Identification of cytosolic protein tyrosine kinases of human prostate by renaturation after SDS/PAGE

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The identification of protein tyrosine kinases (PTKs) was successfully achieved by renaturation in gels after SDS/PAGE. To this effect, samples were mixed with a PTK substrate, namely the polydispersed co-polymer of glutamic acid and tyrosine [poly(Glu,Tyr), M_r from 30 000 to 94 000], and were simultaneously submitted to electrophoresis. Following guanidine hydrochloride denaturation, renaturation and phosphorylation with [γ - 32 P]ATP, kinase activity was detected by autoradiography. When applied to cytosol from human hyperplastic prostate, eleven protein kinases were detected, among which one major (M_r 50 000) and two minor proteins (M_r 40 000 and 38 000) were identified as PTKs by the presence of phosphotyrosine. Incubation of the gel in hot alkali after glutaraldehyde cross-linking almost completely eliminated the detection of non-PTK enzymes. On the other hand, in the absence of poly(Glu,Tyr), no PTK activity was detected. Partial purification of cytosolic PTKs indicates that the native M_r of the major phosphotransferase was 44 000, as estimated by gel filtration following ammonium sulphate precipitation and anion-exchange chromatography. Upon renaturation after electrophoresis, this fraction showed only one major band active on poly(Glu,Tyr) which was associated with the polypeptide of M_r 50 000. This enzyme was also identified following two-dimensional electrophoresis and renaturation in the presence of poly(Glu,Tyr), allowing the determination of a pI in the range 7.5–7.8. Thus PTKs can be easily renatured following electrophoresis and rapidly identified on the basis of their M_r and pI in both crude or partially purified preparations. With the crucial role played by PTKs in the activation of cell function and carcinogenesis, this procedure could be useful in the identification of such enzymes and in distinguishing them from their substrates in gels.

INTRODUCTION

Many extracellular stimuli in eukaryotic cells are mediated through the reversible activation of protein kinases associated with the plasma membrane [1,2]. These enzymes turn on several intracellular signalling cascades of events implicated in various metabolic pathways. The central role of protein kinases in cell homeostasis has justified considerable work in order to purify, characterize and identify these enzymes in many cell types and tissues. The recent improvement of renaturation methods allowing the detection of enzyme activity after protein separation by SDS/PAGE has largely facilitated the identification of Ser/Thr kinases in both crude and purified enzyme extracts [3], but not of protein tyrosine kinases (PTKs). The importance of the latter enzymes in the regulation of cell proliferation, however, justifies the development of sensitive methods for the detection of their activity after SDS/PAGE. In the present investigation, the polydispersed synthetic substrate poly(Glu,Tyr) (M_r extending from 30 000 to 94 000) was used to identify such enzymes in a cytosolic fraction from human hyperplastic prostate; among eleven renatured protein kinases, three PTKs of M_r 50 000, 40 000 and 38 000 were identified.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP (7000 Ci/mmol) was purchased from ICN (Costa Mesa, CA, U.S.A.). Imidazole, iminodiacetic acid, guanidine-HCl (grade I), pepstatin, phenylmethanesulphonyl fluoride (PMSF), poly(Glu-Na,Tyr) (4:1) [poly(Glu,Tyr)] and poly(Glu,Glu-*O*-methyl) (4:1) [poly(Glu,Glu^{met})] were from

Sigma (St. Louis, MO, U.S.A.). Acrylamide and *NN*-bisacrylamide were purchased from LKB, Bromma, Sweden. SDS, Tween 20, Ampholytes of pH 3–10 and Unisep ultrafilters (30 000- M_r cut-off) were obtained from Bio-Rad (Mississauga, Ontario, Canada); aprotinin, leupeptin, CHAPS and α_2 -macroglobulin were from Boehringer Mannheim (Laval, Québec, Canada). Glutaraldehyde [50% (w/w) in water] and cellulose thin-layer plates were purchased from Eastman Kodak (Rochester, NY, U.S.A.) and Sequanal-grade 6 M-HCl and BSA protein standard solution (fraction V; 2 mg/ml) were from Pierce (Rockford, IL, U.S.A.). P-989 scintillation fluid was obtained from Dupont (Boston, MA, U.S.A.), and Accell quaternary methyl amine (QMA) gel matrix, QMA and C₁₈ Sep-Pak cartridges were from Waters (Millipore Corp., Milford, MA, U.S.A.). Sephacryl S300HR was purchased from Pharmacia (Montréal, Québec, Canada); Whatman 934-AH glass microfibre filters (2.4 cm) were from Fisher (Montréal, Québec, Canada) and benzamidine was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Preparation of prostatic cytosol and determination of PTK activity

