MEMBRANE PHOSPHATASE ACTIVITY IN SOMATIC CELL HYBRIDS

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SUMMARY The dephosphorylation of membrane proteins by an endogenous phosphatase has been studied both in A9 and TLX5 cells and in hybrids between them. These cells differ both in growth rate and saturation density achieved in vitro. The activity of the phosphatase seems to parallel the growth rate of these cell lines. It is considered that this phosphatase is part of an ATPase enzyme system. The enzyme from TLX5 cells is stimulated by cyclic adenosine 3'5'-monophosphate (cAMP) and inhibited by zinc and fluoride ions. Prostaglandin E_1 (PGE_1) has been found to have no effect on the activity of the phosphatase.

The level of cAMP in a cell is determined by its rate of production by adenylate cyclase, its rate of breakdown by cAMP phosphodiesterase and its loss by leakage to the external medium. As adenylate cyclase is located in the plasma membrane it is ideally suited to receive the stimuli of density dependent inhibition of growth.

The stimulatory effects of fluoride on mammalian adenylate cyclase (1) have led to the suggestion that the enzyme exists in two forms, a less active or inhibited phosphorylated form and a more active dephosphorylated form (2). Conversion from the inactive to the active form is thought to occur either by direct nucleophilic displacement of phosphate by a hormone or fluoride ion, or by the action of a neighbouring phosphatase (3). Thus a possibility exists that membrane adenylate cyclase exists in the inactive phosphorylated form during logarithmic growth and is activated by a phosphatase on contact inhibition.

To investigate the possible involvement of phosphorylation in the control of cell growth and in the activation of adenylate cyclase, the activity of membrane phosphoprotein phosphatase has been measured both in A9 and TLX5 cells and in hybrids between them. The relationship between the growth characteristics of these cells and the activity of adenylate cyclase has been described (4).

METHODS

Cell culture Cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Cultures were gassed with 10% CO_2 in air. Cell lines were routinely passaged in Falcon plastic flasks, but cells were grown in 9cm plastic dishes for experimental purposes.

An inoculum of A9 cells was kindly supplied by Professor H. Harris. Lines of TLX5 lymphoma cells were established from CBA/LAC mice bearing routine passages of the tumour.

Hybridization The techniques used to induce cell fusion and to isolate hybrids will be described in a forthcoming publication (5). A9H/TLX was a clone isolated from a hybrid colony which arose after fusing A9 cells with TLX5 cells. A9H/TLX(tumour) was derived from the fourth passage of a transplanted A9H/TLX tumour grown in immune-deprived mice.

The karyotypic characterization of the parent and hybrid cells is to be published (5). Both types of hybrid cell possessed approximately the full chromosome complement of one A9 and one TLX5 cell.

Membrane phosphatase assay Cells were allowed to grow to confluence in plastic Petri dishes and removed with a rubber policeman. Cells (0.1ml) were suspended in 1ml of 40mM Tris-HCl, pH 7.2, containing 110mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.2% glucose, 0.3mCi of [^{32}P]inorganic phosphate (sp.act. 206mCi/mmole) and either with or without 1mM dibutyryl cAMP (db cAMP), and were incubated at 37° for 1h. After incubation, the cells were washed twice with 40mM Tris-HCl, pH 7.2 containing 110mM NaCl, centrifuging (300 x g for 3 min) between each wash. All subsequent operations were carried out at 4°. The cells were washed once more with 0.34 M sucrose, resuspended in the sucrose solution and homogenized in a Teflon-glass homogenizer. A membrane pellet was then sedimented by centrifugation at 600 x g for 3 min, after which it was resuspended and washed three times in 40mM Tris-HCl, pH 7.2 containing 1mM $MgCl_2$.

For the determination of phosphatase activity, membrane fragments (300-400µg of protein/ml) were suspended in 40mM Tris-HCl, pH 7.2, containing 10mM $MgCl_2$ at 37°. At various time intervals aliquots (400µl) of the membrane suspension were removed, centrifuged (3000 x g) for 10 min and 200µl of the supernatant was counted in 10ml of scintillation fluid (naphthalene, PPO and diMe POPOP, in ethanol, dioxan and toluene) in a liquid scintillation counter (Packard, Tricarb model 3375).

Protein was estimated by the method of Lowry et al. (6) using bovine serum albumin as standard.

RESULTS

Growth behaviour of cell lines in vitro Growth curves for the four cell lines used are shown in Fig. 1. TLX5 cells had the highest growth rate (doubling time 16 h) and reached the highest maximum density (11,000/mm^2). The cell number declined very rapidly after this density was achieved and most of the cells appeared to be dead after about 7 or 8 days. Medium removed from cultures at day 5 supported only one division of fresh TLX5 cells at low density. These results suggested that TLX5 cells were unresponsive to density dependent growth inhibition and continued dividing until the medium was exhausted or toxic.

