Journal of Cellular Biochemistry 46:291–301 (1991)

Prostatic Epithelial Cells in Culture: Phosphorylation of Protein Tyrosyl Residues and Tyrosine Protein Kinase Activity

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Abstract The ability of dividing canine prostatic epithelial cells in primary monolayers to phosphorylate protein tyrosyl residues was evaluated by metabolic studies performed through incorporation of [^{32}P]-phosphate into alkali-resistant phosphoproteins and by the assay of their tyrosine protein kinase activity. The presence of sodium orthovanadate during cell incubation with [^{32}P]-phosphate greatly enhanced the relative labelling intensity of a 44 kDa alkali-resistant phosphoprotein and the total cellular content of phosphotyrosine in proteins; in this respect, growth factors such as epidermal growth factor, insulin, and insulin-like growth factor I, and the steroids dihydrotestosterone and estradiol were inactive. When the cells were solubilized, sodium orthovanadate stimulated their tyrosine protein kinase activity and inhibited their phosphotyrosine phosphatase activity. To characterize the tyrosine protein kinase of these cultured cells, conditions for optimal activity were established using the substrate poly [Glu^{80}Na, Tyr^{20}]. The subcellular localization of the enzyme was determined upon cell fractionation: 88% of the kinase activity was associated with the particulate fraction and 30% of this activity was partially solubilized with 0.5% Triton X-100; this solubilization was improved to 83% in the presence of 0.25 M KCl. The enzyme directly solubilized from prostatic cells with Triton X-100 (38% of activity) mainly catalyzed the alkali-resistant phosphorylation of pp63, pp59, and pp44, which contained phosphotyrosine. These proteins were also phosphorylated by the major peak of kinase activity which was eluted at an apparent molecular weight of 300–350 kDa upon gel filtration. On a cell basis, the kinase activity was four- to eleven-fold higher in the dividing epithelial cells in culture compared to quiescent secretory and non-secretory epithelial cells, freshly isolated from dog prostates. It is proposed that this tyrosine protein kinase is implicated in the regulation of the proliferation of prostatic epithelial cells.

Key words: protein phosphorylation, phosphotyrosine, phosphotyrosyl protein phosphatase, orthovanadate, cell proliferation, canine prostate

In the aging man and dog, prostatic cancer arises mainly from the proliferation of epithelial cells [1,2]. The activation of glandular epithelial cells also leads to benign prostatic hyperplasia (BPH) in the dog while fibromuscular and epithelial elements contribute in varying degrees to human BPH [3]. The development of both diseases requires testicular steroids but their exact role as inductive or progression factors is not fully understood [3–6]. Surgical procedures are widely used as treatment for human BPH [7] and although the combined therapy with analogs of luteinizing hormone releasing hormone and anti-androgens is gradually replacing other endocrine treatments, prostatic cancer still cannot be cured [6]. Essentially, the endocrine therapy affects androgen-dependent cells but the disease eventually recurs as a steroid-independent tumor; its growth has been attributed to androgen-independent cells already present in the tissue prior to treatment [6,8,9].

The question of androgen-independent growth of prostatic cells has been raised for several years. Our in vitro studies on epithelial cells from normal and hyperplastic human and canine prostates have shown an androgen-independent growth stimulated by non-steroidal growth-promoting factor(s) (GF) present in serum and prostatic extracts [10,11]. In view of the possibility that GF may modulate prostatic cell growth [10–12], in vitro model systems represent useful

Received September 6, 1990; accepted March 22, 1991.

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tools to study the proliferation of androgen-independent but GF-dependent prostatic epithelial cells.

Tyrosine protein kinases (TPKs) are intimately involved in cellular responses to GF and their activation is a prerequisite for the transmission of the mitogenic signals of several GFs [13]. In addition, alterations of TPKs, either in growth factor receptor complexes or as products of cellular protooncogenes or viral oncogenes, frequently lead to cell transformation [14,15]. The present investigation was undertaken to study the levels of tyrosine phosphorylation of proteins in dividing canine prostatic epithelial cells in culture and to characterize their TPK with respect to subcellular localization as well as certain kinetic and molecular properties.

MATERIALS AND METHODS

Tissue culture plasticware and reagents for cell isolation and culture were purchased from Gibco Canada. N-2 hydroxymethylpiperazine-N'-2 ethanesulfonic acid (Hepes), 2 [N-morpholino] ethanesulfonic acid (MES), ethylenediaminetetraacetic acid (EDTA), [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), 2-mercaptoethanol, dithiothreitol, Coomassie brillant blue G, L-phosphoserine (pSer), D-L phosphothreonine (pThr), L-phosphotyrosine (pTyr), epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), benzamidine, poly[$Glu^{80}Na$, Tyr^{20}] or (poly GT) and bovine serum albumin (Fraction V, BSA) were obtained from Sigma (St. Louis, MO). Insulin (beef and pork) was purchased from Novo Laboratories Ltd. (Canada). Triton X-100, 934 AH glass fiber filters, and sodium orthovanadate (Na_3VO_4) also referred to as vanadate were obtained from Fisher Scientific Ltd. (Montreal, Canada). Leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and Pronase (7,000 U/g) were purchased from Boehringer-Mannheim (Montreal, Canada). Sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylenebisacrylamide, bromophenol blue, tris (hydroxymethyl aminomethane), and molecular weight standard proteins were obtained from Bio-Rad (Mississauga, Canada). Cellulose thin layer plates (20 × 20 cm) and glutaraldehyde 50% were purchased from Eastman Kodak Company (Rochester, NY) and Sephacryl S-300 from Pharmacia (Dorval, Canada). Formula 963 scintillation liquid and [^{32}P]phosphate or [^{32}P]-Pi (7,000 Ci/mmol) were obtained from Dupont Canada Inc. (Dorval, Canada) and [γ-^{32}P]ATP (7,000 Ci/mmol) from ICN (Canada). Trichloroacetic acid (TCA) was purchased from BDH (Montreal, Canada). Kodak XAR and Fuji X-ray films were obtained from Picker Int. (Montreal, Canada). Dihydrotestosterone (DHT) and estradiol (E_2), purchased from Steraloids, were crystallized from methanol-water and dissolved in absolute ethanol prior to use. Other reagents were of analytical grade.

