:1) (molecular mass 20–50 kDa) and other

Abbreviations used: PTPP, phosphotyrosyl protein phosphatase (protein-tyrosine-phosphatase, EC 3.1.3.48); DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; LAR, leucocyte antigen-related protein; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ultrogel AcA 44, DEAE-TSK 545, f.p.l.c. Mono S HR 5/5, f.p.l.c. Superose 6 HR 10/30 and wheat-germ lectin-agarose were purchased from Pharmacia-LKB (Uppsala, Sweden). [γ - 32 P]ATP was from Amersham International (Amersham, Bucks., U.K.). Dulbecco's modified Eagle's medium (DMEM) and fetal-calf serum were from Gibco (Paisley, Renfrewshire, Scotland, U.K.).

Isolation of protein fraction 67E from pig lung

Protein 67E was isolated as described previously [21]. Briefly, this included extraction by EGTA of proteins adsorbed on membranes from a pig lung homogenate prepared in the presence of 1 mM-CaCl₂ by the method of De *et al.* [22]. The EGTA extract in buffer A [20 mM-Hepes buffer, pH 7.0, containing 150 mM-KCl, 2 mM-MgCl₂, 2 mM-EGTA, 0.5 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-dithiothreitol (DTT) and 5 μ g of leupeptin/ml] was then loaded on to a column (21.5 mm $\times$ 150 mm) of DEAE-TSK 545 equilibrated with buffer B (20 mM-Hepes buffer, pH 7.0, containing 1 mM-DTT and 0.5 mM-PMSF), and the flow-through fraction was recovered. Further steps involved gel filtration on AcA 44, to isolate proteins with an apparent molecular mass of 67 kDa, followed by affinity chromatography on a polyacrylamide-immobilized phosphatidylserine column. Proteins excluded in the presence of Ca²⁺ were pooled and stored at -20 °C. As described before [21], SDS/PAGE revealed the presence of a broad band with an apparent molecular mass of 55–67 kDa. This fraction was used in most experiments dealing with the characterization of phosphatase activity.

Further purification of PTPP

This was achieved as described above with the following modifications: starting from 80 g wet wt. of lung, EGTA extract was prepared in the absence of KCl, under which conditions phosphatase activity was still excluded from DEAE-TSK 545. The gel filtration on AcA 44 was then omitted, and the fraction excluded from phosphatidylserine column in the presence of 20 mM-Hepes buffer, pH 7.0, containing 1 mM-DTT, 0.5 mM-PMSF and 3 mM-CaCl₂ was adjusted to pH 5.5 with 0.02 M-HCl. It was then loaded on to a Pharmacia f.p.l.c. Mono S HR 5/5 cation-exchange column equilibrated with buffer C (20 mM-Mes buffer, pH 5.5, containing 1 mM-DTT and 0.5 mM-PMSF). After being washed with 4 ml of buffer C, the adsorbed proteins were eluted with 40 ml of a linear gradient of 0–0.5 M-NaCl in buffer C at a flow rate of 1 ml/min. As described in the Results section, a final step involved gel filtration of the most pure fraction on Pharmacia f.p.l.c. Superose 6 HR 10/30 equilibrated with buffer C in the presence of 0.1 M-NaCl. The enzyme fractions were stored at -20 °C.

Phosphatase assay with *p*-nitrophenyl phosphate

Enzyme activity was assayed in a reaction mixture (0.2 ml) containing 10 mM-*p*-nitrophenyl phosphate in 100 mM-sodium acetate buffer for incubations at pH 3.0–6.0 or in 100 mM-Tris/HCl buffer for reactions at pH 7.0–9.0. Assays were initiated by addition of enzyme fractions and, after 30 min incubation at 37 °C with shaking, terminated by addition of 1 ml of 0.02 M-NaOH. The absorbance at 410 nm of the mixture was measured spectrophotometrically by using a sample lacking enzyme as a blank (the molar absorption coefficient for the *p*-nitrophenolate anion was $1.75 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Phosphatase activity with phospho amino acids

Incubations were performed as described above in an identical reaction mixture containing 10 mM of either phosphotyrosine or

phosphoserine. The rate of dephosphorylation was determined by the rate of inorganic phosphate production, which was measured as described elsewhere [23].

