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ENHANCED EXPRESSION OF MEMBRANE PHOSPHOPROTEINS TYROSINE PHOSPHORYLATION IN ESTROGEN-INDUCED KIDNEY TUMORS

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Summary: We demonstrate for the first time that the expression of tyrosine containing membrane phosphoproteins is elevated in estrogen-induced kidney tumors, which is evident from both the types of experiments, i.e., alkali-resistant phosphorylation of membrane proteins and immunoprecipitation of tyrosine containing phosphoproteins. Tyrosine phosphorylation of proteins or peptides was modulated by the growth factors (EGF, IGF-I) and by the inhibitors of tyrosine protein kinase(s). The kinetic analyses revealed that tumor membranes have high affinity and catalytically more efficient tyrosine phosphorylating kinase enzyme(s) compared to that of normal membranes which have low affinity and catalytically less efficient kinase enzyme(s). It is proposed that overexpression of tyrosine containing membranal phosphoproteins may be involved in the induction and growth of estrogen-induced renal neoplasm.

Chronic exposure to estrogens leads to the development of neoplasms at various organ sites in animals as well as humans (1-3). The mechanism of estrogen-induced carcinogenesis is not clear. Phosphorylation of oncoproteins, particularly retroviral oncogene products, at tyrosine residues by protein kinases have been shown to play a critical role in the control of cell growth and cell transformation (4). Membrane receptors for a variety of growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), and insulin like growth factor-1 (IGF-1), have been shown to possess protein tyrosine kinase activity (5). Enhanced expression of membrane receptor protein kinase has been shown to correlate with the stimulation of cell proliferation (4,5). Increased protein phosphorylation at tyrosine residues in

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chemical-induced hepatic tumors has been reported (6) and it was suggested that activation of phosphoprotein is associated with chemical hepatocarcinogenesis initiation. Whether this type of phosphorylation is also enhanced in hormone-induced tumors and plays a role in hormonal carcinogenesis induction is unclear.

In the present study we have examined the possibility of enhanced expression of membrane phosphoproteins which were phosphorylated by protein tyrosine kinase(s) in diethylstilbestrol (DES)-induced kidney tumors in Syrian hamsters. This model of hormonal carcinogenesis has been used in the present work because (i) DES treatment to hamster for 7-9 months induces almost 100% tumors (3), (ii) DES acts as both tumor initiating and promoting agent (iii) and growth of tumor is estrogen-dependent (3). In this study we have investigated the expression and characteristics of membrane by protein tyrosine kinases in DES-induced tumors and in untreated control kidneys.

MATERIALS AND METHODS

DES, N-ethylmaleimide, quercetin, Poly (Glu,Na-Tyr) 4:1 (PGT), and monoclonal antiphosphotyrosine-agarose were purchased from Sigma. Agarose wheat germ lectin was purchased from Pharmacia. [γ-^{32}P]ATP (specific activity 4500 Ci/mmol) was obtained from ICN Biomedical. All other chemicals were of analytical grade.

Tumors in male Syrian hamsters (6-8 wks from Harlan Sprague Dawley, Houston, TX) were induced by s.c. implant of DES (7). Kidneys from treated hamsters were separated into tumor and tumor free tissues (surrounding the tumor). Plasma membranes were prepared (8). Partial purification of phosphoproteins from solubilized membranes was carried out using agarose wheat germ lectin (9).

Protein tyrosine kinase activity was assayed at room temperature by using poly PGT as a phosphorylatable substrate (10). The membrane autophosphorylation was performed as described by Cooper et. al. (11). The samples were fractionated on 9.5% SDS-PAGE. The gels were treated with 1 N KOH at 55°C for 2 hr. Gels were neutralized, dried under vacuum and autoradiography was performed with Kodak X-Omat™ AR-5 films with intensifying screens at -70°C. The immunoprecipitation was performed as described previously (12). Samples were treated with an equal volume of monoclonal anti-phosphotyrosine-agarose overnight at 4°C. The immunoprecipitated proteins were fractionated on 9.5% SDS-PAGE followed by autoradiography as described above.