Cell Culture

The methods used have been described previously [10,16]. In brief, normal and hyperplastic prostates (10–30 g) from adult mongrel dogs (15–30 kg) were minced and dispersed by collagenase treatment (0.25%, w/v). Primary monolayers of epithelial cells were established by plating freshly dispersed cells (2×10^5 per ml of minimum essential medium (MEM) supplemented with 10% dialyzed fetal bovine serum and 1% antibiotic solution (containing 10,000 units of penicillin and 10,000 μg of streptomycin per ml.) in polystyrene dishes, flasks, or plates. The cells were allowed to attach over a 3-day period at 37°C in a humidified chamber under 5% CO_2-95% air, and were further cultured for up to one week following the replacement of the medium by MEM supplemented with 10% charcoal-treated dog serum with periodical replacement of the medium; these conditions are known to favor their proliferation [10].

Analysis of Cellular Proteins Labelled With [^{32}P]-phosphate

Cells in monolayers were rinsed twice with phosphate-free MEM supplemented with 1 mM Hepes, pH 7.4 (MEM/Hepes), and then incubated for 2 h at 37°C in phosphate-free MEM/Hepes containing 100 μCi of [^{32}P]-Pi per ml in the presence or absence of Na_3VO_4 at the indicated concentrations. The medium was then quickly removed and the cells were washed twice with phosphate buffered saline (PBS), scraped from the dishes in buffer A, pH 6.8 (0.06 M Tris, 1.25% SDS, 5% 2-mercaptoethanol, 12% glycerol, and 1 mM PMSF), transferred to microtubes and precipitated by the addition of TCA to a final concentration of 10%. The mixture was allowed to stand overnight at 4°C prior to the collection of the precipitate by centrifugation. The pellets were neutralized with saturated Tris and then dissolved in buffer A before being heated at 100°C for 5 min. Following centrifuga-

tion (10,000*g* , 10 min), the protein concentration of the solubilized material was determined by the method of Bradford [17] using BSA as the standard. Bromophenol blue (0.01% w/v) was added and samples, equivalent to 80 μg of protein, were analyzed by SDS-PAGE either through 7.5% (20 V, 20 h) or 7–15% (40 V, 20 h) linear gradients of polyacrylamide [18]. The gels were then stained with Coomassie brillant blue G (0.15%, w/v, in 10% methanol and 7% acetic acid).

Total ^{32}P-labelling of phosphoproteins in wetted gels was detected by autoradiography at room temperature with Kodak XAR-5 or Fuji Rx films for 24–48 h. The relatively high stability of phosphotyrosyl bonds in proteins to alkali-treatment as compared to phosphoseryl and to a lesser extent phosphothreonyl residues [19] was used as a criterion to detect those phosphoproteins likely to be enriched in phosphotyrosine. After fixation with glutaraldehyde [20], parallel gels were soaked in 1 N KOH at 56°C for 2 h, stained with Coomassie blue, dried, and submitted to autoradiography at −70°C for 48–72 h in the presence of intensifying screens to detect alkali-resistant phosphorylation [20]. Gels and autoradiograms were scanned with a Bio-Rad densitometer, model 1650 (light source: 580 nm; slit width: 0.25 mm). The surface area under each peak was integrated to measure the level of phosphorylation of each protein band whose molecular weight was estimated by comparison with standard proteins analyzed in parallel.

Phosphoamino Acid Analysis

To estimate the cellular content of phosphotyrosine, protein samples were digested with Pronase according to the method described by Ushiro and Cohen [21]. Radiolabelled prostatic phosphoproteins were first precipitated with 10% TCA (60 min on ice), recovered by centrifugation (900*g* for 10 min), and washed three times with 0.5 ml of cold 10% TCA and twice with 2 ml of anhydrous ether. The samples were resuspended in 0.4 ml of 25 mM ammonium acetate containing 2 mM calcium acetate and 100 μM Na_3VO_4. The pH was then adjusted to 7.5 with 20 mM NH_4OH. Proteolytic digestion was initiated by adding 200 μg of Pronase in ammonium acetate buffer. An aliquot of 1 μl of toluene was added to each sample as a preservative and incubations were carried out at room temperature for 24 h. Samples were lyophilized and phosphoamino acid analysis was then performed by two-dimensional electrophoresis on thin layer cellulose plates [19].

Individual alkali-resistant phosphoprotein bands were also extracted from parallel polyacrylamide gels, not treated in alkali, and hydrolyzed at 112°C in 6 N HCl for 1.5 h as described earlier [20] in order to analyze their phosphoamino acid content by two-dimensional electrophoresis.

Cell Fractionation

All procedures were carried out at 4°C. Cells were recovered from monolayers by scraping and washing the plates in PBS, pH 7.4, containing 10 μg/ml Aprotinin, 1 μg/ml Leupeptin, 0.4 μg/ml Pepstatin, 0.2 mM PMSF, 1 mM EDTA, and 100 μM Na_3VO_4 (unless otherwise indicated). They were recovered by centrifugation at 600*g* for 5 min and resuspended in five volumes of buffer B, pH 7.4 (20 mM Hepes, 100 mM sucrose, 70 mM NaCl, 5 mM 2-mercaptoethanol, 50 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1 mM PMSF, 10 mM benzamidine, 1 mM EDTA, 1 mM EGTA, 100 μM Na_3VO_4), and homogenized with a Polytron. The suspension was centrifuged for 10 min at 1,000*g* to remove nuclei and other cell debris. The supernatant was recentrifuged for 60 min at 105,000*g* to obtain a soluble fraction referred to as the cytosolic fraction and a particulate fraction which was resuspended in buffer C (buffer B without benzamidine, sucrose, and NaCl). Protein concentrations were determined and aliquoted preparations were frozen at −70°C until their assay.

