Comparison of tyrosine phosphorylation of proteins by membrane fractions from mouse liver, Ehrlich ascites tumor and MH134 hepatoma

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When membrane fractions from mouse liver, Ehrlich ascites tumor and MH134 hepatoma were incubated with [γ-^{32}P]ATP at 0°C in the presence of $MnCl_2$, $ZnCl_2$ and $NaVO_3$, proteins were phosphorylated on tyrosines to a larger extent in liver membranes than in tumor membranes. Separation of labelled proteins by SDS-gel electrophoresis showed phosphorylated alkali-resistant bands of 170, 140, 130, 80, 56, 53 and 46 kDa proteins in Ehrlich ascites tumor membranes; liver membranes exhibited more strongly phosphorylated bands of 170, 56, 53 and 46 kDa proteins. Epidermal growth factor stimulated the tyrosine phosphorylation of only a 170 kDa protein, which was more significant in liver membranes. Liver membranes exhibited slightly higher levels of tyrosine protein kinase activity compared to tumor membranes.

Membrane protein *Phosphorylation* *Phosphotyrosine* *EGF receptor* *Cancer*

1. INTRODUCTION

The tyrosine phosphorylation in proteins is known to be catalyzed by specific tyrosine protein kinases which have been found to reside in oncogene products [1] and membrane receptors of various growth factors [2–5]. Although the exact physiological significance of the tyrosine phosphorylation is not known, the reaction has been suggested to correlate with cell transformation and proliferation [6,7]. To find a clue as to the roles of the reaction in the control of cell transformation, phosphorylation of tyrosine in endogenous proteins and exogenous tyrosine-glutamate copolymers by membrane fractions from mouse liver and ascites tumor cells was compared.

Abbreviations: EGF, epidermal growth factor; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. EXPERIMENTAL

Membrane fractions (P2 fractions) were prepared from male *ddy* and C3H mouse livers as in [8] except that all buffer solutions contained 1 mM EDTA and 0.5 mM PMSF. Ehrlich ascites tumor and MH134 hepatoma cells were inoculated intraperitoneally to *ddy* and C3H mice, respectively. The tumor cells were washed 3–4 times with 0.137 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 0.84 mM Na_2HPO_4, 0.5 mM PMSF, 1 mM EDTA (pH 7.4), and cells were homogenized with 5 vols of 0.02 M boric acid, 0.5 mM PMSF and 1 mM EDTA (pH 10.2) in a Dounce homogenizer (loose-fitting, 60 strokes). Membrane fractions were prepared from the homogenate as in [9]. The membrane fractions were suspended in 20 mM Hepes, 1 mM EDTA and 0.5 mM PMSF (pH 7.4) (7–12 mg protein/ml) and stored at −80°C and used within a week. Judging from the increase in the specific activity of 5′-nucleotidase, plasma membranes were purified 10-fold from the

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homogenates. Electron microscopic examination of the membrane fractions and measurements of marker enzymes indicated that the fractions contained primarily vesicular elements derived from the plasma membrane and smooth endoplasmic reticulum and little nonmembranous material.

For phosphoamino acid analysis, membrane phosphorylation with [γ-^{32}P]ATP (8–20 × 10^3 cpm/pmol) was carried out in 0.42 ml of the mixture as in fig.1 and stopped by making the mixture 25% in trichloroacetic acid. The insoluble material was collected by centrifugation, washed with ethanol-ether (1 : 1) and chloroform-methanol (2 : 1), and dried. The phosphorylated 170-kDa protein was extracted from the gel after SDS-PAGE as in [10] except that 2-mercaptoethanol treatment was omitted. The dried samples were suspended in 0.3 ml of 0.05 M NH_4HCO_3 and digested with trypsin (1/10 (w/w) of protein) for 2 h at 30°C. Acid hydrolysis in the presence of 4 mM phenol and analysis of hydrolysates by electrophoresis on Whatman 3MM paper at pH 3.5 for 70 min at 3 kV followed by ascending paper chromatography were performed as in [11]. Each ninhydrin spot of phosphoamino acid standards was excised, destained as in [12] and the ^{32}P count measured by Cerenkov radiation. The breakdown of phosphoserine (24.7%), phosphothreonine (6.3%) and phosphotyrosine (52.3%) during the hydrolysis, was taken into account in the determination of the relative percent of these phosphoamino acids.

