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# The major form of protein tyrosine kinase in the dog prostate is expressed by a 50 kDa polypeptide

Linh T. Nguyen, Yves Durocher, Alcide Chapdelaine and Simone Chevalier

*Departments of Biochemistry and of Medicine, University of Montreal and Maisonneuve-Rosemont Research Center, Montréal, Canada*

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We have already reported that the protein tyrosine kinase (PTK) activity in the dog prostate is distributed in cytosolic (75%) and particulate (Triton X-100-solubilized) fractions and that upon gel filtration, both PTKs migrate as entities of $M_r$ 44 000 [(1991) Biochem. Cell. Biol. 69, 146–153]. Herein we demonstrate by immunoprecipitation with anti-phosphotyrosine antibodies that the soluble PTK has the ability to undergo self-phosphorylation. In addition, the polypeptide responsible for that enzymatic activity has been identified by 2 approaches: (1) a two-dimensional electrophoresis, in which the first dimension performed in non-denaturing conditions allowed the localization of the native enzyme, while the second dimension (SDS-PAGE) permitted the analysis of alkali-resistant phosphoproteins corresponding to the activity; (2) protein renaturation after SDS-PAGE followed by in situ phosphorylation (with [$\gamma$-$^{32}$P]ATP) of polyGT electrophoresed together with the enzyme preparation; the exclusive presence of the radiolabeled phosphotyrosine in the renatured protein confirmed its enzymatic nature. Using these methods, the major form of PTK in the dog prostate was shown to be expressed by a 50 kDa polypeptide which possesses autophosphorylation sites and which is present in the cytosol as an active monomer.

Canine prostate; Tyrosine phosphorylation; Autophosphorylation; Renaturation

## 1. INTRODUCTION

The essential role of protein tyrosine kinases (PTKs) in the control of cell proliferation has clearly been demonstrated by studies on several growth factor systems as well as on oncogenes. Indeed, most growth factors mediate their mitogenic signal through the intrinsic PTK activity of their receptor [2] while several oncogenes encoding PTKs induce a transformed phenotype when expressed in cells [3,4]. More recently, the association of low-$M_r$ PTKs with specific cell surface proteins (e.g. lck-CD4/CD8; lck-interleukin-2 receptor; fyn-T cell antigen receptor; lyn-B cell antigen receptor) was demonstrated to play an important role in the activation of the immunologic response of lymphocytes [5–8]. Numerous PTKs with unknown functions are also distributed elsewhere than in membranes [9–11]; this is the case of human and dog prostates whose PTK activity, mainly distributed in cytosolic fractions, migrates upon gel filtration as entities of 44–50 kDa [1,12]. Since the identification of PTKs in the prostate may represent an important step in our understanding of the mechanisms regulating its growth and differentiation, we have, in the present investigation, combined in situ renaturation experiments with the resolution power of SDS-PAGE, along with 2D electrophoresis in non-denaturing conditions, and affinity for anti-phosphotyrosine antibodies to demonstrate that the major form of PTK in the canine prostate possesses autophosphorylation sites and is expressed by a polypeptide of 50 kDa.

*Abbreviations:* PTK, protein tyrosine kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; polyGT, polymer of sodium glutamate and tyrosine; pY, phosphotyrosine; pS, phosphoserine; pT, phosphothreonine; QMA, quaternary methyl amine; $C_{18}$, octadodecyl; 2D, two-dimensional.

*Correspondence address:* S. Chevalier, Maisonneuve-Rosemont Research Center, 5415 l'Assomption Blvd., Montréal, Québec, H1T 2M4 Canada.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Prostates (10–30 g) were excised from adult mongrel dogs (15–30 kg) as already described [1]. Polyclonal anti-phosphotyrosine (anti-pY) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). All protease inhibitors were obtained from Boehringer-Mannheim (Laval, Canada); [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was purchased from ICN Biomedicals (Mississauga, Canada); the polybuffer 74 was obtained from Pharmacia (Québec, Canada) and the substrate polyGT (4:1) from Sigma Chemical Co. (St.-Louis, MO).