Tyrosine Protein Kinase Assay

Standard procedure: the TPK activity of 20 μl of prostatic fractions (10 μg of proteins) was determined by phosphorylation at room temperature for 20 min of 50 μg of poly GT in phosphorylation buffer, pH 7.4, which contained 20 mM Hepes, 20 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM 2-mercaptoethanol, 100 μM Na_3VO_4 (unless otherwise indicated), 0.1% (v/v) Triton X-100, and 20 μM [γ-^{32}P]ATP (2–3 × 10^3 cpm/pmol) in a final volume of 100 μl. The reaction was stopped by the addition of unlabelled ATP (20 mM final concentration) and NaOH (0.9 N final concentration) followed by heating at 90°C for 10 min [22]. The proteins and poly GT were then precipitated (30 min on ice) by the addition of TCA to attain, after neutralization, a final concentration of 10%. The precipitate was recovered on a Whatman 934 AH disc filter, washed 4 times with 5 ml of a mixture of ice-cold 5% TCA that contained 10

mM NaH_2PO_4 and 10 mM $Na_4P_2O_7$, and 3 times with 5 ml of ice-cold 95% ethanol. The filters were counted in Formula-963 scintillation fluid. Parallel assays where enzyme preparations or poly GT were deleted were used as blanks. Results of TPK activity were expressed in pmol of Pi transferred from ATP to poly GT per min.

The tyrosine protein kinase activity was also determined by the detection of the alkali-resistant phosphorylation of endogenous proteins. Fractions (10 μg of proteins) were incubated for 20 min at room temperature in the same phosphorylation buffer as above but containing 2–10 × 10^3 cpm/pmol [γ-^{32}P]ATP and no poly GT. The reaction was stopped by the addition of 20 mM unlabelled ATP. After the addition of saturated Tris to neutrality, the proteins were resuspended in buffer A and separated by SDS-PAGE; alkali-resistant phosphoproteins were revealed by autoradiography as described above.

Solubilization of TPK

To fixed volumes of particulate fractions (105,000*g* pellet), equal volumes of buffer C containing 1.0% (v/v) of Triton X-100 and in some instances 0.5 or 1.0 M of KCl were added and the resulting samples were homogenized in a Dounce homogenizer with 10 strokes of a tight fitting pestle and maintained on ice for 45 min before recentrifugation for 60 min at 105,000*g*. The supernatants were recovered and the pellets were resuspended with a Dounce homogenizer in equal volumes of buffer C containing 0.5% (v/v) Triton X-100.

Gel Filtration

The material solubilized from the particulate fraction in the presence of 0.5% Triton X-100 and 0.25 M KCl was chromatographed on a Sephacryl S-300 column (1 × 50 cm) equilibrated in elution buffer, pH 7.4, containing 20 mM Tris-HCl, 10% glycerol, 0.5 mM dithiothreitol, 10 μg/ml Aprotinin, 1 μg/ml Leupeptin, 0.4 μg/ml Pepstatin, 0.2 mM PMSF, 100 μM Na_3VO_4, 0.2 mM EDTA, 0.2 mM EGTA, 0.5% (v/v) Triton X-100, and 0.25 M KCl.

Fractions of 0.45 ml were collected and their TPK activity was assayed by the phosphorylation of poly GT. The proteins present in the peak fractions were allowed to undergo endogenous phosphorylation by incubation with [γ-^{32}P]ATP and the resulting alkali-resistant phosphoproteins were analyzed by autoradiography after SDS-PAGE, as described above.

Determination of Phosphatase Activity

Phosphatase activities were assayed at pH 7.4 using phosphoamino acids (pSer, pThr, and pTyr at 10 mM) as substrates in the presence and in the absence of Na_3VO_4 (at indicated concentrations) under optimal conditions established for prostatic TPK but without poly GT and ATP. Since prostatic acid phosphatase (PAP) is a phosphotyrosine phosphatase [23], the hydrolysis of phosphoamino acids was also determined in 50 mM sodium citrate buffer, pH 4.8, as already reported [23]. In all instances, the release of inorganic phosphate (Pi) was determined according to the method of Ames [24].

RESULTS

Protein Phosphorylation: Effects of Orthovanadate, Growth Factors, and Steroids

The labelling kinetics of alkali-resistant prostatic phosphoproteins following incubation of cultured prostatic epithelial cells with [^{32}P]-Pi in the presence or absence of Na_3VO_4 indicated that, at zero time, no radiolabelled phosphoproteins were detected and that the labelling intensity was proportional to the time of incubation up to 180 min while the patterns of alkali-resistant phosphorylation remained the same after 60 min of incubation (not shown). As shown in Figure 1, the presence of Na_3VO_4 at concentrations varying from 5 to 100 μM had no effect on total phosphorylation while at a concentration as low as 5 μM, it increased the relative labelling intensity of a 44 kDa alkali-resistant phosphoprotein (pp) with no significant effect on the labelling intensity of the other alkali-resistant phosphoproteins. Following the densitometric analysis of autoradiograms, it was calculated that at 120 min of labelling, pp 44 was increased by 147 ± 75% (mean of four preparations) compared to an average of 28 ± 25% for the other major alkali-resistant bands. In contrast, when the growth factors, EGF and insulin at 50 nM, IGF-I at 10 nM, and steroids, DHT and E_2 at 10 nM, were incubated with prostatic cells under similar conditions, no effect on both the total as well as alkali-resistant phosphorylation was observed (not shown).

The effect of Na_3VO_4 on the cellular phosphotyrosine content of prostatic proteins is illustrated in Figure 2 where the liberated phosphoamino acids were analyzed following protein digestion with Pronase; as already observed by Ushiro and Cohen [21], Pronase liberated very