RESULTS

Characterization of the optimal conditions for protein tyrosine kinase activity revealed that the protein tyrosine kinase enzyme present in both normal kidney and tumor membranes is dependent on the concentration of substrate, membrane proteins, incubation period and the concentration of cofactors $MgCl_2$ and $MnCl_2$ (Fig. 1-4). Using the optimal conditions, phosphorylating potential of enzyme(s) present in the

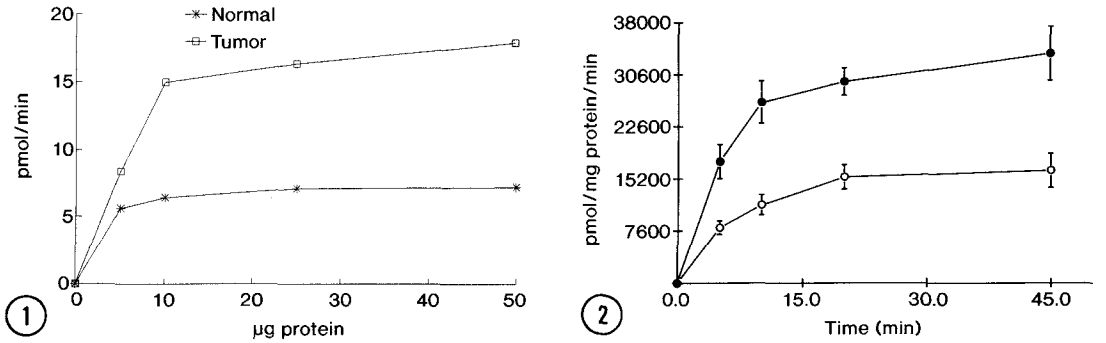


Figure 1. Effect of increasing concentrations of membrane proteins on the activity of protein tyrosine kinase. Each value is a mean of 2-3 measurements.

Figure 2. Effect of incubation time on the activity of protein tyrosine kinase activity. Each value is a mean of 2-3 measurements. Open circle represents the data obtained form control kidney and closed circle represents the data obtained from kidney tumors.

membranes of normal kidney, surrounding kidney tissue, and tumors was compared by using PGT as a substrate. Tumor membranes had 50 and 100% more phosphorylating activity than surrounding tissue and normal kidney membranes, respectively (Table 1). Tissue surrounding kidney tumor showed significantly higher phosphorylating activity than that of normal kidney.

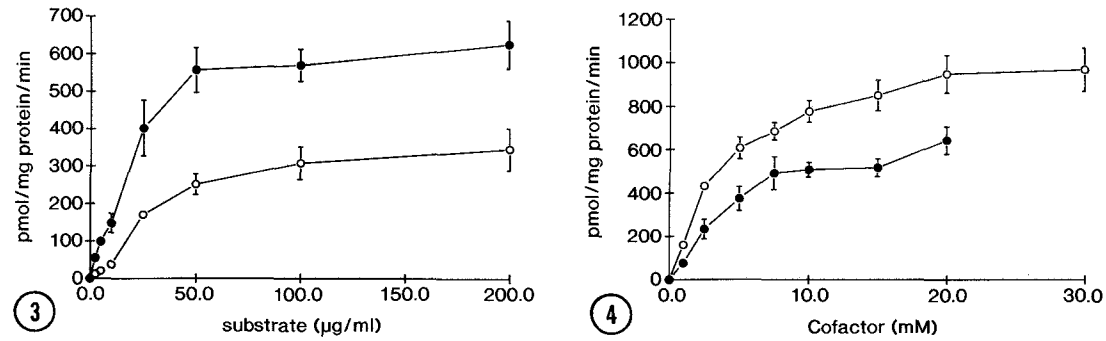


Figure 3. Effect of increasing concentrations of substrate on the activity of protein tyrosine kinase activity. Each value is a mean of 2-3 measurements. Open circle represents the data obtained from control kidney and closed circle represents the data obtained from kidney tumors.