### 2.2. *Protein tyrosine kinase assay*

The PTK activity was measured by an incubation of enzyme preparations with 20 $\mu$M [$\gamma$-$^{32}$P]ATP (2000 cpm/pmol) and 50 $\mu$g polyGT for 20 min at 20°C in 100 $\mu$l of a reaction medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.4, 10 mM $MnCl_2$, 20 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM $\beta$-mercaptoethanol, 100 $\mu$M $Na_3VO_4$ and 0.5% Triton X-100. The reaction was stopped by the addition of 100 $\mu$l of a solution containing 10 mM unlabeled ATP, 10 mM $Na_4P_2O_7$ and 2 N NaOH, followed by heating at 90°C for 10 min [13]. The

radioactivity of polyGT was determined following TCA precipitation and filtration on Whatman filter discs as described elsewhere [12].

2.3. *Tissue fractionation and partial purification of PTK*

The soluble or cytosolic fractions used as a source of PTK were obtained by differential centrifugation of homogenates prepared with prostate minces [1]. Gel filtration was performed on a Sephadex G-100 column (1.5 × 85 cm) equilibrated and eluted (1.32-ml fractions) with 20 mM HEPES buffer, pH 7.4, containing 0.1 M KCl, 10% glycerol, 0.5 mM DTT, 0.1% $NaN_3$, 0.2 mM EDTA, 1 mM benzamidine, 10 μg/ml aprotinin, 0.4 μg/ml pepstatin, 0.4 μg/ml leupeptin and 0.2 mM PMSF. Fractions corresponding to the peak of activity were pooled and concentrated by ammonium sulfate precipitation at 75% saturation (16 h at 4°C). The precipitate was redissolved and desalted by chromatography on a Sephadex G-25 column (2.5 × 15 cm) equilibrated and eluted with a buffer, pH 7.0 containing 25 mM imidazole, 14 mM iminodiacetic acid, 2% taurine (w/v) and 10 mM β-mercaptoethanol. The resulting material was applied onto a Mono P HR 5/20 column to perform chromatofocusing through a fast protein liquid chromatography (FPLC) system. The proteins were eluted by creating a linear pH gradient using as the eluent 10% polybuffer 74 containing 2% taurine and adjusted to pH 4.0 with iminodiacetic acid.

2.4. *Immunoprecipitation*

A peak fraction from Sephadex G-100 was used as the starting material. Phosphorylation of endogenous proteins was first carried out by a 30-min incubation in the reaction medium (100 μl) described above containing 10 μM unlabeled ATP but no β-mercaptoethanol. Polyclonal anti-pY antibody (5 μg/ml) in 50 μl of buffer B (3×) (150 mM Tris-HCl pH 7.4, 450 mM NaCl, 30 mM EDTA, 30 mM $Na_2PO_7$, 300 mM NaF, 300 μM $Na_3VO_4$, 3% Triton X-100 and the cocktail of protease inhibitors mentioned above) was then added and the mixture was incubated at room temperature with gentle shaking for 2 h. Parallel assays containing either phosphotyrosine (pY) or phosphoserine (pS) at 20 mM were also performed to evaluate the specificity of the reaction. The immunocomplexes were precipitated by a 90-min incubation at 4°C in the presence of a second polyclonal anti-IgG antibody (45 μg/ml) and polyethylene glycol (6000) at a final concentration of 3% (w/v) in a total volume of 450 μl. The mixtures were then centrifuged at 6000 × *g* for 20 min at 4°C. Supernatants were carefully aspirated and pellets, washed once with 20 mM HEPES buffer, pH 7.4, were finally resuspended in phosphorylation medium as described above and containing 20 mM β-mercaptoethanol, 50 μg polyGT and 20 μM [$\gamma$-$^{32}$P]ATP (2000 cpm/pmol). An incubation of 30 min was carried out and the radiolabeled polyGT was analyzed by autoradiography after SDS-PAGE.