Preparation of [32 P]phosphotyrosyl proteins

Histones (Sigma type II A) or poly(Glu,Tyr) were phosphorylated on tyrosine residues by using the EGF-receptor tyrosine kinase of A431-cell membranes according to the procedure described by Swarup *et al.* [7]. A431 cells were cultured in DMEM containing 5% (v/v) fetal-calf serum, 100 μ g of penicillin/ml and 100 μ g of streptomycin/ml in a gas phase of 5% CO₂ in air. The A431-cell membrane fraction was prepared as previously described by Fava & Cohen [24].

Preparation of [32 P]phosphoseryl histones

Histones (Sigma type II A) were phosphorylated on serine residues with the catalytic unit of cyclic AMP-dependent protein kinase according to the procedure described by Swarup *et al.* [7].

Protein phosphatase assays

[32 P]Phosphotyrosyl histones (15000–20000 d.p.m., 2.2–3.3 μ M), [32 P]phosphotyrosyl poly(Glu,Tyr) (60000–600000 d.p.m., 0.5–5 μ M) or [32 P]phosphoseryl histones (72600–74400 d.p.m., 2.2–3.3 μ M) were incubated in 0.2 ml of 100 mM-Tris/HCl buffer, pH 7.0, at 37 °C for 5–60 min with gentle shaking. Reaction was blocked by addition of 0.8 ml of 50% (w/v) trichloroacetic acid and 0.2 ml of 0.625% (w/v) BSA. After 10 min on ice, the mixture was centrifuged at 15000 *g* for 6 min. The [32 P]phosphate released during the reaction was determined directly on the supernatant [with poly(Glu,Tyr) as substrate] or after addition of an acidic ammonium molybdate solution as previously described [25]. The 32 P-labelled phosphomolybdic acid was extracted with 0.5 ml of 2-methylpropan-1-ol/toluene (1:1, v/v) [26] and radioactivity was determined by liquid-scintillation counting. Concentrations of [32 P]protein phosphate were calculated by taking account of the specific radioactivity of [γ - 32 P]ATP used in the phosphorylation reactions.

Dephosphorylation of [32 P]phosphotyrosyl EGF receptor from A431-cell membranes

The reaction mixture containing A431-cell membranes (5 μ g of protein), 20 mM-Hepes buffer, pH 7.4, 20 mM-MgCl₂, 2 mM-MnCl₂ and 1 mM-CaCl₂ was preincubated at 4 °C for 15 min with or without 500 nM-EGF. Receptor phosphorylation was initiated by the addition of [γ - 32 P]ATP (25 μ M, 2.5 μ Ci) in a final volume of 0.02 ml. The mixture was then incubated for 20 min at 4 °C, followed by the addition of 1 μ g of 67E protein fraction. The reaction was allowed to continue for 10 min at 4 °C and stopped by the addition of electrophoresis sample buffer and heating for 3 min at 100 °C. The mixture was separated by SDS/PAGE, and submitted to autoradiography.

Dephosphorylation of solubilized [32 P]phosphotyrosyl insulin receptor

Solubilized receptor from rat liver was prepared as described by Karasik *et al.* [27]. Portions of lectin-purified solubilized insulin receptor (5 μ g of protein) were preincubated for 15 min at 4 °C in 50 mM-Hepes buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, 4 mM-MnCl₂, 10 mM-MgCl₂ and 0.5 mM-CaCl₂, with or without 0.1 μ M-insulin, and then incubated for 20 min at 4 °C with [γ - 32 P]ATP (25 μ M, 2.5 μ Ci) in a final volume of 0.025 ml. The dephosphorylation reaction was then initiated by adding 1 μ g of 67E protein fraction (in the absence or in the presence of 50 μ M-Na₃VO₄) and allowed to continue for another 20 min at 4 °C. The reaction was terminated by addition of

electrophoresis sample buffer and heating for 3 min at 100 °C. Proteins were separated by SDS/PAGE, and subjected to autoradiography.

Other analytical methods

Protein concentration was determined by the method of Bradford [28], with BSA as standard. SDS/PAGE was performed by the procedure of Laemmli [29], and proteins were detected by silver staining [30] with a kit from Bio-Rad Laboratories. Enzyme activity was recovered from the gel by using the procedure described by Hager & Burgess [31], renaturation of proteins after dilution of guanidinium chloride being performed for 17 h.