Figure 4. Effect of various concentrations of $MgCl_2$ or $MnCl_2$ on the activity of normal kidney membrane protein tyrosine kinase activity. Each value is a mean of 2-3 measurements. Open circle represents the data obtained in the presence of $MgCl_2$ and closed circle represents the data obtained in the presence of $MnCl_2$.

TABLE 1

Protein Tyrosine Kinase Activity in normal and kidney tumor membranes

Conditions	No. of animals	No. of animals bearing tumors	pmol/min PTK activity
Control	8	0	738 ± 54
Surrounding tissues[a]	10		920 ± 66*
Tumor	10	10	1456 ± 121**

[a]Grossly visible tumor free tissues are called surrounding tissue. Significant * at $P < 0.05$; ** $P < 0.02$. PTK = Protein tyrosine kinase.

Kinetic analyses revealed that normal kidney membrane protein tyrosine kinase(s) has a Km = 114 ug/ml and Vmax = 440 pmol/mg protein/min. The corresponding values for tumor membrane enzyme were Km = 29 ug/ml and Vmax = 880 pmol/mg protein/min (figure 5 and table 2). The efficiency of catalyzing the phosphorylation reaction by tumor membrane enzyme(s) was 10-fold higher than that of normal kidney membrane enzyme(s) (catalytic efficiency of enzyme = 3, 30 for normal kidney and tumor enzyme(s), respectively). Thus, kinetic analyses revealed that tumor membranes have high affinity and catalytically more efficient phosphorylating enzyme(s) compared to that of normal kidney membranes which have low affinity and catalytically less efficient enzyme(s).

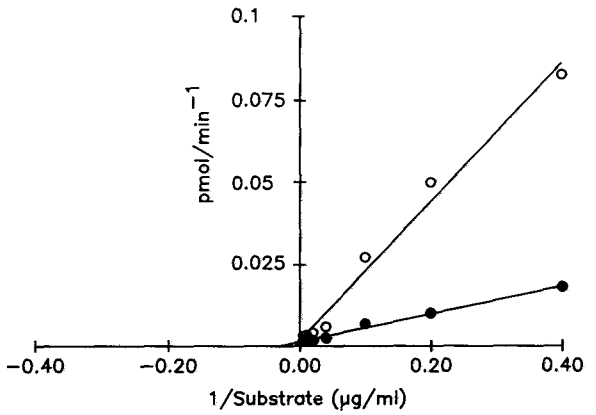


Figure 5. A double reciprocal plot of the phosphorylation of exogenous substrate by membrane fractions from normal kidney (open circle) and kidney tumors (closed circle).

TABLE 2

Kinetic analysis of phosphorylation of exogenous substrate poly (Glu, Na-Tyr) by normal kidney and tumor membrane protein tyrosine kinase(s)

Kinetic Constants	Normal Membranes	Tumor membranes
Km (μg/ml)	114	29
Vmax (pmol/mg protein)	440	880
KCat	3	30

Phosphorylation of PGT by partially purified phosphoprotein from both normal kidney and tumor membranes was significantly enhanced in a dose-dependent fashion in the presence of EGF or IGF-I (Table 3). N-ethylmaleimide and quercetin, known inhibitors of protein tyrosine kinases, inhibited phosphorylation of PGT catalyzed by normal kidney and tumor membrane protein tyrosine kinase(s) in a dose-dependent fashion (Table 4).

Normal kidney membranes revealed at least six major alkali-resistant proteins of Mw approximately 190, 180, 125, 105, 66 and 60 KDa (Fig. 6). In tumor membranes phosphoproteins corresponding to that observed in normal kidney tissue membranes were also present, however, their levels were drastically elevated in the

TABLE 3

Effect of IGF-I and EGF on the activity of WGA-purified normal and estrogen-induced kidney tumor membrane protein tyrosine kinase(s)

Effector	% Control Activity	
	Normal	Tumor
IGF (nM)		
0	100	100
1	121	148
10	142	220
100	158	280
EGF (nM)		
0	100	100
1	117	238
10	156	484
100	254	544

Each value is percent mean of 3-4 experiments.