2.5. *Electrophoresis*

Unless otherwise specified, SDS-PAGE was routinely performed on 10% gels (12 × 15 × 0.15 cm) as described by Laemmli [14]. 2D electrophoresis was used to localize the PTK activity in polyacrylamide gels by performing first an electrophoresis in non-denaturing conditions, and second, an electrophoresis in the presence of SDS to analyze those alkali-resistant phosphoproteins corresponding to the enzyme activity. Prior to the first dimension, 2 sets of phosphorylation assays were performed under the same conditions as described above, except that to the first one was added 0.5 μM [$\gamma$-$^{32}$P]ATP (5 × $10^3$ cpm/pmol) and to the other one, 0.5 μM unlabeled ATP (final volume 50 μl). To all preparations, 25 μl of 0.375 M Tris-HCl, pH 6.8, containing 30% glycerol, 0.3% Triton X-100, 10 mM β-mercaptoethanol were then added and the samples were layered on 5% polyacrylamide gel buffered with 0.375 M Tris-HCl, pH 8.8, containing 0.1% Triton-X-100 and 10% glycerol. Electrophoresis was carried out in a refrigerated mini slab gel apparatus (Bio-Rad) for 70 min at 200 V, with the running buffer, pH 8.3, containing 25 mM Tris-HCl and 0.192 M glycine. Afterwards, gels were soaked for 5 min in 25 mM Tris-HCl buffer, pH 7.4, at 4°C. The gel lane containing unlabeled phosphoproteins was cut into 3-mm slices which were then transferred to test tubes containing the phosphorylation medium (200 μl) with polyGT (100 μg) and 10 nM of [$\gamma$-$^{32}$P]ATP (3000 cpm/fmol). After 90 min of reaction, the gel slices were removed and the phosphorylation of polyGT was determined after TCA precipitation as described earlier. The other gel lane containing the radiolabeled phosphoproteins was equilibrated with SDS-PAGE sample buffer and submitted to a second dimensional electrophoresis as described by O'Farrell [15].

2.6. *Renaturation of protein tyrosine kinase after SDS-PAGE and phosphoamino acid analysis*

The renaturation method used for PTK identification was adapted from Kameshita and Fujisama [16] and will be published separately [17]. Briefly, proteins were first separated by SDS-PAGE in the presence or in the absence of polyGT (50 μg/lane); the gel was then treated with 8 M guanidine-HCl to allow the reversible denaturation of polypeptides and their subsequent renaturation in the presence of 0.04% Tween 20. In situ phosphorylation was initiated by incubating the gel in the phosphorylation medium containing [$\gamma$-$^{32}$P]ATP as described above. To increase the relative proportion of pY-enriched proteins and enhance the detection of phosphorylated polyGT, the latter gel was treated first with glutaraldehyde, then with 1 N KOH at 56°C for 2 h [18]. Following autoradiography, the gel band was directly hydrolyzed in 6 N HCl (110°C, 90 min) and the hydrolyzate partially purified by passing through $C_{18}$ and QMA cartridges [19]. Phosphoamino acids were then analyzed by 2D electrophoresis on cellulose thin layer plates [20].

## 3. RESULTS AND DISCUSSION

To identify the major soluble form of PTK in the canine prostate, we have developed an approach to localize the enzyme in SDS-PAGE which relies on both the autophosphorylation property of the PTK and its ability to phosphorylate the synthetic substrate polyGT. The PTK was first partially purified by gel filtration of the cytosol on Sephadex G-100; as shown in Fig. 1A, the enzyme was eluted at a relative $M_r$ of 52 000 as compared to a reported value of 44 000 on Sephacryl S-300 [1]. The PTK autophosphorylating capacity was then demonstrated with a polyclonal anti-pY antibody, as shown in Fig. 1B and as evidenced by PolyGT-phosphorylation, the enzyme remained active once immunoprecipitated by the antibody. The formation of anti-pY/

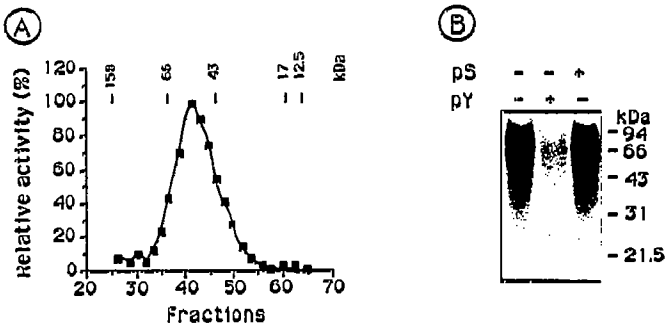


Fig. 1. Gel filtration of a cytosolic fraction from dog prostate, and subsequent immunoprecipitation of PTK activity. (A) Following chromatography on Sephadex G-100, the PTK activity of each fraction was assayed by the phosphorylation of polyGT. (B) Immunoprecipitation was performed with polyclonal anti-pY antibodies on peak fractions obtained from gel filtration, along with the displacement by pY or pS. The PTK activity of immunocomplexes was measured with polyGT and analyzed by autoradiography after SDS-PAGE following glutaraldehyde and KOH treatments of gels.