RESULTS

Effect of protein 67E on phosphorylation of EGF and insulin receptors

A main band with an apparent molecular mass of 170 kDa, corresponding to EGF receptor, was phosphorylated in an EGF-dependent manner in membranes from A431 cells incubated with [γ - 32 P]ATP. Inclusion of protein fraction 67E in the incubation mixture almost abolished phosphorylation of EGF receptor when EGF was omitted, but inhibition was less pronounced in the assay performed in the presence of EGF (results not shown). This suggested that protein fraction 67E contained an inhibitor of EGF-receptor autophosphorylation, which might act by different mechanisms, including interaction with the receptor, direct inhibition of tyrosine kinase, masking of tyrosine residues involved in autophosphorylation or the presence of a PTPP activity. In order to distinguish between these various possibilities, further experiments were performed with insulin receptor isolated from rat liver membranes.

A similar pattern was obtained in this case, as illustrated in Fig. 1. A polypeptide with an apparent molecular mass of 92 kDa, corresponding to the β -chain of insulin receptor, was specifically phosphorylated in response to insulin, and again protein fraction 67E promoted a strong inhibition of the phosphorylation process (70%, as determined from the radioactivity of the 92 kDa polypeptide measured by liquid-scintillation counting of the corresponding band). As shown by Tonks *et al.* [32], two additional bands with apparent molecular masses of 160 and 200 kDa were also phosphorylated in an insulin-independent manner, but this process also displayed some inhibition by protein fraction 67E. As shown in Fig. 1 (compare lanes 4 and 5), the inhibitory effect of protein fraction 67E was reduced by 49% in the presence of 50 μ M- Na_3VO_4 , a well-known inhibitor of PTPP [6]. This prompted us to examine in more detail the possible presence of a phosphatase in protein fraction 67E.

Characterization of phosphatase activity from protein fraction 67E

Protein fraction 67E was found to dephosphorylate various substrates in a pH-dependent manner. As indicated in Table 1, *p*-nitrophenyl phosphate, DL-phosphotyrosine and [32 P]phosphotyrosyl histones were hydrolysed by protein fraction 67E at an optimal pH of 5.5, 6.0 and 7.0 respectively. This phosphatase activity appeared to be rather specific, since phosphoserine, phosphothreonine, [32 P]phosphoserine, ATP and various inositol phosphates remained refractory to the hydrolytic activity of 67E phosphatase.

With [32 P]phosphotyrosyl histones as a substrate, various compounds were examined for their ability to modulate the activity of 67E phosphatase. As shown in Table 2, ATP, sodium pyrophosphate, NaF and MgCl_2 at millimolar concentrations strongly inhibited PTPP activity (around 90%). Similar concentrations of MnCl_2 , CaCl_2 and ZnCl_2 decreased the enzyme

activity by 50–60%, which was also achieved by 50 μ M- Na_3VO_4 . In the presence of 1 mM-spermine the enzyme was only inhibited by 27%, whereas 10 mM-LiCl appeared to be slightly stimulatory (32%). Finally, EDTA up to 5 mM was without effect.

Additional experiments (results not shown) performed with *p*-nitrophenyl phosphate also revealed that 67 kDa phosphatase was relatively insensitive to sodium L(+)-tartrate (23% and 77% inhibition at 5 mM and 20 mM respectively).

Fig. 1. Dephosphorylation of insulin receptor by protein 67E

Partially purified and solubilized insulin receptor from rat liver was incubated as described in the Materials and methods section with [γ - 32 P]ATP and various additions as indicated at the top of the Figure. Proteins were separated by SDS/PAGE, followed by autoradiography. On the left, arrows indicate the positions of molecular-mass standards (values in kDa), as detected by silver staining.

Table 1. Substrate-specificity of partially purified 67E phosphatase

Phosphatase activities were determined at three different pH values for each substrate as described in the Materials and methods section. Data (means of three different determinations, except for ATP and inositol phosphates, two determinations) are expressed in nmol/min per mg for all the substrates, except for [32 P]phosphotyrosyl and [32 P]phosphoserine (pmol/min per mg). Abbreviation: N.D., not determined.