Human hyperplastic prostates (obtained from cadavers within 8 h *post mortem*) were dissected and frozen at -70°C until used. After thawing and mincing, the tissue was homogenized with a Polytron in 4 vol. of ice-cold buffer A [25 mM-Hepes adjusted to pH 7.4 with NaOH, 200 mM-KCl, 5 mM-dithiothreitol (DTT), 2 mM-EDTA, 2 mM-EGTA, 100 μM -Na₃VO₄, 1 mM-PMSF, 10 mM-benzamidine, 5 μg of leupeptin/ml, 5 μg of aprotinin/ml and 2 μg of pepstatin/ml]. Nuclei and cell debris were removed by a

Abbreviations used: PTK, protein tyrosine kinase; PAP, prostatic acid phosphatase; poly(Glu,Tyr), co-polymer of L-glutamic acid and L-tyrosine; poly(Glu,Glu^{met}), co-polymer of L-glutamic acid and L-glutamic acid methyl ester; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; pTyr, phosphotyrosine; pThr, phosphothreonine; pSer, phosphoserine; QMA, quaternary methyl amine.

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30 min centrifugation at 3000 *g* (4 °C), and the cytosolic fraction was obtained after a subsequent centrifugation of the supernatant at 105 000 *g* for 60 min. Protein concentrations were determined by a Coomassie Blue binding assay [4], using BSA as a standard.

Enzyme preparations (25 μ l aliquots) were incubated for 20 min at 22 °C in 25 mM-HEPES buffer, pH 7.4, containing 50 μ g of poly(Glu,Tyr), 20 mM-MgCl₂, 5 mM-MnCl₂, 2 mM-DTT, 100 μ M-Na₃VO₄, 0.5 % Triton X-100 and 20 μ M-[γ -³²P]ATP (1 Ci/mmol) in a final volume of 100 μ l. The reaction was stopped by the addition of 25 μ l of 100 mM-ATP. Endogenous protein phosphorylation on serine and threonine residues was then decreased by a further 10 min incubation in a boiling bath in the presence of 100 μ l of 1 M-NaOH [5]. Poly(Glu,Tyr) and proteins were subsequently precipitated by the addition of 500 μ l of ice-cold 20 % (w/v) trichloroacetic acid. Following a 30 min standing period on ice, precipitates were filtered on glass microfibrils and washed three times with 5 ml of ice-cold 5 % (w/v) trichloroacetic acid containing 10 mM-P_i and 10 mM-PP_i, and twice with 5 ml of 95 % ethanol. The filters were then counted for radioactivity in 5 ml of scintillation fluid and the specific PTK activity was expressed as the pmol of phosphate transferred from ATP to poly(Glu,Tyr) per min per μ g of protein.

Partial purification of PTK

The 105 000 *g* supernatant was precipitated by the slow addition of (NH₄)₂SO₄ up to 30 % saturation. After standing on ice for 30 min and elimination of the insoluble material by centrifugation (10 000 *g* for 20 min), the (NH₄)₂SO₄ concentration was raised to 50 % saturation and the mixture was stirred on ice for 30 min; the protein precipitate, which contained 60–85 % of the initial PTK activity (results not shown), was recovered by centrifugation and used for further purification. After dissolution in buffer B (25 mM-imidazole, 14 mM-iminodiacetic acid, 50 mM-KCl, 10 mM- β -mercaptoethanol, pH 7.0) and desalting on a Sephadex G50 column (2.5 $\times$ 17 cm) equilibrated with buffer B, α_2 -macroglobulin was added (0.2 inhibitory unit/ml) to the pooled PTK fractions before clarification by centrifugation. The sample was then applied at 1 ml/min on to a QMA column (1.5 cm $\times$ 20 cm) equilibrated in buffer B and adapted to an f.p.l.c. system (Pharmacia); elution was performed with 5 column vol. of a linear KCl gradient (50–500 mM) in buffer B. Active fractions (eluted at 150 mM-KCl; not shown) were then pooled and precipitated with (NH₄)₂SO₄ (75 % saturation; 30 min on ice); the protein precipitate was recovered by centrifugation and solubilized in 3 ml of buffer B containing 0.2 inhibitory unit of α_2 -macroglobulin/ml. The preparation was then submitted to gel filtration on a Sephacryl S300HR column (2.6 cm $\times$ 58 cm) equilibrated in buffer B and eluted at a flow rate of 1 ml/min with an f.p.l.c. system. Fractions containing PTK activity were pooled, concentrated by ultrafiltration (30 000-*M_r* (cut-off) and stored in 40 % glycerol at –70 °C.