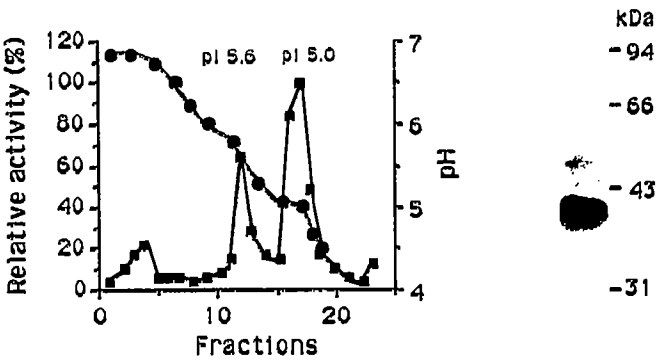


Fig. 2. Chromatofocusing and endogenous alkali-resistant phosphorylation. (Left panel) Pool fractions from the Sephadex G-100 column were submitted to a chromatofocusing column adapted to a FPLC system. The fractions corresponding to the major peak of activity at a pI of 5.0 were pooled, concentrated on an Amicon membrane (cut-off 10 000) and used for endogenous phosphorylation. (Right panel) Endogenous alkali-resistant phosphoproteins separated by SDS-PAGE were detected by autoradiography after glutaraldehyde and alkali treatments.

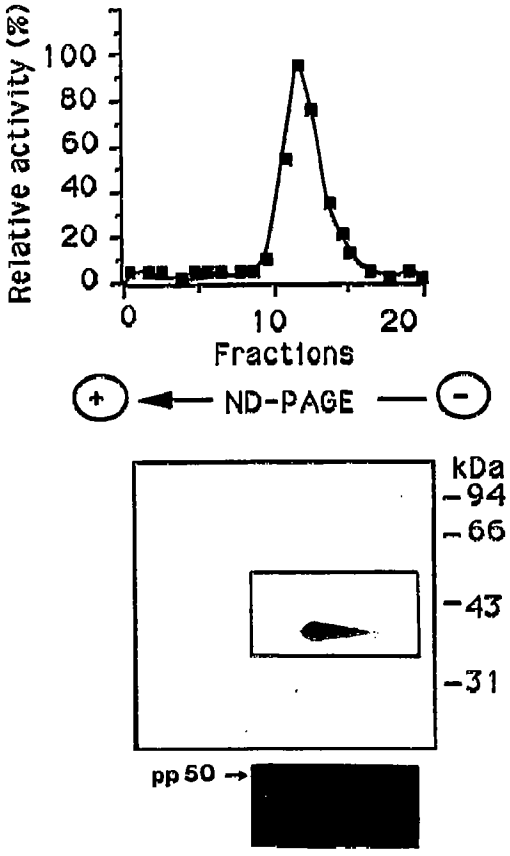


Fig. 3. Two-dimensional electrophoresis. The partially purified PTK fraction after G-100 and chromatofocusing (pI 5.0) was submitted to electrophoresis to separate native phosphoproteins in non-denaturing conditions as the first dimension; PTK activity was then assayed by polyGT phosphorylation with gel slices in test tubes (upper panel) and after SDS-PAGE (second dimension), by the detection of endogenous alkali-resistant phosphoproteins (middle panel). A prolonged exposure (1 month) of the rectangular region was required to ascertain the presence of pp50 (lower panel).

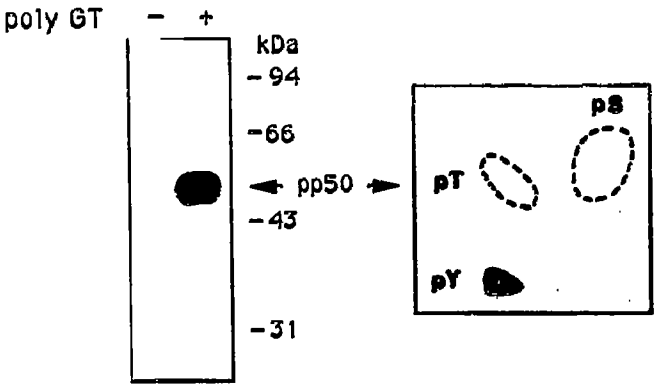


Fig. 4. Renaturation of PTK after SDS-PAGE. The enzyme preparation obtained after chromatofocusing (pI 5.0, 10 μg of protein) was separated by SDS-PAGE in the presence (+) or in the absence (–) of polyGT. The gel was treated in guanidine-HCl and Tween-20 and incubated with [$\gamma$-$^{32}$P]ATP according to Durocher et al. [17]. Renatured kinases (left panel) were revealed by autoradiography after glutaraldehyde and alkali-treatments. The pp50 band (+polyGT) was then excised, hydrolyzed in HCl and, following the addition of internal standards of pY, pS and phosphothreonine (pT) (3 μg), was submitted to phosphoamino acid analysis (right panel) by 2D-thin layer electrophoresis, as described in section 2. Standards were detected by ninhydrin staining and radiolabeled phosphoamino acids by autoradiography.