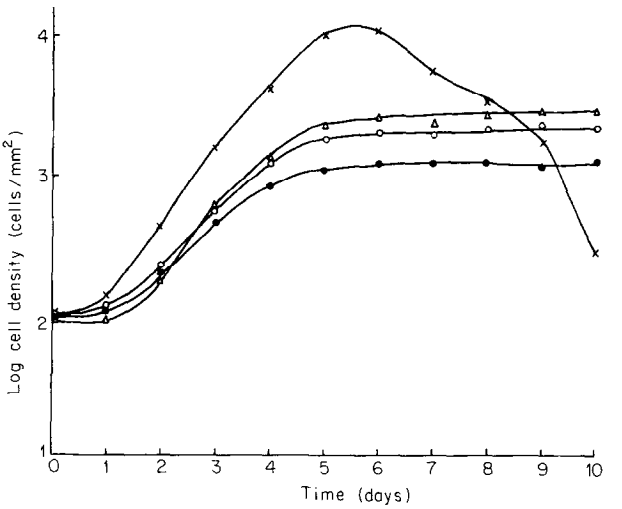

Fig. 1. Growth curves for TLX5, (x—x); A9, (o—o); A9H/TLX (o——o) and A9H/TLX(tumour) (Δ—Δ).

Cells were seeded in a large number of Petri dishes, at a density of 100 cells/mm^2. At daily intervals, a number of dishes from each series was trypsinised and the cells were counted. The data were used to construct growth curves.

In addition, the medium was removed from a number of dishes soon after they had reached maximum cell density and tested for its ability to support the growth of freshly seeded cells.

In marked contrast, A9 and the hybrid cells showed lower rates of division (doubling times of 19h for A9H/TLX(tumour), 21h for A9 and 24h for A9H/TLX), and lower maximum densities. All three cell lines exhibited an extended plateau phase, in which the cells remained viable for several weeks. A9H/TLX(tumour) reached a density of 2,600 cells/mm^2, which was slightly higher than A9 (2,200 cells/mm^2), and considerably higher than A9H/TLX (1,200 cells/mm^2). Medium removed from these cultures at day 7 supported a second complete cycle of culture growth from 100 cells/mm^2 to maximum density.

Characterization of [^{32}P]phosphate incorporated into membranes The effect of enzymatic digestions on the phosphorylated membrane components of the four cell types is shown in Table 1. In all cases, more [^{32}P]phosphate is released in the presence of pronase, than with trypsin or phospholipase C. In some

Table 1

Effect of enzyme digestions, hydroxylamine and organic solvent extraction on ^{32}P incorporated in cell membranes

Cell Type	Control (no treatment)	Trypsin	Pronase	Phospholipase C	Hydroxylamine	Ethanol - ether	Chloroform - methanol - HCl
TLX5	15	22	63	4	84	14	1
A9	18	29	41	21	95	19	2
A9H/TLX	22	23	84	21	95	16	2
A9H/TLX(tumour)	18	57	94	9	89	15	6

Phosphorylated membranes, prepared as described under 'Methods' were incubated for 30 min at 37° in 40 mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$ and 5 mM $ZnSO_4$ with the indicated enzyme (1 mg per ml). With pronase the incubation mixture also contained 10 mM $MgCl_2$ and with phospholipase C, 20 mM $CaCl_2$. Membranes were also incubated at 37° for 30 min in 100 mM sodium acetate buffer (pH 5.6) containing 1 M hydroxylamine. The samples were centrifuged and the radioactivity in the aqueous phase was determined by liquid scintillation counting. Extraction of lipids was carried out with 1.4 ml mixtures of ethanol - ether (1:1) and chloroform - methanol - HCl (20 : 10 : 0.1) and the radioactivity in the organic phase was measured.

cases less [^{32}P] is released in the presence of phospholipase C than is observed in untreated membranes. This may indicate that the phosphatase may require integral phospholipids for its action. In all cases almost 100% of the labelled phosphate is released from the membranes with 1M hydroxylamine. This indicates that the phosphate is associated with membrane proteins through labile acyl phosphate bonds and not through phosphoryl esters.

Extraction of the membranes with the organic solvents ethanol - ether (1:1) or chloroform - methanol - HCl (20:10:0.1) does not remove significant quantities of [^{32}P]. The nature of the extracted product was determined by two dimensional t.l.c. (7) when 90% of the radioactivity remained on the origin. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed the major phosphorylated product migrated to the cathode.