TABLE 4

Influence of inhibitors on normal and tumor membrane protein tyrosine kinase activity

	% Inhibition	
	Normal	Tumor
N-ethylmaleimide		
0 mM	0	0
1 mM	32	42
10 mM	59	78
Quercetin		
0 μM	0	0
100 μM	4	13
500 μM	35	41

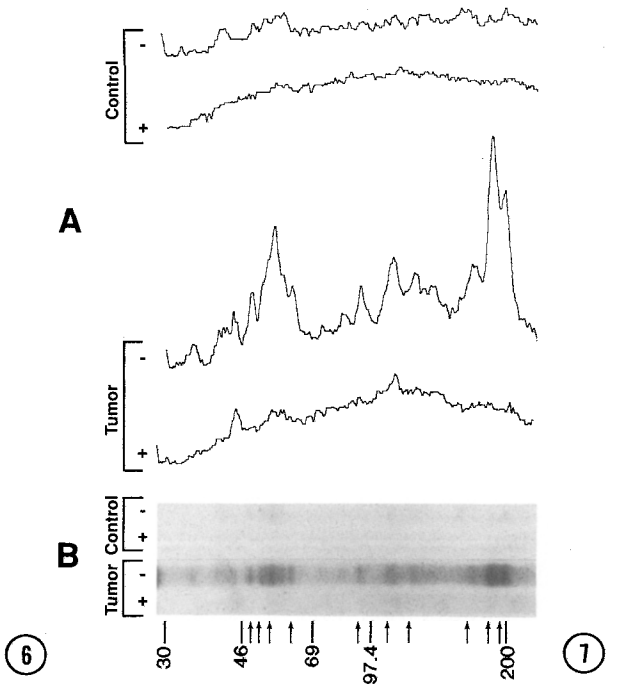
Activity in the absence of inhibitor was taken as a 100%. Each value is percent mean of 3-4 experiments.

tumor membranes (Fig. 6). Addition of protein kinase inhibitor quercetin inhibited the autophosphorylation of both normal kidney and tumor membranes (Fig. 6).

Immunoprecipitation with the monoclonal antibody of phosphotyrosine revealed that six common tyrosine containing phosphoproteins are present in both normal kidney and tumor membranes (Fig. 7), which is in agreement with the results obtained by alkali-resistant autophosphorylation studies. The levels of these common proteins in tumor membranes were very high than that found in control kidney membranes. Among these 190, 180, and 66 KDa proteins were about 5 fold more phosphorylated in tumor membranes as compared to that of normal kidney membranes. The phosphorylation on tyrosine residues on other proteins (i.e., 125, 105, and 60 KDa) was 2-3 fold higher than that found in normal kidney membranes (figure 7).

DISCUSSION

Present study demonstrated for the first time that the expression of tyrosine kinase containing membrane phosphoproteins is elevated in DES-induced kidney tumors, which is from all the three types of experiments i.e., phosphorylation of exogenous substrate, alkali-resistant phosphorylation of membrane proteins and immunoprecipitation of tyrosine containing phosphoproteins. The kinetic analyses revealed that the tyrosine phosphorylating enzyme(s) in tumor membranes has high affinity and catalytically more efficient than that of normal kidney membranes which has low affinity and catalytically less efficient kinase activity.