Tyrosine protein kinase was assayed in 60 μl mixture containing 20 mM Hepes (pH 7.4), 3 mM $MnCl_2$, 10 μM $ZnCl_2$, 30 μM $NaVO_3$, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, 0.1 mg/ml

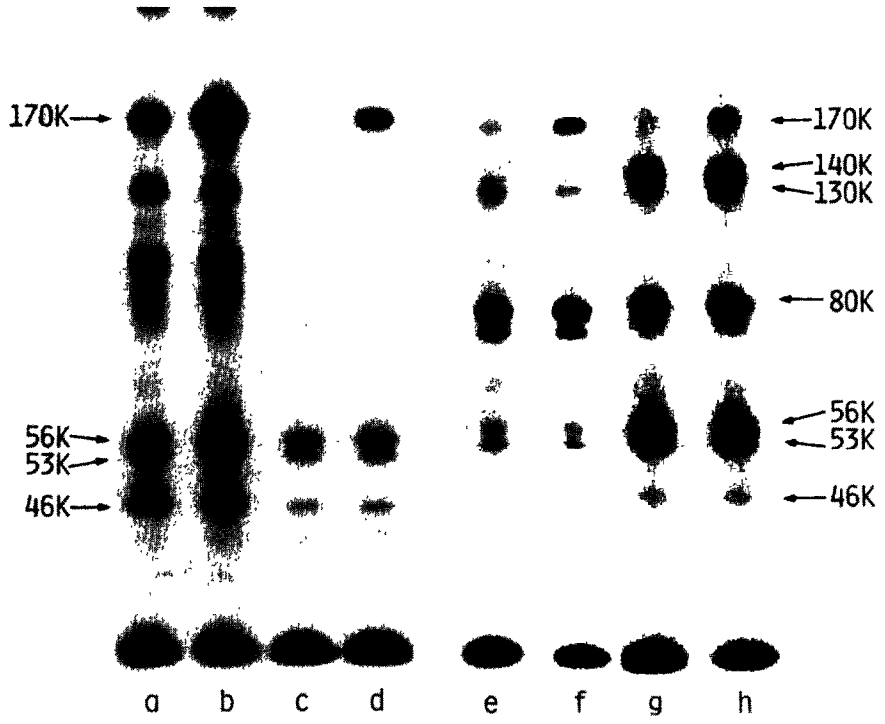

Fig.1. Phosphorylation of membrane proteins from *ddy* mouse liver (a–d) and Ehrlich ascites tumor (e–h). Phosphorylation was carried out at 0°C for 10 min in a 60 μl mixture containing: 20 mM Hepes (pH 7.4), 1 mM $MnCl_2$, 10 μM $ZnCl_2$, 30 μM $NaVO_3$, 50 μg membrane protein and 15 μM [γ-^{32}P]ATP (3 × 10^3 cpm/pmol) in the absence (a, c, e, g) or presence (b, d, f, h) of 0.22 μM EGF. Before adding [γ-^{32}P]ATP, the rest of the mixture (40 μl) was incubated at 0°C for 10 min. The reaction was stopped and applied to SDS-PAGE as in [16]. The gels were stained with Coomassie blue, dried and autoradiographed before (a, b, e, f) and after (c, d, g, h) alkali treatment [17]. Exposure times were 3 days (a, b, e, f), 7 days (c, d) and 21 days (g, h) with an intensifying screen at −80°C.

tyrosine-glutamate (1:4) copolymers (M_r 28000, Sigma) [13], 15 μM [γ-^{32}P]ATP (800 cpm/pmol), and 1.4 μg protein of membrane fractions. After 10 min at 30°C, a 50 μl aliquot was spotted on Whatman 3MM paper, and further processed as in [14]. The control value without substrate was subtracted from the complete value.