Gel electrophoresis in one or two dimensions and renaturation of protein kinases

When renaturation of protein kinases was carried out after one separation step by SDS/PAGE, enzyme preparations (25–50 μ g of protein) in electrophoresis buffer (2 % SDS, 5 % β -mercaptoethanol, 10 mM-Tris/HCl, pH 6.8, 15 % glycerol, 0.01 % Bromophenol Blue) were mixed or not with 50 μ g of poly(Glu,Tyr) and incubated for 3 min at 100 °C. The mixtures were then submitted to electrophoresis on slab gels (8 cm $\times$ 7 cm $\times$ 0.15 cm; acrylamide/bisacrylamide, 30:0.8; stacking, 4 %, separation, 9 %) at 25 mA/gel.

For two-dimensional electrophoresis, duplicate samples of enzyme fraction (32 μ g of protein of partially purified PTK)

containing 4.5 M-urea, 1 % Triton X-100, 0.5 % CHAPS, 1 % β -mercaptoethanol and 0.4 % Ampholytes 3–10 were submitted to electrophoresis on 6 % acrylamide/0.16 % bisacrylamide gel rods (3.5 mm $\times$ 7.5 cm) containing 8 M-urea, 0.5 % Triton X-100 and 2 % Ampholytes 3–10 for 60 min at 200 V and then overnight at 800 V (15 °C). The anolyte was 10 mM-H₃PO₄ and the catholyte was 20 mM-NaOH. Gels were equilibrated for an initial 30 min period in 100 mM-Tris/HCl buffer, pH 6.8, and for another 30 min in 100 mM-Tris/HCl, pH 6.8, containing 3 % SDS and 5 % β -mercaptoethanol. One gel was applied on to a slab gel, where poly(Glu,Tyr) was added to the separation gel at a concentration of 0.1 mg/ml prior to polymerization, while the other was applied on to a slab gel lacking poly(Glu,Tyr) (control gel). Electrophoresis in the second dimension was carried out as described above.

For renaturation of protein kinases, gels were treated as described by Kameshita & Fujisawa [6], with some modification. Briefly, SDS was removed from gels by two successive washes (100 ml, 60 min each) in 20 % propan-2-ol/50 mM-imidazole/28 mM-iminodiacetic acid (pH 7.0), followed by one wash (250 ml, 60 min) with buffer C (50 mM-imidazole, 28 mM-iminodiacetic acid, 10 mM- β -mercaptoethanol, pH 7.0), all at 22 °C. To further the unfolding of proteins within the gel matrix, gels were incubated in a plastic bag in the presence of 10 ml of 8 M-guanidine-HCl in buffer C containing 50 mM- β -mercaptoethanol (90 min at 22 °C); proteins were then allowed to renature at 4 °C by four incubations under gentle shaking in buffer B containing 0.04 % Tween 20 and 10 % sucrose (first, second and fourth washes, 200 ml for 90 min; third wash, 400 ml for 16 h). Gels were subsequently equilibrated (60 min at 22 °C) in 200 ml of buffer D (10 mM-Hepes adjusted to pH 7.4 with NaOH, 10 mM- β -mercaptoethanol, 20 mM-MgCl₂, 5 mM-MnCl₂ and 100 μ M-Na₃VO₄) and phosphorylation was carried out in a plastic bag (2 h at 22 °C) in 5 ml of buffer D containing 100 μ Ci of [γ -³²P]ATP (1500–7000 Ci/mmol). Gels were then successively washed with 5 % (w/v) trichloroacetic acid containing 1 % (w/v) P_i, 1 % (w/v) PP_i and 1 mM-ATP (first, second and fourth washes, 200 ml for 90 min; third wash, 400 ml for 16 h) and then with 10 % methanol (250 ml, 60 min). Following protein cross-linking within the gel matrix by 10 % (v/v) glutaraldehyde treatment (100 ml, 30 min), and rinsing with distilled water (3 $\times$ 250 ml; 20 min each), gels were stained with Coomassie R250, inserted into a plastic bag and exposed to Fuji X-ray film at –70 °C in order to obtain the global (or total) phosphorylation pattern of renatured kinases.