Membrane phosphatase activity Under the conditions described, the rate of dephosphorylation of endogenous substrates by membrane phosphatase was proportional to elapsed time and to membrane protein concentration. Table 2 shows the results of the incorporation of [^{32}P] from inorganic phosphate into the total membrane fraction of whole cells incubated with or without db cAMP,

Table 2. Rate of incorporation and liberation of radioactive inorganic phosphate from cell membranes labelled with ^{32}P

Cell Type	Treatment*	^{32}P incorporation pmole/mg protein/h	^{32}P release ± SEM pmole/mg protein/min x 10^3
A9	None	0.90	6 ± 0.5
	+	1.11	6 ± 0.5
A9H/TLX	None	0.84	3 ± 0.2
	+	0.84	3 ± 0.2
A9H/TLX (tumour)	None	2.11	18 ± 1.5
	+	1.86	9 ± 2
TLX5	None	6.4	28 ± 2
	+	10.4	72 ± 5

* + refers to incubation with ^{32}P inorganic phosphate in the presence of 1 mM db cAMP

and the unstimulated phosphatase activity of isolated membrane fragments of the four cell lines. The addition of 1mM db cAMP to the incubation medium caused a 63% stimulation of $[^{32}P]$ incorporation into TLX5 membranes, a 22% stimulation into A9 and a 12% stimulation into A9H/TLX(tumour). The incorporation of $[^{32}P]$ into A9H/TLX cell membranes was unaffected by cAMP.

When the four cell types were ranked in order of increasing rate of incorporation of phosphate into membranes, or of increasing rate of phosphate release, the order obtained was the same as that given when the cells were ranked in order of increasing growth rate.

Cyclic AMP causes an 80% increase in the rate of release of $[^{32}P]$ from TLX5 membranes as shown in Table 3. The effects of various ions on the activity of membrane phosphatase is also shown in Table 3. Thus, while $CaCl_2$ caused a slight stimulation of the enzyme, $ZnSO_4$ inhibited it almost completely, and NaF caused a 25% inhibition at a concentration of 5mM. PGE_1 had no effect at the concentration tested, neither did it reverse the slight stimulation produced by calcium ions.

DISCUSSION

Membrane phosphatase activity was measured after incorporation of $[^{32}P]$ phosphate by the intact cells. Enzymatic digestion studies showed that the major portion of the phosphate was incorporated into membrane proteins,

Table 3. Effect of various agents on the activity of membrane phosphatase from TLX5 lymphoma

Agent	Concentration	Relative Activity*
Cyclic AMP	1 mM	179
$CaCl_2$	1 mM	113
PGE_1	5 µM	100
$CaCl_2$ + PGE_1	1 mM + 5 µM	117
$ZnSO_4$	1 mM	19
NaF	5 mM	75

either in the presence or absence of db cAMP. Since 1M hydroxylamine removed 90% of the labelled phosphate from membrane protein, probably part of the phosphate is incorporated in the membrane as an acyl phosphate group. Protein-bound acyl phosphates are considered by many workers as intermediates in an ATPase reaction (8).

A four- to five-fold greater activity of Na^+-K^+-activated ATPase has been observed in transformed fibroblasts compared with the normal non-transformed cell line (9). However, the levels of ATPase in both normal and transformed cells have also been correlated with growth rate (10). The present results show that TLX5 cells (doubling time 16h) have a high endogenous phosphatase activity and that there is a decrease in phosphatase activity with decreasing growth rate such that A9/TLX(tumour) (19h) > A9 (21h) > A9H/TLX (24h).

A phosphoprotein phosphatase which is activated by fluoride and PGE_1 has been found in membrane preparations of polymorphonuclear granulocytes and blood platelets (2). Using membrane preparations from TLX5 lymphoma we have observed no activation of phosphatase activity with PGE_1. Fluoride ion caused a 25% reduction in the activity of this enzyme, which is similar to the observed 50% inhibition of protein phosphatases from cerebral cortex by 10mM NaF (11). A number of phosphoprotein phosphatases are inhibited by Zn^{2+} (11) in agreement with our result with TLX5 membrane phosphatase. cAMP has been shown to cause a two-fold stimulation of the activity of TLX5 phosphatase. This may be important physiologically in the regulation of the phosphorylation of adenylate cyclase.

For the cell lines TLX5, A9, A9H/TLX(tumour) and A9H/TLX there appears to be an inverse correlation between the activity of the membrane phosphatase and the activity of the adenylate cyclase. Thus the most malignant cell line, TLX5, which shows the highest phosphatase activity has both the lowest basal and fluoride stimulated enzyme activity (4). Therefore, the relationship between enzyme dephosphorylation and activity of the adenylate

cyclase would not appear to be indicated by the present studies.

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