3. RESULTS AND DISCUSSION

When membrane fractions were incubated with [γ-^{32}P]ATP at 0°C for 10 min in the presence of $MnCl_2$, $ZnCl_2$ and $NaVO_3$, 15–25 pmol of P_i was incorporated per mg of membrane protein with mouse liver and 8–16 pmol with tumor cells (table 1). Phosphoamino acid analysis revealed that 66–78% of the P_i incorporated into mouse liver membrane proteins and 28–38% of P_i incorporated into tumor membrane proteins were recovered as phosphotyrosine. Ten times more P_i was incorporated into tyrosines in *ddy* mouse liver membrane proteins than in Ehrlich ascites tumor membrane proteins. Little difference in the amount of P_i incorporated into serine and threonine was observed in these same fractions. Also, more tyrosine phosphorylation was observed in C3H liver membranes than in MH134 hepatoma membranes. It is not due to procedural differences in preparation of the membranes, since *ddy* mouse membranes prepared by the same procedure as for Ehrlich ascites tumor membranes also showed greater tyrosine phosphorylation (not shown). It is also not due to the differences in the dephosphorylation of phosphoproteins. $^{32}P_i$ release from the phosphorylated proteins was measured by adding 100 μM cold ATP after phosphorylation of the membrane proteins with 5 μM [γ-^{32}P]ATP at 0°C for 5 min. Not more than 7% of the incorporated ^{32}P was released at 0°C for 5 min from membrane proteins of either *ddy* mouse liver or Ehrlich ascites tumor. The lower tyrosine phosphorylation in the tumor membranes is probably not due to the high content of endogenous phosphotyrosines. Since alkaline

Table 1

Phosphorylation and phosphoamino acid analysis of membrane proteins

Membrane	Addition	P_i incorporated into protein (pmol/mg protein)	P_i incorporated (pmol/mg protein) into		
			P-Serine	P-Threonine	P-Tyrosine
Liver (*ddy*)	None	24.6 ± 0.1	3.73 ± 0.61 (15)	1.60 ± 0.06 (7)	19.25 ± 0.75 (78)
Liver (*ddy*)	EGF	35.0 ± 6.9	3.93 ± 0.72 (11)	1.11 ± 0.15 (4)	30.00 ± 6.40 (85)
Liver (C3H)	None	14.5 ± 0.9	4.21 ± 1.70 (28)	0.80 ± 0.06 (6)	9.50 ± 0.80 (66)
Liver (C3H)	EGF	24.7 ± 1.9	3.29 ± 1.16 (13)	0.68 ± 0.09 (3)	20.78 ± 0.82 (84)
Tumor (Ehrlich)	None	8.07 ± 0.50	4.04 ± 0.25 (50)	1.79 ± 0.15 (22)	2.24 ± 0.09 (28)
Tumor (Ehrlich)	EGF	9.43 ± 1.16	3.61 ± 0.28 (39)	2.12 ± 0.56 (22)	3.70 ± 0.33 (39)
Tumor (MH134)	None	16.4 ± 4.4	8.03 ± 1.91 (49)	2.24 ± 0.98 (13)	6.14 ± 1.49 (38)
Tumor (MH134)	EGF	19.1 ± 4.1	8.98 ± 2.18 (47)	2.69 ± 0.63 (14)	7.44 ± 1.29 (39)