Substrate	Specific activity		
	pH 5.5	pH 6.0	pH 7.0
<i>p</i> -Nitrophenyl phosphate (10 mM)	171	103.5	71.3
Phosphotyrosine (10 mM)	199	230	60
Phosphothreonine (10 mM)	N.D.	6.5	4
Phosphoserine (10 mM)	N.D.	10.7	7.2
ATP (5 mM)	0	N.D.	0
Inositol hexakisphosphate (100 μ M)	0	N.D.	0
Inositol 1,4,5-trisphosphate (50 μ M)	0	N.D.	0
Inositol 1,4,5-trisphosphate (50 μ M) + MgCl_2 (2 mM)	0	N.D.	0
Inositol 1-phosphate (50 μ M)	0	N.D.	0
Inositol 1-phosphate (50 μ M) + MgCl_2 (2 mM)	0	N.D.	0
[32 P]Phosphotyrosyl histones (3.3 μ M)	120	116	185
[32 P]Phosphoserine (3.3 μ M)	0	0	0.013

Table 2. Sensitivity to various compounds of phosphotyrosyl histone phosphatase activity

Phosphatase activity was determined as described in the Materials and methods section with 1 μ g of partially purified 67E phosphatase and 2.2 μ M-[32 P]phosphotyrosyl histones as substrate at pH 7.0. Data (means of two determinations) are expressed as percentages of control values, i.e. in the absence of any factor. Specific activity of the enzyme under these conditions was 185 pmol/min per mg.

Compound	Concentration (mM)	PTPP activity (%)
Control		100
ATP	5	10
Sodium pyrophosphate	5	7
NaF	10	13
MgCl ₂	1	10
Na ₃ VO ₄	0.05	46
EDTA	5	104
ZnCl ₂	1	52
LiCl	10	132
MnCl ₂	10	50
CaCl ₂	5	42
Spermine	1	73

Table 3. Purification of 67E phosphatase from pig lung

Purification	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (-fold)
EGTA extract	45	1629	36	100	1
DEAE-TSK	7.4	386	52	24	1.4
Polyacrylamide-phosphatidylserine	3.77	270	72	17	2
Mono S (fraction 25)	0.004	39.3	9825	2.4	273

From a physicochemical point of view, the phosphatase activity also displayed the same behaviour as that previously described for protein fraction 67E and could be detected throughout the purification procedure. In particular, phosphatase activity was eluted as a single symmetrical peak at an apparent molecular mass of 67 kDa on gel filtration on AcA 44 (results not shown). However, owing to the heterogeneity of protein fraction 67E, further purification of the enzymic protein was then attempted and is reported below.

Purification of 67E phosphatase

Table 3 summarizes the various purification steps used for 67E phosphatase starting from an EGTA extract from pig lung, and resulting in a final purification of 273-fold over the EGTA extract. This is representative of three different purifications with final products displaying specific activities of between 3 and 10 μ mol/min per mg with *p*-nitrophenyl phosphate as substrate. It is clear that DEAE-TSK and phosphatidylserine exclusion did not produce significant purification of the enzyme, owing partially to low stability of the protein. As shown in Fig. 2, the protein was eluted as a single peak at 0.1 M-NaCl from an f.p.l.c. Mono S cation-exchange column. Examination of the most pure fractions by SDS/PAGE indicated a good coincidence between phosphatase activity, which was maximal in fractions 24, 25 and 26 (Fig. 2), and the presence of a thin polypeptide band detected

Fig. 2. Elution profile of 67E phosphatase on chromatography on a f.p.l.c. Mono S HR 5/5 cation-exchange column

Proteins excluded from a column of polyacrylamide-immobilized phosphatidylserine (3.77 mg; see Table 3) were separated as described in the Materials and methods section with 40 ml of a linear NaCl gradient (0–0.5 M) (.....). Phosphatase activity (●) was determined with *p*-nitrophenyl phosphate as substrate and is given in munits/fraction (1 unit $\equiv$ 1 μ mol/min). —, A_{280} .