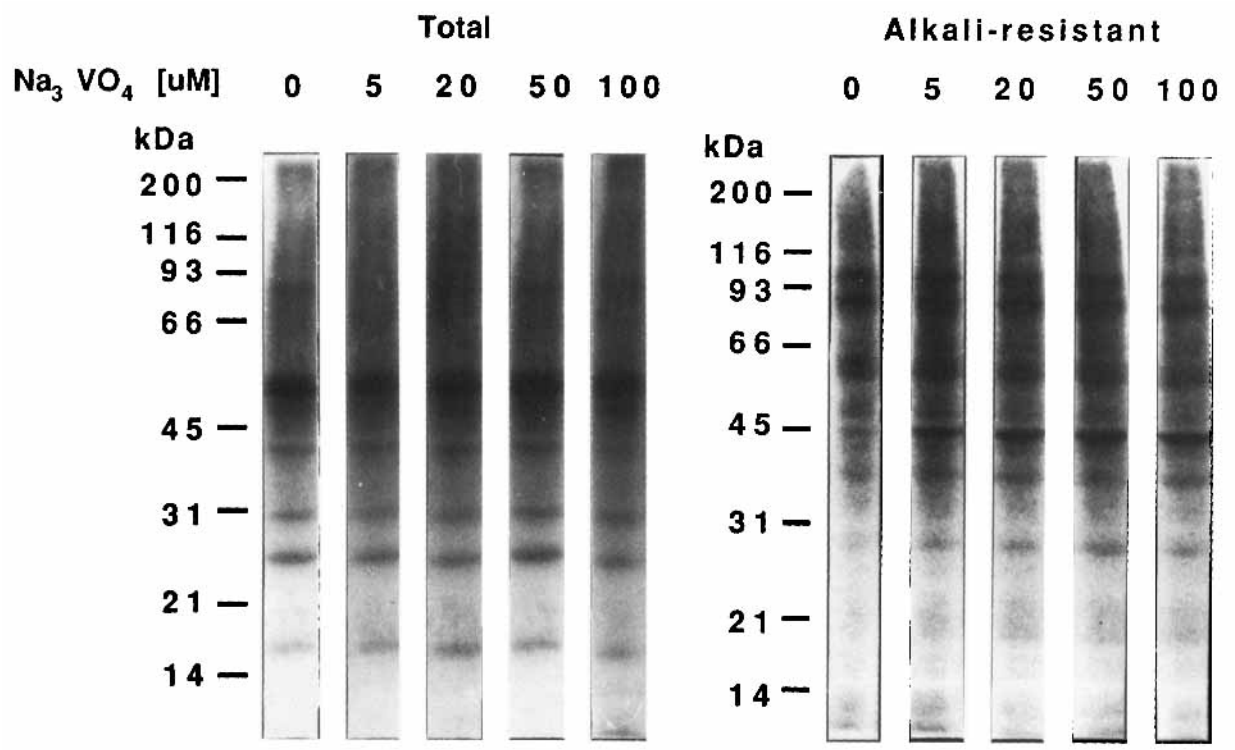

Fig. 1. Effect of Na_3VO_4 on the incorporation of [^{32}P]-phosphate into prostatic phosphoproteins. Epithelial cells cultured for 3 days were incubated with [^{32}P]-Pi for 2 h in the presence or in the absence of increasing concentrations of Na_3VO_4 up to 100 μM. Cellular proteins were processed through SDS-PAGE and the gels, untreated (total phosphorylation), or treated with glutaraldehyde and KOH (alkali-resistant phosphorylation), were submitted to autoradiography as described in Methods.

little phosphoserine and phosphothreonine and a trace quantity of phosphotyrosine; the latter was greatly enhanced when Na_3VO_4 was present during the incubation of prostatic cells with [^{32}P]-Pi (Fig. 2).

In phosphorylation studies performed with whole cells, Na_3VO_4 may affect several enzymes involved in Pi transport and metabolism [25]. As in the present system, total phosphorylation was not altered in its presence, its effects were likely to occur through the tyrosine protein kinase–phosphotyrosyl protein phosphatase pathway rather than on serine/threonine kinases and phosphatases [19]. Tyrosine protein kinase and phosphatase activities of prostatic cells were therefore measured in a cell-free system in the presence and in the absence of Na_3VO_4. As shown in Table I, the basal level of activity (16.9 pmol/min/mg protein) measured in the absence of Na_3VO_4 during both cell solubilization and assay of TPK activity was increased by almost two-fold when it was added either during solubilization (final concentration: 25 μM in the assay mixture) or in the enzymatic assay. A higher level of TPK activity (2.6-fold increase) was also observed when Na_3VO_4 was present at all times (Table 1). In those conditions used to measure TPK activity (pH 7.4), phosphotyrosine was preferentially hydrolyzed as compared to pSer and no activity toward pThr was detected (Table 1); Na_3VO_4 inhibited this pTyr phosphatase activity by 55% and 90% when present at concentrations of 100 uM and 10 mM, respectively. When phosphoamino acid hydrolysis was measured at acid pH (i.e., under optimal conditions for PAP), similar results were obtained but with higher phosphatase activity (Table 1).

Partial Characterization of TPK

Kinetics. Preliminary experiments performed to assay the TPK activity with the synthetic substrate poly GT indicated that the activity could only be measured following cell solubilization with detergents. As illustrated in Table II, those cells resuspended in the phosphorylation medium only (i.e. in the absence of Triton X-100) exhibited minimal TPK activity (11%) when compared to control cells resuspended in 0.5% (v/v) Triton X-100, referred to as 100%. The activity was completely abolished by