Phosphorylation and SDS-PAGE of membrane proteins were carried out as in fig.1. After staining, all protein bands in a gel were excised and the ^{32}P count was measured. Phosphoamino acid analysis was performed as described in section 2. The values in parentheses indicate relative percent of phosphorylated serine, threonine and tyrosine in the sample. The average with the range of values for two separate experiments is given

phosphatase effectively removes P_i from tyrosine hydroxyl of proteins at neutral pH [15], these membrane fractions (50 μg proteins) were incubated with alkaline phosphatase (0.25 μg, Sigma type VII-S) at 30°C for 10 min in 60 μl of 20 mM Hepes (pH 8.0) (this treatment removed 50–60% $^{32}P_i$ from prelabelled membrane fractions). After removing the phosphatase by centrifugation followed by washing, the treated membranes were phosphorylated with [γ-^{32}P]ATP under the conditions as described in fig.1 in the absence of EGF. The alkaline phosphatase treatment increased the phosphorylation of membrane proteins by only 5–10% with either liver or Ehrlich ascites tumor membranes. Since the P_i incorporation reached a plateau and at least 30% of the added [γ-^{32}P]ATP remained unhydrolyzed under these conditions, these differences in tyrosine phosphorylation may reflect the availability of tyrosines in these membrane preparations.

The phosphorylated membrane proteins were next applied to SDS-PAGE (8% acrylamide) to determine whether or not liver and ascites tumor cells have distinct tyrosine phosphoproteins. Since the phosphotyrosyl bond is more stable to alkali treatment than the phosphoseryl or phosphothreonyl bonds [17], gels were treated with 1 M KOH for 2 h at 55°C and the remaining radioactive protein bands were detected as tyrosine phosphoproteins by autoradiography (fig.1). In *ddy* mouse liver membranes, protein bands of 170, 56, 53 and 46 kDa seemed to be phosphorylated at tyrosine. Molecular masses of proteins which contained alkali-stable phosphate and migrated near the dye front, were determined to be 20, 11 and 10 kDa by SDS-PAGE (15% acrylamide) (not shown). In addition to these bands, other alkali-stable bands of 140, 130 and 80 kDa were detected in Ehrlich ascites tumor membranes. ^{32}P counts in these alkali-stable bands were about 10 times higher in liver membranes than in tumor membranes.

Incubation of these membranes with EGF increased the P_i incorporation by 42–71% with liver membranes and by 17% with tumor membranes. Phosphoamino acid analysis indicated that only the phosphorylation of tyrosine was significantly increased by EGF (table 1).

The EGF pretreatment caused an increase in the phosphorylation of a 170-kDa protein (p170) without significant increases in the phosphorylation of other proteins (fig.1). Densitometric analysis of the autoradiograms of KOH untreated gels showed that EGF stimulated p170 phosphorylation of liver membranes 4–7-fold and of tumor membranes 3-fold. Mouse liver p170 has been known to be an EGF receptor which is autophosphorylated at tyrosines [18], so that Ehrlich tumor p170 was extracted from a KOH untreated gel and the phosphoamino acids were analyzed. Relative percents of $^{32}P_i$ incorporated into serine, threonine and tyrosine were 29, 5 and 66%, respectively. These results suggest that the p170 in the tumor membranes is also an EGF receptor.

Tyrosine protein kinase activities (pmol/min per mg protein, mean ± SD) towards tyrosine-glutamate (1:4) copolymers were 229 ± 94 (*ddy* liver), 285 ± 40 (C3H liver), 185 ± 16 (Ehrlich ascites tumor) and 162 ± 57 (MH134 tumor). The enzyme activity was not affected by 0.22 μM EGF. The slightly higher activity in liver membranes than in tumor membranes may reflect in part on the differences in the amount of tyrosine phosphorylation of these membrane proteins.

The lower levels of tyrosine phosphorylation in tumor membranes than liver membranes support the notion that increased phosphorylation of tyrosine, which has been observed in transformed cells after Rous sarcoma virus infection [11], is neither a universal mechanism of transformation nor an inevitable secondary cellular response to transformation [19]. Tyrosine protein kinase activity towards a synthetic tyrosine-containing peptide, has been found to be markedly lower in the leukemic cell lines and in proliferating normal bone marrow cells than in normal lymphocytes [20]. High percentages of phosphorylated tyrosine were also found in the particulate fractions from non-proliferating anuclear cells, platelets and red blood cells [21].

These results implicate that a high phosphotyrosine content in membranes, at least after in vitro phosphorylation, is not necessarily associated with cancer.

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