Alkali treatments were then performed by soaking glutaraldehyde-fixed and Coomassie-stained gels in 1 M-KOH at 56 °C for 2 h in order to decrease the relative proportion of phosphoserine (pSer) and to a lesser extent of phosphothreonine (pThr) as compared with phosphotyrosine (pTyr) [8,9]. Following neutralization in 10 % acetic acid/3 % glycerol for 2 h, alkali-treated gels were dried and submitted to autoradiography in order to detect alkali-resistant phosphorylation.

Phosphoamino acid analysis

³²P-labelled polypeptides were excised from the gel, minced and partially hydrolysed in 6 M-HCl at 110 °C for 90 min. Hydrolysates were evaporated under nitrogen, resuspended in 100 mM-NH₄OH and applied on to C₁₈ Sep-Pak environmental cartridges equilibrated in the same buffer; unretained fractions which contained phosphoamino acids were then applied on to QMA Sep-Pak light cartridges equilibrated in 100 mM-NH₄OH. After washing with 10 ml of water, phosphoamino acids were eluted from the column with 1 ml of 100 mM-HCl and evaporated under nitrogen. Unlabelled phosphoamino acids (pSer, pThr and pTyr; 2 μ g of each) were then added to the residues, which were

dissolved in 100 mM-NH₄OH and applied to cellulose thin-layer plates for two-dimensional electrophoresis [9].

RESULTS

A three-step procedure was used to obtain a partially purified cytosolic PTK fraction from human prostate, which was used together with the crude cytosol for renaturation of PTKs after SDS/PAGE. As summarized in Table 1, (NH₄)₂SO₄ precipitation was followed by two chromatographic steps, namely anion exchange (quaternary amine), which retained 75% of the total activity, and size exclusion on a Sephacryl S300HR column, from which the PTK activity was eluted with an *M_r* of 44000 (as estimated with the *M_r* markers used for column calibration; results not shown). The overall purification factor was 42-fold, with 20% recovery of PTK activity.

Fig. 1 shows the autoradiograms obtained when renaturation experiments were performed with gels containing crude (lanes 1 and 2) or partially purified (lanes 3–5) cytosolic PTK fractions from human prostate that were submitted to SDS/PAGE in the

Table 1. Partial purification of cytosolic PTK from human prostate

Purification was performed as described in the Materials and methods section. (NH₄)₂SO₄ represents 30–50%-satd. (NH₄)₂SO₄ fraction; QMA represents pooled active fractions eluted from QMA column at 150 mM-KCl; S300HR represents active fractions eluted from Sephacryl S300HR with an *M_r* of 44000.

Fraction	Volume (ml)	Protein (mg)	Activity	
			Total (pmol/min)	Specific (pmol/min per mg)
Cytosol	87	428	24610	57
(NH ₄) ₂ SO ₄	19	107	15387	144
QMA	24	8.8	7049	801
S200HR	18	2.1	4850	2425

Fig. 1. Renaturation of cytosolic protein kinases from human prostate

Crude (lanes 1 and 2) or partially purified (lanes 3–5) PTK fractions were submitted to electrophoresis without exogenous substrate (lanes 1 and 3) or with 50 µg of either poly(Glu,Tyr) (lanes 2 and 4) or poly(Glu,Glu^{met}) (lane 5). Proteins within the gel were then allowed to renature and were phosphorylated with [γ-³²P]ATP as described in the Materials and methods section. Radiolabelled proteins were revealed by autoradiography. (a) Total phosphorylation pattern (60 min of exposure at room temperature with an intensifying screen); (b) residual phosphorylation after glutaraldehyde cross-linking and alkali treatment of the same gel (90 min exposure at -70 °C with an intensifying screen). On the far left are indicated the protein kinases (PK) that were selected for phosphoamino acid analysis (see Fig. 2).