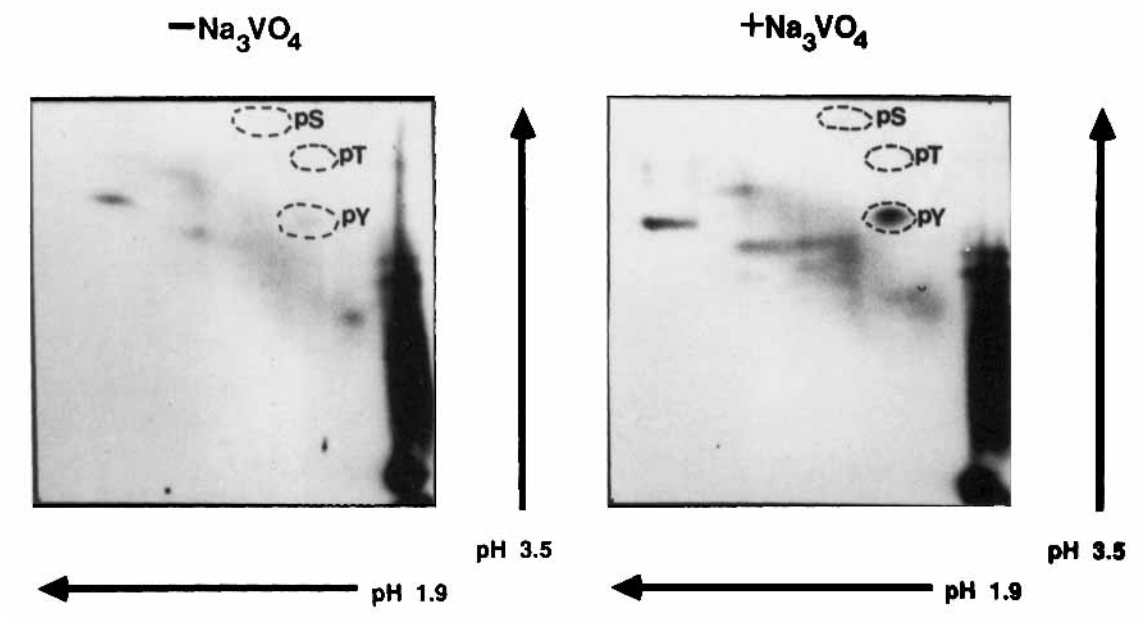

Fig. 2. Effect of Na_3VO_4 on the cellular phosphotyrosine content of prostatic proteins. After a 2 h incubation of epithelial cells (cultured for 3 days) with [^{32}P]-Pi in the presence (+) or in the absence (−) of 100 μM Na_3VO_4, cellular proteins were digested with Pronase. Following the addition of internal standards of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), phosphoamino acids were analyzed by two-dimensional electrophoresis (first dimension pH 1.9; second dimension pH 3.5) and revealed by Ninhydrin staining and autoradiography.

a preincubation of the suspension at 100°C for 10 min or by the deletion of $MnCl_2$ and $MgCl_2$ from the incubation mixture (Table II). The addition of reducing agents such as dithiothreitol (5 mM) and 2-mercaptoethanol (5 or 10 mM) enhanced the phosphorylation of poly GT by 35 to 165% while the presence of NaCl (0.1–0.2 M) or KCl (0.2 M) in the reaction mixture reduced the activity by 38–55% (Table II). As shown in Figure 3A, the optimal pH was 7.2–7.4. The enzyme required either Mg^{++} or Mn^{++} as divalent cations but was more active in the presence of $MgCl_2$ than in the presence of $MnCl_2$; their optimal concentrations were 20 mM and 10 mM, respectively (Fig. 3B). However, the highest TPK activity (ten-fold higher) was measured in the

TABLE I. Effect of Sodium Orthovanadate on TPK and Phosphoamino Acid Phosphatase Activities*

	Specific activity of TPK (pmol/min/mg) Na_3VO_4 present during cell solubilization	
	0	100 μM
Na_3VO_4 in TPK assay mixture		
0	16.9 ± 4.3 (100%)	30.6 ± 1.9 (180%)
100 μM	32.9 ± 8.4 (190%)	44.4 ± 5.7 (260%)

	Relative hydrolysis of phosphoamino acids at pH 4.8			pH 7.4		
Vanadate	0	100 μM	10 mM	0	100 μM	10 mM
Substrate						
pTyr	100	80 ± 4 (20%)	18 ± 4 (82%)	14.5 ± 1.5	6.5 ± 1.5 (55%)	1.5 ± 0.5 (90)
pSer	5 ± 4	—	—	1.5 ± 1.5	—	—
pThr	0	—	—	1.5 ± 1.5	—	—

*Results from two cell preparations are presented (average ± SD). Numbers in parentheses either indicated stimulatory effect of Na_3VO_4 on TPK activity with reference to basal level observed in the absence of Na_3VO_4 or its inhibitory effects on pTyr phosphatase activity measured at pH 7.4 and 4.8, respectively. The pTyr phosphatase activity is expressed relatively to the hydrolysis of pTyr at pH 4.8 referred to as 100.

TABLE II. Effect of Cell Solubilization, Reducing Agents, and Ionic Strength on TPK Activity*

		TPK activity (%)
Control		100
Without Triton X-100		11 ± 3
100°C, 10 min.		0
Without Mg^{++} and Mn^{++}		0
Dithiothreitol, [mM]	5	135 ± 10
2-mercaptoethanol, [mM]	5	265 ± 35
	10	255 ± 63
NaCl, [M]	0.1	62 ± 2
	0.2	45 ± 7
KCl, [M]	0.2	42 ± 10

*Prostatic cells were solubilized with non-reducing buffer C containing 0.5% (v/v) of Triton X-100. Unless indicated, all assays were performed in the presence of a final concentration of 0.1% (v/v) Triton X-100, 10 mM each of $MgCl_2$ and $MnCl_2$, and 100 μM Na_3VO_4. The TPK activity was measured by the phosphorylation of poly GT as described in Materials and Methods.

presence of both cations at a concentration of 20 mM (Fig. 3C). Under those conditions that favored the optimal TPK activity (0.1% (v/v) Triton X-100, 5 mM 2-mercaptoethanol, 100 μM Na_3VO_4, 20 mM of both $MgCl_2$ and $MnCl_2$ at pH 7.4), the estimated Km's for ATP and poly GT (Eadie-Hofstee plots, not shown) were 4.0 ± 0.3 μM and 30 ± 2 μg/ml (N = 2), respectively. The transfer of ^{32}Pi from [γ-^{32}P]ATP to poly GT increased linearly with the time of incubation for at least 40 min and with up to 20 ug of prostatic proteins (not shown). The level of TPK activity per 10^6 prostatic epithelial cells cultured for three days was 31 ± 14 pmol/min with a specific activity of 25.6 ± 13.6 pmol/min/mg protein (N = 6).