PTK complexes was also specific for pY-enriched proteins; it was displaced by pY, but unaffected by pS, confirming the specificity of the antibody (Fig. 1B). These results suggest that the prostatic PTK possesses autophosphorylation sites and is likely to catalyze the phosphorylation of its tyrosyl residues. It is noteworthy that autophosphorylation is a characteristic of almost all described PTKs with few exceptions, such as a p36–40 PTK from HL-60 cells [21] and a p50 CSK from neonatal rat brain [22]. To identify the PTK, enzyme fractions eluted from Sephadex G-100 were pooled and further purified by chromatofocusing which, instead of the single peak of activity observed at a pI of 5.5 as reported previously [1], yielded a major peak characterized by a pI of 5.0, and a minor one by a pI of 5.6 (Fig. 2). This difference may be explained by the better resolution obtained with chromatofocusing (pH gradient 7–4) compared to isoelectric focusing (pH gradient 10–3). When in vitro phosphorylation was studied using the enzyme fraction of pI 5.0, 2 alkali-resistant phosphoproteins (pp) were detected, namely a major one, pp41 and a faint band, pp50 (Fig. 2). Upon 2D electrophoresis with the first dimension run in non-denaturing conditions and assay of PTK activity on polyGT with gel slices, the migration of the native PTK could be determined and is shown in Fig. 3 (upper panel); when the alkali-resistant phosphoproteins corresponding to the PTK activity located in the first dimension on non-denaturing PAGE were revealed after the second dimension, pp41 and pp50 were both present but with a significant shift between the 2 phosphoproteins, so that pp50 fitted better with the PTK activity (Fig. 3,

lower panel). This is also supported by results from renaturation of the enzyme activity after protein separation by SDS-PAGE which is shown in Fig. 4; indeed, when the partially purified PTK fraction (gel filtration in G-100 and chromatofocusing, pI 5.0) was allowed to migrate within the gel together with the polydispersed substrate polyGT ($M_r$ range 30 000–90 000 as shown in Fig. 1B) and these proteins were submitted to a denaturation/renaturation process prior to the incubation with the phosphorylation medium, only 1 band at a position corresponding to an alkali-resistant phosphoprotein of 50 kDa was detected (Fig. 4, left panel). The fact that the phosphoamino acid analysis (Fig. 4, right panel) showed only the presence of pY linked to the presence of polyGT, confirmed that a PTK was indeed present and renaturated. The possibility that the detection of pp50 as a PTK would be artefactual is unlikely since our results of both 2D electrophoresis and in vitro phosphorylation indicate the presence of an alkali-resistant phosphoprotein of 50 kDa which corresponds to the PTK activity detected by renaturation. When similar experiments were carried out with the minor peak obtained from chromatofocusing, a PTK of 50 kDa was also evidenced by renaturation (not shown), suggesting that these proteins may represent isoenzymes. Whether the difference in their isoelectric point can be accounted for by post-translational modifications such as endogenous phosphorylation or sulfation remains to be established. One of these possibilities i.e. the in vivo phosphorylation of pp50 on tyrosyl residues is most likely since 40% of PTK activity can be immunoprecipitated by anti-pY without prior in vitro phosphorylation (not shown). With respect to the highly labeled alkali-resistant pp41 band (Figs. 2B and 3), its phosphoamino acid analysis revealing the presence of pS (not shown) indicates that it is not a PTK; in addition, no such activity was associated with a pp41 band upon renaturation. Considering that the $M_r$ of the major soluble form of PTK in the canine prostate ranges from 44 000 (Sephacryl S-300) to 52 000 (Sephadex G-100), that a polypeptide of 50 kDa expresses the enzymatic activity and that the latter enzyme can be immunoprecipitated with an anti-pY antibody, it is concluded that this PTK is a monomer of 50 kDa with at least 1 autophosphorylation site. Whether this soluble PTK is implicated in a signal transducing pathway playing a role in prostatic cell proliferation is currently under investigation.

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