Fig. 3. Analysis by SDS/PAGE of proteins eluted from an f.p.l.c. Mono S HR 5/5 cation-exchange column

Equal volumes (100 μ l) of fractions 20–27 from Fig. 2 were freeze-dried, dissolved in 10 μ l of water and submitted to SDS/PAGE as described in the Materials and methods section. They were detected by silver staining. Arrows indicate the positions of molecular-mass standards (values in kDa).

by silver staining at an apparent molecular mass of 67 kDa (Fig. 3). However, these fractions were still contaminated by a polypeptide running at an apparent molecular mass of 55 kDa as well as by low-molecular-mass peptides (Fig. 3).

After f.p.l.c. Mono S cation-exchange chromatography, part of the purified phosphatase was chromatographed on Superose 6 HR 10/30 (Fig. 4). Here again, the protein did not appear to be homogeneous. However, the phosphatase activity was still eluted at an apparent molecular mass of 67 kDa.

As shown in Fig. 5, phosphatase activity could be recovered from polyacrylamide gels run in the presence of SDS under non-denaturing conditions. Hydrolysis of *p*-nitrophenyl phosphate was catalysed by the protein fraction eluted close to 67 kDa.

Activity of purified 67E phosphatase towards phosphoproteins

In agreement with the data of Table 1, purified phosphatase was inactive towards [32 P]phosphoserine, but hydrolysed [32 P]phosphotyrosyl histones, suggesting that it could be a typical PTPP. However, the activity against the latter substrate

Fig. 4. Elution profile of 67E phosphatase on gel filtration on f.p.l.c. Superose 6 HR 10/30

Fraction 24 from Fig. 2 was chromatographed as described in the Materials and methods section. Arrows show the positions of molecular-mass standards: BSA (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa); cytochrome *c* (12.5 kDa). Phosphatase activity (●) was determined with *p*-nitrophenyl phosphate as substrate and is given in munits/fraction (1 unit = 1 μ mol/min). —, A_{280} .

Fig. 5. Detection of phosphatase activity after SDS/PAGE under non-reducing conditions

(a) Partially purified 67E phosphatase (2 munits) was submitted to electrophoresis in 12% (w/v) polyacrylamide in the presence of SDS under non-reducing conditions. Silver staining was used. (b) Gel pieces (4 mm) were cut out and treated by the procedure of Hager & Burgess [31] as described in the Materials and methods section and the resulting renatured extracts were assayed for *p*-nitrophenyl phosphate phosphatase activity. Arrows show the positions of molecular-mass standards (kDa).

(1.4 nmol/min per mg) remained 2000-fold lower than that determined with *p*-nitrophenyl phosphate (3.08 μ mol/min per mg). Such a low activity was probably related to the basic substrate histone, since the same phosphatase preparation displayed a V_{max} of 1.93 μ mol/min per mg, with a K_m of 10 μ M, with

Fig. 6. Phosphatase activity of purified protein 67E towards different substrates as a function of pH

Phosphatase activity was determined as described in the Materials and methods section with *p*-nitrophenyl phosphate (□), DL-phosphotyrosine (■) or [32 P]phosphotyrosyl poly(Glu,Tyr) (▼). Data are expressed as percentages of maximal activity obtained at optimal pH with each of the substrates, i.e. 3.4 μ mol/min per mg for *p*-nitrophenyl phosphate, 2.8 μ mol/min per mg for DL-phosphotyrosine and 1.5 μ mol/min per mg for [32 P]phosphotyrosyl poly(Glu,Tyr). Data are means from four determinations for *p*-nitrophenyl phosphate and DL-phosphotyrosine, and from two experiments for [32 P]phosphotyrosyl poly(Glu,Tyr).

[32 P]phosphotyrosyl poly(Glu,Tyr) as substrate. As shown in Fig. 6, an optimal pH of 7.0 was observed with the latter substrate, whereas both *p*-nitrophenyl phosphate and DL-phosphotyrosine were maximally hydrolysed at pH 6.5. This was somewhat different from the data obtained for the partially purified enzyme (Table 1), suggesting that an acid phosphatase might have been eliminated during the purification.