Subcellular Distribution and Solubilization of TPK. The results of the distribution of TPK in cultured prostatic epithelial cells are presented in Table III. The particulate fraction contained most of the enzyme with 88% of the total activity and a specific TPK activity that was 17-fold higher than that of the cytosolic fraction (65.2 compared to 3.9 pmol/min/mg protein). These results are also reported in Figure 4 (lanes 1 to 3) together with those of the solubilization of the enzyme from the particulate fraction. In lanes 4 and 5, it is shown that 30% of the kinase could be extracted at a concentration of 0.5% (v/v) of Triton X-100; higher concentrations (1.0 and 2.0%) did not improve this yield (not shown). On the other hand, when 0.25 M

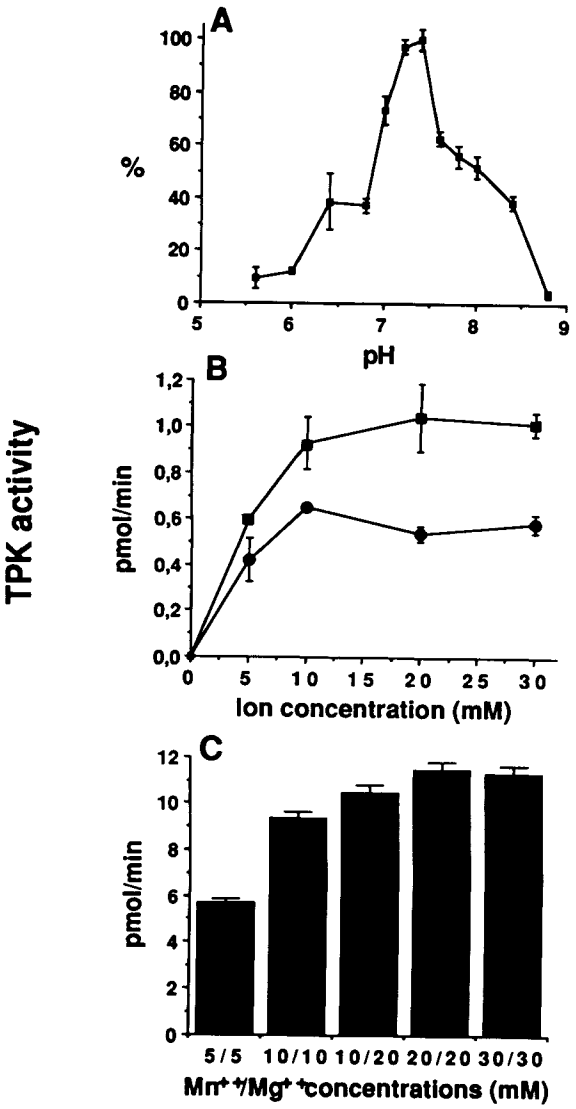

Fig. 3. Kinetic properties. Prostatic cells resuspended in buffer B containing 0.5% Triton X-100 were used for the phosphorylation of poly GT under optimal conditions as described in Methods. **A:** Effect of pH on TPK activity. The buffers, 2-[N-morpholino]ethane sulfonic acid (pH 5.5, 6.0), Hepes (pH 6.5 to 7.4), or Tris (pH 7.8 to 9.2), were used at a concentration of 50 mM. In **B**, the effects of $MgCl_2$ (■) or $MnCl_2$ (●) on TPK activity were studied separately and in **C**, the effects of a combination of both cations are presented.

KCl was used along with 0.5% Triton X-100, 83% of the activity was solubilized (lanes 6 and 7); a higher KCl concentration (0.5 M) did not result in any further solubilization (not shown). Since most of the enzyme activity was found in the particulate fraction, this solubilization protocol was also applied to whole cells; with 0.5% (v/v) Triton X-100, 38 ± 4% of TPK activity was extracted and the addition of 0.25 M KCl improved the solubilization to 85 ± 5% (N = 2).

TABLE III. Subcellular Distribution of Tyrosine Protein Kinase Activity

Cell[a] preparation	Total activity[b] (%)		Specific activity pmol/min/mg	
	Cytosolic	Particulate	Cytosolic	Particulate
1	14.0	83.0	4.6	102.0
2	10.9	91.1	8.0	101.9
3	13.7	85.3	2.3	32.0
4	3.8	93.2	0.8	24.7
X ± s.d.	10.6 ± 4.7	88.2 ± 4.8	3.9 ± 3.1	65.2 ± 43[c]

[a]Prostatic epithelial cells cultured for three days in primary monolayers were used for fractionation.
[b]Activity (%) was calculated relatively to the cellular homogenate.
[c]Average value (X ± s.d., standard deviation) of particulate fractions significantly different from corresponding average value for cytosolic fractions (Student's t test, $P < 0.001$).

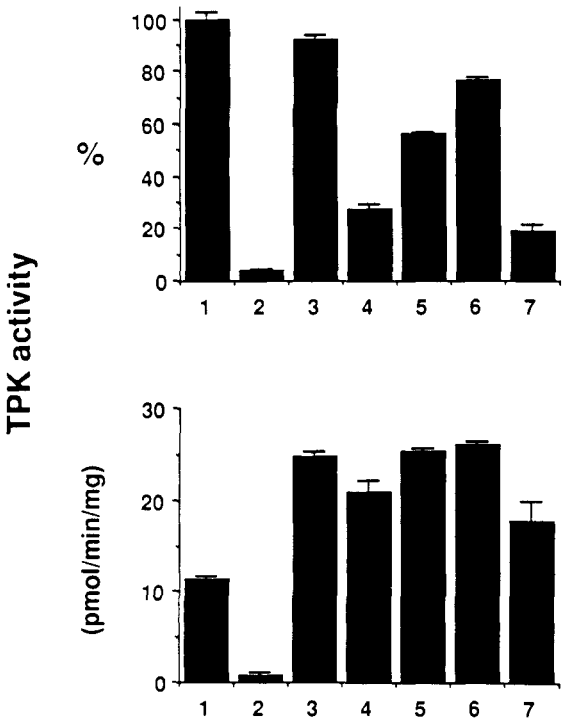

Fig. 4. Subcellular distribution and solubilization of prostatic TPK. Cytosolic and particulate fractions were isolated from epithelial cells cultured for 3 days. The particulate fraction was solubilized with 0.5% (v/v) Triton X-100 or with the combination of 0.5% (v/v) Triton X-100 and 0.25 M KCl prior to recentrifugation at 105,000*g* for 60 min at 4°C. The TPK activities and proteins of supernatants and pellets were then measured as described in Methods. Results from two cell preparations (average value ± standard deviation of the mean) are presented. **1.** Supernatant after centrifugation at 1,000*g* for 10 min of homogenized cells, referred to as 100% of activity. **2.** Cytosolic fraction. **3.** Particulate fraction. Material solubilized with Triton X-100 (**4**) or Triton X-100 and KCl (**6**). Residual insoluble material to Triton X-100 (**5**) or to Triton X-100 and KCl (**7**). **Upper section:** TPK activity expressed as % of the activity of fraction 1. **Lower section:** Specific TPK activity expressed per mg of protein.