Fig. 2. Phosphoamino acid analysis of renatured ³²P-labelled proteins

Bands corresponding to the major protein kinase (PK) activities indicated by arrows in Fig. 1 were excised, hydrolysed in HCl and analysed by two-dimensional thin-layer electrophoresis following their partial purification on C₁₈ and QMA cartridges as described in the Materials and methods section [8]. Autoradiograms of the separated ³²P-phosphoamino acids are shown. (pS, pSer; pT, pThr; pY, pTyr). (a) and (b) Excised bands from Fig. 1(a), lanes 1 and 2 respectively; (c) excised bands from Fig. 1(b), lane 2.

absence (lanes 1 and 3) or in the presence of poly(Glu,Tyr) (lanes 2 and 4) or poly(Glu,Glu^{met}) (lanes 5). The total phosphorylation pattern (Fig. 1a) shows that in the crude fraction at least six major protein kinases (PK; *M_r* values ranging from 38000 to 120000) were active after renaturation. The inclusion of poly(Glu,Tyr) together with the cytosol prior to electrophoresis (Fig. 1a, lane 2) increased the labelling intensity of PK210, PK50, PK40 and PK35, while decreasing the activity of PK94, PK60 and PK38. However, alkali treatment of the gel (Fig. 1b) considerably decreased the ³²P labelling in bands corresponding to all kinases present in the crude cytosolic fraction, leaving two major labelled bands in the gel, PK60 and PK52, together with minor ones, namely PK210, PK120 and PK94. When poly(Glu,Tyr) was present (Fig. 1b, lane 2), an intensively labelled alkali-resistant band migrating at *M_r* 50000 was observed, along with minor bands corresponding to PK210, PK60, PK40, PK38 and PK35.

Phosphoamino acid analysis of six ³²P-labelled bands observed in total phosphorylation (Fig. 1a, lane 1), and corresponding to major kinases renatured in the absence of poly(Glu,Tyr) (PK120, PK94, PK60, PK52, PK40 and PK38), showed that they were all specific for serine and threonine residues, since no pTyr was detectable (Fig. 2a). Even though the presence of poly(Glu,Tyr) increased ³²P labelling in regions of the gel corresponding to PK210 and PK35 (Fig. 1a, lane 2), no pTyr was detected (Fig. 2b), suggesting that poly(Glu,Tyr) may help the renaturation of or activate these Ser/Thr kinases. In addition, phosphoamino acid analysis of the three major alkali-stable ³²P-labelled bands (PK210, PK60 and PK52) renatured in the absence of poly(Glu,Tyr) (Fig. 1b, lane 1), revealed mainly the presence of pThr, with no detectable pTyr (results not shown). On the other hand, as demonstrated by the presence of pTyr (Fig. 2c), a PTK activity was indeed associated with the PK50, PK40 and PK38 bands detected in the presence of poly(Glu,Tyr) (Fig. 1b, lane 2),

Fig. 3. Renaturation of the partially purified PTK after two-dimensional electrophoresis

The partially purified enzyme (32 μ g of protein) was first separated by isoelectrofocusing in the presence of urea as described in the Materials and methods section. After equilibration in SDS sample buffer, the gel was layered on to a slab gel containing 0.1 mg of poly(Glu,Tyr)/ml and submitted to SDS/PAGE. Proteins were subsequently renatured and the gel was incubated in phosphorylation buffer containing [γ - 32 P]ATP as described in the Materials and methods section. After drying, the gel was submitted to autoradiography.

and PK50 was by far the major renatured phosphotransferase. When renaturation experiments were performed on the partially purified PTK fraction and enzyme activity revealed by autoradiography of either untreated gels (Fig. 1a) or glutaraldehyde-crosslinked and alkali-treated gels (Fig. 1b), one heavily

phosphorylated polypeptide of M_r 50000 was observed (Fig. 1, lane 4). As expected, and in agreement with its high alkali stability (Fig. 1b, lane 4), phosphoamino acid analysis of PK50 revealed the presence of radiolabelled pTyr only (results not shown). In the absence of poly(Glu,Tyr) (Fig. 1, lane 3) or in the presence of poly(Glu,Glu^{met}) (Fig. 1, lane 5), no phosphotransferase activity was detected.

Renaturation of the partially purified PTK fraction was also performed after two-dimensional electrophoresis and is shown in Fig. 3. When poly(Glu,Tyr) was included in the second-dimension gel, one major phosphotransferase activity of M_r 50000 with a pI of 7.5–7.8 was observed. In agreement with the result obtained in Fig. 1(a) (lane 3), no activity was detected when the second dimension was run without poly(Glu,Tyr) (results not shown).