DISCUSSION

The present study provides good evidence for the presence of a PTPP in the EGTA extract of pig lung membranes currently used for the isolation of annexins. Indeed, the kinetic parameters determined with [32 P]phosphotyrosyl poly(Glu,Tyr) as substrate are of the same order of magnitude as those reported for other purified PTPPs with the same [33] or other protein substrates [32,34–36]. Although it could not be purified to homogeneity, the enzyme was shown to display an apparent molecular mass of 67 kDa on gel filtration, which coincided with the presence of a 67 kDa protein on electrophoresis under denaturing conditions. Moreover, experiments depicted in Fig. 5 unequivocally indicated that phosphatase activity coincided with the position around the 67 kDa protein in the gel. This suggests that the isolated enzyme corresponds to a monomer of 67 kDa. However, any attempts to separate 67E phosphatase from the contaminating 55 kDa polypeptide remained unsuccessful. The latter has been identified as keratin 8, on the basis of molecular cloning of the corresponding cDNA and with the use of a polyclonal antibody recognizing specifically the 55 kDa band (R. Y. Li & J. M. F. Ragab-Thomas, unpublished work).

The phosphatase displayed a good specificity for phosphotyrosine residues, either as free phospho amino acid or as an integral part of various phosphotyrosyl proteins, including histones, EGF and insulin receptors or the amino acid heteropolymer poly(Glu,Tyr). Like other enzymes with similar properties [32,37,38], the phosphatase was also active towards *p*-nitrophenyl phosphate, which was used as a convenient substrate

to monitor the enzyme purification. However, the phosphatase remained without effect on various other substrates such as ATP, pyrophosphate, phosphoserine, phosphothreonine and inositol phosphates. It can thus be described as a typical PTPP. The protein is different from the acid phosphatase isolated from human lung by Efstratiatis & Moss [39], which is fully resistant to L(+)-tartrate and displays an apparent molecular mass of 37 kDa on gel filtration. Brunati & Pinna [40] have isolated a similar 65 kDa cytosolic PTPP from rat spleen, which was also excluded from DEAE-cellulose at pH 7.0. However, in contrast with 67E phosphatase, it was totally resistant to 50 μ M- Na_3VO_4 , did not display any interaction with membranes and was inactive against *p*-nitrophenyl phosphate.

In addition to its inhibition by Na_3VO_4 , ZnCl_2 and L(+)-tartrate (well-known characteristic inhibitors of PTPP), 67E phosphatase was sensitive to fluoride, a potent phosphoserine protein phosphatase inhibitor [41]. However, several other PTPPs, in particular those characterized as acid phosphatases, were found to be sensitive to fluoride [37,42–45]. From this point of view, 67E phosphatase shares many properties with the prostatic PTPP, but this protein appears as a dimer of two 50 kDa subunits [43,44].

The 67E phosphatase displayed an optimal pH of 6.5 when *p*-nitrophenyl phosphate was used as substrate. This was shifted to 7.0 with phosphotyrosyl poly(Glu,Tyr), as also shown for skeletal acid phosphatase [38], PTPP from human placenta [7] and acid phosphatase from human astrocytoma [37]. This indicates that phosphotyrosyl proteins might represent the physiological substrate for all of these enzymes.

A main characteristic of 67E phosphatase is its presence in EGTA extracts from pig lung. Although this might simply be fortuitous, it suggests some kind of Ca^{2+} -dependent interaction with pig lung membranes. As previously shown for protein fraction 67E [21], this interaction does not involve a specific Ca^{2+} -dependent adsorption on anionic phospholipids similar to that of annexins, as revealed by the affinity chromatography on polyacrylamide-immobilized phosphatidylserine. However, other possibilities still remain open, since keratin 8 was also detected in the EGTA extract, suggesting some interaction with the cytoskeleton, particularly intermediate filaments. Also, despite the use of various proteinase inhibitors, it cannot be excluded that 67E phosphatase could be part of an intrinsic protein released by proteolytic cleavage during the Ca^{2+} precipitation step of membranes. The origin of the localization of 67E phosphatase in EGTA extracts might give some clue to the understanding of the mechanism responsible for the targeting of this enzyme towards specific membrane substrates. Indeed, with the exception of membrane-bound PTPP [10–13], no mechanism responsible for a selective action of soluble PTPP on specific substrates has yet been proposed. This should justify further studies on the PTPP described in the present investigation, in order to define better its structural features, subcellular localization and physiological function.

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