Figure 5 shows that the cellular Triton extract had the ability to phosphorylate three major alkali-resistant proteins, pp63, pp59, and pp44; in addition, when extracted from untreated gels, hydrolyzed, and submitted to phosphoamino acid analysis, each of these proteins was shown to contain phosphotyrosine (Fig. 5). The deletion of DTT during the cell solubilization and phosphorylation processes did not alter the alkali-resistant phosphorylation pattern as revealed by SDS-PAGE (not shown).

Estimation of Apparent Molecular Weight. The result of gel filtration on Sephacryl S-300 of the solubilized material from the particulate fraction (Triton-KCl) is presented in Figure 6; one major peak of TPK activity with an apparent Mr of 310 kDa was observed and the enzyme in this peak yielded the phosphorylation of alkali-resistant proteins of 78, 63, 59, and 44 kDa with no radiolabelled phosphoprotein of high Mr remaining in the stacking gel. In addition, when SDS-PAGE was performed under conditions to detect high Mr proteins with more accuracy (5% acrylamide), no alkali-resistant phosphoprotein of 300–350 kDa was observed (not shown). Similar results (apparent Mr of 300 to 350 kDa for the TPK activity) were observed when Triton extracts from whole cells (38% of the enzyme) or Triton-KCl extracts of the remaining portion of the enzyme (47% of the enzyme) were used for gel filtration (not shown). The addition or deletion of dithiothreitol and of 0.25 M KCl in the sample or column buffers did not alter the apparent Mr of the enzyme. All attempts to chromatograph cytosolic fractions from cultured cells on Sephacryl S-300 resulted in the complete loss of enzyme activity.

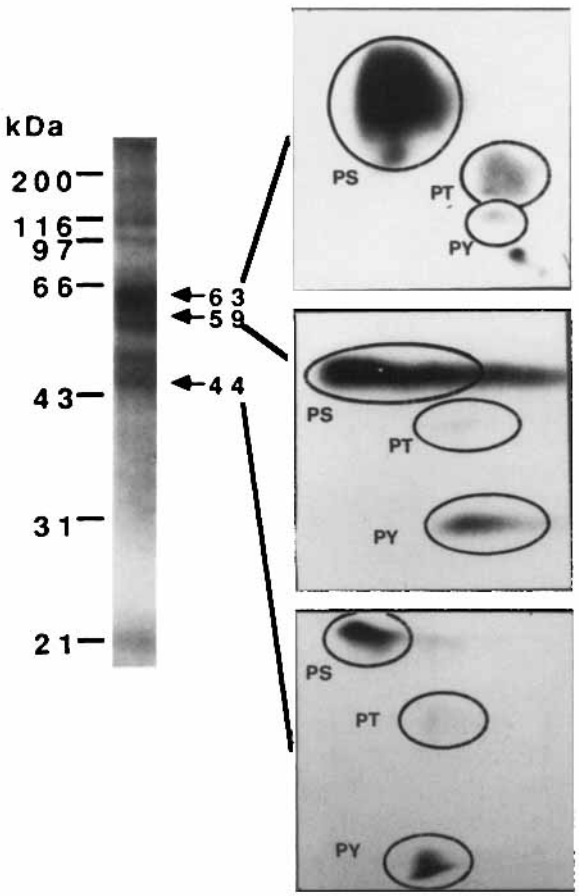

Fig. 5. Alkali-resistant phosphorylation and detection of phosphoamino acids in prostatic proteins. Prostatic cells (cultured for 3 days) were directly extracted with Triton X-100 by the procedure described in Methods for the solubilization of TPK from the particulate fraction. The extract (10 μg of proteins) was then phosphorylated by incubation with [γ-^{32}P]ATP, and processed through SDS-PAGE, glutaraldehyde cross-linking, alkali-treatment, and autoradiography. Labelled phosphoproteins (pp63, pp59, and pp44) were identified by autoradiography in the alkali-treated gel (left portion of figure), extracted from parallel untreated gels, hydrolyzed in HCl, and submitted to two-dimensional thin layer electrophoresis as already reported [20]. Phosphoamino acid standards were located by Ninhydrin staining and their labelling was detected by autoradiography as described in Methods and in the legend of Fig. 2.

DISCUSSION

In a previous methodological report on the refinement of the alkali-treatment of phosphoproteins in polyacrylamide gels to reveal those enriched in phosphotyrosine, it was demonstrated that dividing canine prostatic epithelial cells in primary culture do indeed have the ability to phosphorylate several endogenous proteins on their tyrosyl residues [20]. The present metabolic studies on alkali-resistant protein phosphorylation and characterization of TPK show several interesting features of this cell system which in some aspects are common to those reported for freshly dispersed secretory and non-secretory epithelial cells isolated from normal and metaplastic canine prostates while in others are strikingly different. One of the common features is the cell sensitivity to the presence of Na_3VO_4 during metabolic studies which not only enhances the total cellular content of phosphotyrosine in prostatic proteins but increases the relative labelling of only one alkali-resistant phosphoprotein of 44 kDa containing pTyr [20]. Interestingly, this pp44 is intensively labelled in non-secretory epithelial cells obtained from metaplastic glands which also actively synthesize DNA [26,27], suggesting that this protein may be important in dividing prostatic cells. The situation of cells in culture resembles the in vivo situation with respect to their acid phosphatase activities on phosphoamino acids [23,26]; indeed, pTyr is the substrate that is hydrolyzed to a greater extent compared to pSer and pThr and this pTyr phosphatase activity is sensitive to inhibition by Na_3VO_4. Since the pTyr phosphatase activity of prostatic epithelial cells has already been attributed to PAP [23], it is suggested that its residual activity at pH 7.4 would partly explain the stimulatory effect of Na_3VO_4 observed in metabolic studies. On the other hand, contrary to the situation observed for freshly dispersed cells of the canine prostate [26], Na_3VO_4 stimulates the TPK activity of cultured cells in a cell-free system. Further investigation of the purified TPK is necessary to determine whether this enzyme is indeed stimulated by vanadate as reported for the TPK associated with the insulin receptor [28].