To optimize the procedure for the detection of PTK, the effect of increasing concentrations of poly(Glu,Tyr) (Fig. 4a) and proteins (Fig. 4b) (crude cytosolic fraction) applied to the gels was studied. In all instances, alkali treatments were necessary in order to clearly detect specific poly(Glu,Tyr) phosphorylation, since several Ser/Thr kinases present in this fraction were also renatured (Fig. 1a, lanes 1 and 2). At least 25 μ g of poly(Glu,Tyr) per lane of 370 mm³ was necessary for a clear and rapid detection of PK50 activity (Fig. 4a), and as observed in the right panel, varying the concentration of poly(Glu,Tyr) between 5 and 100 μ g per lane increased its labelling linearly.

With respect to protein concentration, 5 μ g was the minimum amount of cytosolic protein applied to the gel, and this quantity was sufficient to allow a clear detection of PK50 activity (Fig. 4b). Poly(Glu,Tyr) phosphorylation also increased rapidly up to 25 μ g and reached a plateau at 100 μ g of protein (Fig. 4b, right panel). When sodium orthovanadate was omitted from the

Fig. 4. Linearity of poly(Glu,Tyr) phosphorylation by PK50 with respect to poly(Glu,Tyr) and protein concentrations

(a) Cytosolic proteins (50 μ g) were mixed with various amounts of poly(Glu,Tyr) and submitted to electrophoresis. After renaturation, phosphorylation, glutaraldehyde cross-linking, alkali treatment and autoradiography (left panel), gel bands corresponding to PK50 were excised and counted in order to quantify the amount of 32 P incorporated into poly(Glu,Tyr) (right panel). (b) Same protocol as in (a), but performed with various amounts of cytosolic proteins mixed with 50 μ g of poly(Glu,Tyr). After autoradiography (left panel), the labelling of poly(Glu,Tyr) was quantified by Čerenkov counting (right panel).

equilibration and phosphorylation buffers, no significant difference in poly(Glu,Tyr) labelling by PK50 was observed (results not shown).

DISCUSSION

Renaturation after SDS/PAGE has recently been used to identify several protein kinases in various cell extracts, including human platelets [10], fungal and red blood cell membranes [11,12,14] and fibroblast lysates [13]. A few known protein kinases, such as the calmodulin-dependent protein kinase II [6], cyclic AMP-dependent kinase and casein kinase II [14] have also been successfully renatured. In some instances the inclusion of a substrate in the separating gel greatly increased their detection [6,11,14]. In addition, a Ser/Thr/Tyr kinase from murine erythroleukaemia cells obtained by gene fusion and expressed in bacteria was successfully renatured [15]. However, no naturally occurring PTK has been identified so far upon renaturation after SDS/PAGE [10–14,18], even if such experiments were performed with preparations known to contain PTKs [10]. This may be explained either by the specific nature of the catalytic domain, which could be more difficult to renature than other types of kinases, or by their low degree or lack of autophosphorylation activity after renaturation. To circumvent these problems, we have mixed a polydispersed synthetic substrate for tyrosine kinases, poly(Glu,Tyr), with enzyme samples prior to SDS/PAGE, and have identified PTK of M_r 50000 which corresponds to the major form of the native enzyme present in the cytosol from human prostate. This native enzyme is thus a monomeric protein. The renaturation protocol also allowed the identification of two other minor forms of PTK, of M_r 38000 and 40000, which have not been detected previously by gel filtration of the crude prostatic cytosol [19], indicating a high sensitivity of this procedure. Another advantage of the present method is that it is possible to run control lanes (without substrate) in the same gel instead of performing two gels, one with and another without poly(Glu,Tyr). On the other hand, since poly(Glu,Tyr) is dispersed over a limited range of relative M_r [30000–94000, as indicated by autoradiography of ^{32}P -poly(Glu,Tyr) in gels (results not shown)], the detection of PTKs of higher M_r in the cytosolic fraction might not have occurred. To overcome this problem, poly(Glu,Tyr) was added to the gel mixture (at 0.1 mg/ml) prior to polymerization, and the experiment was carried out as described for renaturation and phosphorylation. The same ^{32}P labelling pattern as in Fig. 1 was observed after autoradiography (results not shown), suggesting that no other PTK of higher or lower M_r active on poly(Glu,Tyr) is present in this fraction, unless it is resistant to renaturation. Whether PTKs specific for substrates other than poly(Glu,Tyr) are present but not detected remains to be established.