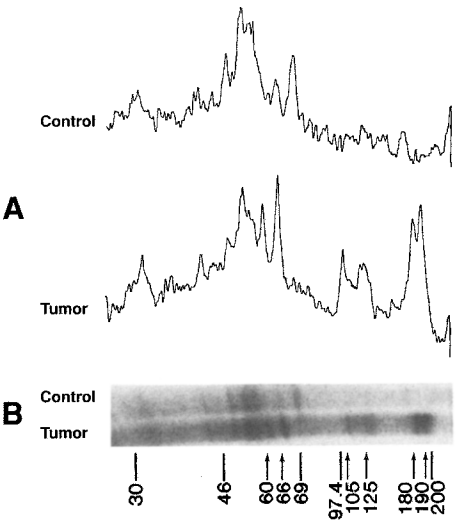


Figure 6. Panel A is autoradiograph of alkali-resistant phosphoprotein labeled by protein tyrosine kinase(s) from normal kidney and tumor membrane fraction after SDS-PAGE. Panel B is scanning of autoradiogram shown in panel A. Plus represents the membrane phosphorylation in the presence of 500 μM quercetin, whereas minus indicates the absence of quercetin. Phosphoproteins are indicated by arrows.

Figure 7. Autoradiograph of phosphoprotein labeled by protein tyrosine kinase(s) form antiphosphotyrosine antibody precipitated extract from normal kidney and tumor membranes after SDS-PAGE. Panel B is scanning of autoradiogram shown in panel A. Phosphoproteins are indicated by arrows.

Phosphorylation of proteins catalyzed by protein tyrosine kinases play an important role in proliferation of both normal and neoplastic cells and in retroviral- and chemical-induced cell transformation (4,6). Both initiation and promotion of estrogen-induced neoplasm require cell proliferation. Elevated expression of tumor membrane protein kinases resulting in an increased phosphorylation of tyrosine specific phosphoproteins may play an important role in estrogen-induced carcinogenesis.

Estrogens can increase the expression of several protein tyrosine kinase containing oncoproteins and receptors of growth factors in human breast tumor cells (13). Human breast tumor tissues have been reported to possess higher levels of protein tyrosine kinases (14). Recently, estrogen-induced kidney tumors have shown to have higher level of c-fos, c-myc and c-jun oncogenes (15). It is therefore possible

that the phosphoprotein in tumor membranes are non-receptor proteins i.e., oncogene products. Furthermore, the overexpression of cellular onco-phosphoprotein viz., src family oncoproteins or receptors of growth factors, have been reported to induce cell transformation (4,5). Stimulation of phosphorylation of substrate by growth factors, EGF and IGF-I, in both normal and tumor kidney membranes was observed in this study. It is therefore possible that estrogen treatment causes overexpression of onco-phosphoprotein and/or growth factor receptor proteins in the hamster kidney, and this in turn plays a critical role in the hormone-induced renal carcinogenesis. This possibility is consistent with the observation that phosphoproteins levels were enhanced in surrounding tissues of estrogen treated hamsters.

Alternatively, the increased expression of protein tyrosine phosphorylation of kidney tumor membranes may not play a causative role in tumorigenesis. It is known that hamster kidney tumors require estrogen for continued growth (3). Thus, the enhanced levels of phosphoprotein in tumor membranes may simply be required for the stimulation of tumor growth by estrogen but may not be a primary carcinogenic event(s) produced by the estrogen. However, the increase in the levels of phospho-protein in surrounding kidney tissue from estrogen treated hamsters argues against this hypothesis. Studies in our laboratory are in progress to ascertain the role of these phosphoprotein in initiation and promotion of hormone-induced carcinogenesis.

Our findings are significant because the results demonstrate for the first time that the expression of tyrosine containing phosphoproteins is elevated in estrogen-induced kidney tumors, which is one of the major animal models of hormonal carcinogenesis. Consequently, our results provide specific markers for evaluating the similarities and differences of estrogen-induced hamster tumors with human and other animal cancers on the basis of tyrosine containing phosphoprotein expression. However, further studies are needed to elucidate the relationship between the molecular basis of protein tyrosine kinase activation, the expression of various membrane phosphotyrosine containing proteins and hormone-induced renal carcinogenesis.

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