The TPKs of dividing prostatic epithelial cells in primary monolayers show important differences with those characterized from the normal canine prostate [29] and/or its freshly dispersed non-secretory epithelial cells (unpublished data); indeed, while 75% of the enzyme is located in the cytosol of normal glands, and that in this system both cytosolic and particulate TPKs have an apparent Mr of 44 kDa [29], the present data show that in cultured cells, most of the TPK activity is located in the particulate fraction and has an apparent Mr of 300–350 kDa. Interestingly, both the 300 and 44 kDa TPKs catalyzed the alkali-resistant phosphorylation of low Mr

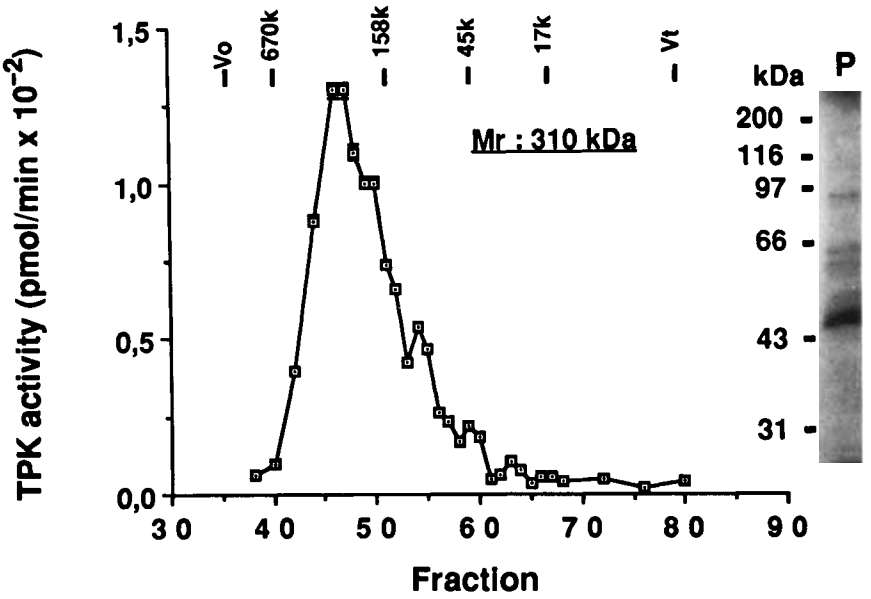

Fig. 6. Gel filtration on Sephacryl S-300. The extract solubilized from the particulate fraction (0.5% Triton X-100 and 0.25 M KCl; Fig. 4, lane 6) was chromatographed on a Sephacryl S-300 column and the TPK activity of column fractions was assayed by the phosphorylation of poly GT (⊡). The peak fraction (P) was used for phosphorylation of endogenous proteins in the presence of [γ-^{32}P]ATP which were submitted to SDS-PAGE, alkali-treatment and autoradiography.

polypeptides (SDS-PAGE), the major one being a p44 protein which contains phosphotyrosine. Whether this protein is the same protein increased by Na_3VO_4 in metabolic studies remains to be established. The possibility that it is related to TPKs is also unknown. Due to the autophosphorylation properties of most TPKs [13,15,30], the enzyme may be present among alkali-resistant phosphoproteins shown to contain phosphotyrosine. However, none of them has a Mr of 300–350 kDa and corresponds to the size of TPKs associated with known growth factor receptors. These data indicate that in the present system, the TPK activity is unlikely to be due to a high Mr protein, or to growth factor receptors such as those for EGF [31] and insulin (unpublished data) which could be readily detected in parallel control studies performed with extracts from rat liver membranes. The present findings are consistent with our data showing that EGF, insulin, IGF-I, and steroids (dihydrotestosterone and estradiol) had no effect on the growth of canine prostatic epithelial cells in culture [10,11, and unpublished data]; the metabolism of phosphoproteins measured via the incorporation of [^{32}P]-Pi; and their tyrosine kinase activity as assayed by the phosphorylation of both poly GT and alkali-resistant endogenous phosphoproteins. The possibility that other TPKs are expressed in prostatic cells is also not excluded but their activity toward the substrate poly GT would be very low.

That the TPK activity of prostatic epithelial cells in culture is related to their proliferation is suggested by its higher level, 31 ± 14 pmol/min/10^6 cells, compared to those activities measured in freshly isolated cells which vary from 2.7 ± 2.0 pmol/min per 10^6 secretory epithelial cells (normal glands) to 7.6 ± 5.3 pmol/min per 10^6 non-secretory epithelial cells (metaplastic prostates) [27]. In human prostatic carcinoma cell lines, it has been reported that DU145 cells which grow more rapidly than LNCaP cells also have a higher TPK activity [32].

The association of the TPK activity of cultured prostatic cells with the particulate fraction supports the hypothesis that its interaction with the plasma membrane may play a role in the regulation of their division. It is well established that TPKs expressed as receptors for growth factors at the cell surface or coded by several oncogenes must be associated with plasma membranes in order to transmit the mitogenic signals of growth factors or transform cells [30]. From the present data, it is suggested that the portion of TPK activity (30%) readily extracted with Triton X-100 represents the enzyme anchored in the membrane while the re-

maining activity, solubilized with a combination of Triton X-100 and KCl, is likely to be retained by both hydrophobic and electrostatic forces within other structures.

In view of the fact that some GF receptors have the propensity to form large Mr aggregates [33,34] and that in certain other systems, soluble TPKs of low Mr may associate with other cell surface proteins [35], it is tempting to propose that in dividing cultured prostatic epithelial cells, TPK appears as a high molecular weight complex at the membrane level either by association with other proteins or by self-association. Whether the proliferation of prostatic epithelial cells involves a change in the distribution of a pre-existing TPK or is due to the induction of a new enzyme is currently under investigation.

ACKNOWLEDGMENTS

The secretarial assistance of C. Plante and M. Boire is gratefully acknowledged. C.B. received a Studentship from the Cancer Research Society Inc. and the University of Montreal. Y.D. and S.C. hold a Studentship and a Scholarship, respectively, from Le Fonds de la Recherche en Santé du Québec. This research was supported by the Medical Research Council of Canada, Le Fonds pour la Formation de Chercheurs et l'aide à la Recherche of Quebec, and CEDAR of the University of Montreal.

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