From the present data, it appears that the identification of prostatic PTKs depends upon the presence of poly(Glu,Tyr) within the gel, since in its absence and under the conditions used, no radiolabelled band in the gels contained detectable pTyr. This is in contrast to the situation encountered with Ser/Thr kinases, the detection of which could easily be achieved without the addition of exogenous substrates. These results suggest that the autophosphorylation of renatured PTKs is low or absent, or that the PTKs are present in minute amounts in the prostate. However, PK50 may possess an autophosphorylating activity, since a longer autoradiographic exposure period to X-ray film (5 weeks) resulted in the detection of a faint band of M_r 50000 when the partially purified PTK fraction was renatured in the absence of poly(Glu,Tyr) (results not shown). Cationic polymers, such as poly(L-lysine), were reported to activate the autophosphorylation (PTK activity) of the insulin receptor [20]. To verify whether the

presence of an anionic polymer such as poly(Glu,Tyr) would activate the autophosphorylation of prostatic PTKs, renaturation experiments were carried out with an analogue, the polyanion poly(Glu,Glu^{met}). The fact that no phosphotransferase activity was observed with this substrate is in agreement with a low autophosphorylation activity of prostatic PTKs. These data also indicate that the pTyr hydrolysed from PTK gel bands originates mainly from the substrate poly(Glu,Tyr).

If alkali treatments of gels greatly simplified the search for PTKs, gel bands showing increased labelling in the presence of poly(Glu,Tyr) still have to be analysed for the presence of pTyr for definitive proof. Indeed, the presence of poly(Glu,Tyr) can increase the efficiency of renaturation and/or stimulate the activity of Ser/Thr kinases, such as PK210 and PK40. On the other hand, as observed in the present study for PK94 and PK38, and in agreement with other reports [16,17], poly(Glu,Tyr) may also inhibit some Ser/Thr kinases. As seen for PK210, PK60 and PK52, and as previously reported [8,9], the relatively high stability of pThr residues in hot alkaline conditions also indicate that phosphoamino acid analysis should be performed on hydrolysates from protein kinases detected by renaturation in the presence of poly(Glu,Tyr). Caution should also be taken when crude fractions containing multiple PTKs active on poly(Glu,Tyr) are used in renaturation experiments. Indeed, different kinases may have different potentials for renaturation, and the major enzyme active on poly(Glu,Tyr) in a test tube assay may be the most difficult one to renature, or may not be renatured at all. In addition, the presence of other enzymes migrating with PTKs should also be considered. These possibilities were examined in the present system, and renaturation experiments performed with the partially purified PTK fraction confirmed that PK50 was the major PTK in human prostatic cytosol. Interestingly, the purification factor for PK50 activity measured after renaturation (determination of the radioactivity present in PK50 bands) was estimated to be 89-fold, which is twice that obtained when the activity was measured in a test tube assay (as monitored during the purification procedure). This discrepancy may be partly explained by the presence of PTK molecules that have been partially denatured during the purification steps, but which remain able to renature correctly after SDS/PAGE. The role of human prostatic acid phosphatase (PAP), a pTyr protein phosphatase [21,22], also appears to be minimal; indeed, PAP is composed of two 50000- M_r subunits [22] which are likely to migrate with PK50 when prostatic cytosol is used as a source of PTK and thus possibly interfere with detection of the latter. This possibility is however unlikely, since the omission of vanadate, an inhibitor of PAP [23], from equilibration and phosphorylation buffers did not alter the level of poly(Glu,Tyr) phosphorylation by cytosolic PK50 upon renaturation (results not shown), suggesting that PAP was either inactive and/or not properly renatured.

In conclusion, as demonstrated by renaturation after SDS/PAGE, one major PTK, expressed as a 50000- M_r polypeptide, together with two other minor forms of M_r 38000 and 40000, are present in the cytosol from human prostate. Whether these enzymes play a role in prostatic physiopathology is not yet known. The present method could be very useful in the screening and identification of PTKs in various cellular systems, thus avoiding their laborious purification. It should also help us to understand the key role played by PTKs in cellular signalling, leading to growth and differentiation as well as carcinogenesis.

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