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(^{32}P)Phosphoryl Transfer by Endogenous Protein Kinase at the Ehrlich Cell Surface into Extrinsic Acceptor Proteins

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ABSTRACT

The presence of protein kinase(s) at the outer surface of Ehrlich mouse ascites tumour cells has been established. Endogenous protein of the cell surface as well as extrinsic proteins such as phosvitin and histone can act as acceptors for the (γ^{32}P)-phosphoryl group of ATP. Phosvitin is much more effective as acceptor protein than either the endogeneous protein or the histone protein. The transfer of phosphoryl groups into the phosvitin molecule by the membrane-associated protein kinase is not further stimulated by cyclic AMP. The preferential transfer into phosvitin, rather than into histone, is discussed.

INTRODUCTION

We have published in a series of papers (3, 4, 5, 11) from our laboratory evidence for the presence of one or more protein kinase(s) (ATP: protein phosphotransferase, E.C.2.7.1.37) at the surface of the intact Ehrlich tumour mouse ascites cells. The (γ^{32}P)-phosphoryl group of ATP has been recovered in at least four different phosphorylated oligopeptides isolated after partial acid hydrolysis of the membrane material from Ehrlich cells. One of these oligopeptides consisted mainly of (^{32}P)phosphorylserine, another mainly of (^{32}P)phosphorylthreonine. From a complete amino acid analysis of a third oligopeptide we have reason to believe that this peptide consists mainly of (^{32}P)phosphoryloxyproline.

The experiments so far performed and reported have dealt with the endogenous protein kinase(s) of the external surface of the Ehrlich cell as well as endogenous acceptor protein(s) of the membrane.

However, rat liver plasma membrane has been reported to be phosphorylated in the presence of (γ^{32}P)ATP and an extrinsic protein kinase (12). This reaction was also claimed to be sensitive to cyclic AMP. Furthermore, in a recent paper (7) the enzymatic phosphorylation of surface proteins of HeLa cells using an exogenous protein kinase and (γ^{32}P)ATP has been described.

In the present paper we report results from an experimental system with endogenous protein kinase(s) from the intact cell surface of the Ehrlich cell and an extrinsic acceptor protein in the presence of (γ^{32}P)ATP in the external incubation medium.

MATERIAL AND METHODS

The Ehrlich mouse ascites tumour cells were grown for 7–8 days in 5-week-old Swiss albino mice obtained from the Anticimex breeding farm, Norrviken, Stockholm. The tumour cells were separated by centrifugation of the ascitic fluid which had been diluted without delay severalfold with ice-cold Krebs-Ringer bicarbonate medium in order to diminish the tendency to cell agglutination. The cells were washed twice in the Krebs-Ringer bicarbonate medium. Incubation at 37°C followed immediately after preparation of cells in order to maintain cell integrity. Under the experimental conditions used the cells can be maintained at 37°C for up to 3 hours with glucose supplementation without any visible change in cellular function, as measured by trypan blue uptake and tendency to agglutination (6).

For incubation, the cells were suspended in a buffered isotonic medium, pH 7.5, containing 130 mM NaCl and 25 mM KCL and with a final cytocrit value of 10%. The incubation medium also contained 350 μmoles of Tris-acetic acid buffer, pH 7.5, 5 μmoles of glutathione (reduced form), 20 μmoles of $MgCl_2$, 10 μmoles of Na_2HPO_4 (unlabelled), 0.5 μmoles of (γ^{32}P)ATP (sodium form). In addition, 2.5 μmoles of either histone or phosvitin was included in some experiments with or without 1×10^{-5} M of cyclic AMP.

In order to exclude any contamination of protein kinase in the commercial histone and phosvitin preparations, experiments were performed in the same incubation medium, minus Ehrlich cells.

Incubation was terminated at 1 min by a rapid centrifugation. The entire centrifugation procedure did not exceed 30 sec. The supernatant representing the external medium including the acceptor protein in most experiments was immediately precipitated with trichloroacetic acid (10% final concentration). In order to obtain a complete precipitation of the low molecular weight acceptor protein, 50 mg of bovine albumin had been added to the precipitating agent. Concomitantly the cell pellets were precipitated with the trichloroacetic acid.

The precipitation was allowed to proceed for at least 48 hours. The precipitates were washed once with 10% trichloroacetic acid containing 7 and 2 μmoles, respectively, of unlabelled orthophosphate and ATP.

In order to remove nucleic acid and phospholipids the precipitate was treated according to Schneider (13). Partial hydrolysis of the Schneider protein was performed using the method of Lipmann (9). Labelled phosphopeptides were isolated by ion exchange chromatography as previously described (4).

All chemicals were of analytical grade, (γ^{32}P)ATP was purchased from NEN Chemicals, GmbH, Frankfurt am Main, Germany.

Unlabelled ATP (sodium form), cyclic AMP, histone (Type II-A) and phosvitin were all obtained from Sigma Chemical Company, St. Louis, Mo., USA.

RESULTS

Table I illustrates the phosphoryl transfer into the extrinsic acceptor protein in the presence and absence of 1×10^{-5} M cyclic AMP. In addition, one series of experiments was run without any extrinsic acceptor protein (control I).

Finally, a second control was performed without cells but in the presence of (γ^{32}P)ATP and phosvitin.

Table I. *Incorporation of terminal phosphoryl group of (γ^{32}P)ATP into phosphorylserine (SerP), phosphorylthreonine (ThrP) and phosphopeptides of either endogenous or extrinsic acceptor protein of Ehrlich cells*

	SerP	ThrP	Phospho-peptides
1. Histone	2.97	0.84	0.12
2. Histone+cyclic AMP	7.80	2.45	0.38
3. Control I (ATP+ cells)	10.6	1.20	0.84
4. Phosvitin	58.1	37.5	7.59
5. Phosvitin+cyclic AMP	61.8	26.5	7.72
6. Control II (ATP+ phosvitin)	0	0	0

The results given are phosphoryl groups in pmol/5×10^{8} cells/min transferred into acceptor protein at 37°C.

Table II. *Residual concomitant incorporation of (γ^{32}P)ATP into Ehrlich cell fraction after removal of corresponding supernatants (Table I)*

	SerP	ThrP	Phospho-peptides
1. Histone	7.48	1.04	0.43
2. Histone+cyclic AMP	7.90	1.79	0.49
3. Control I (ATP+ cells)	3.70	1.64	0.22
4. Phosvitin	4.95	1.98	0.43
5. Phosvitin+cyclic AMP	3.42	1.36	0.29

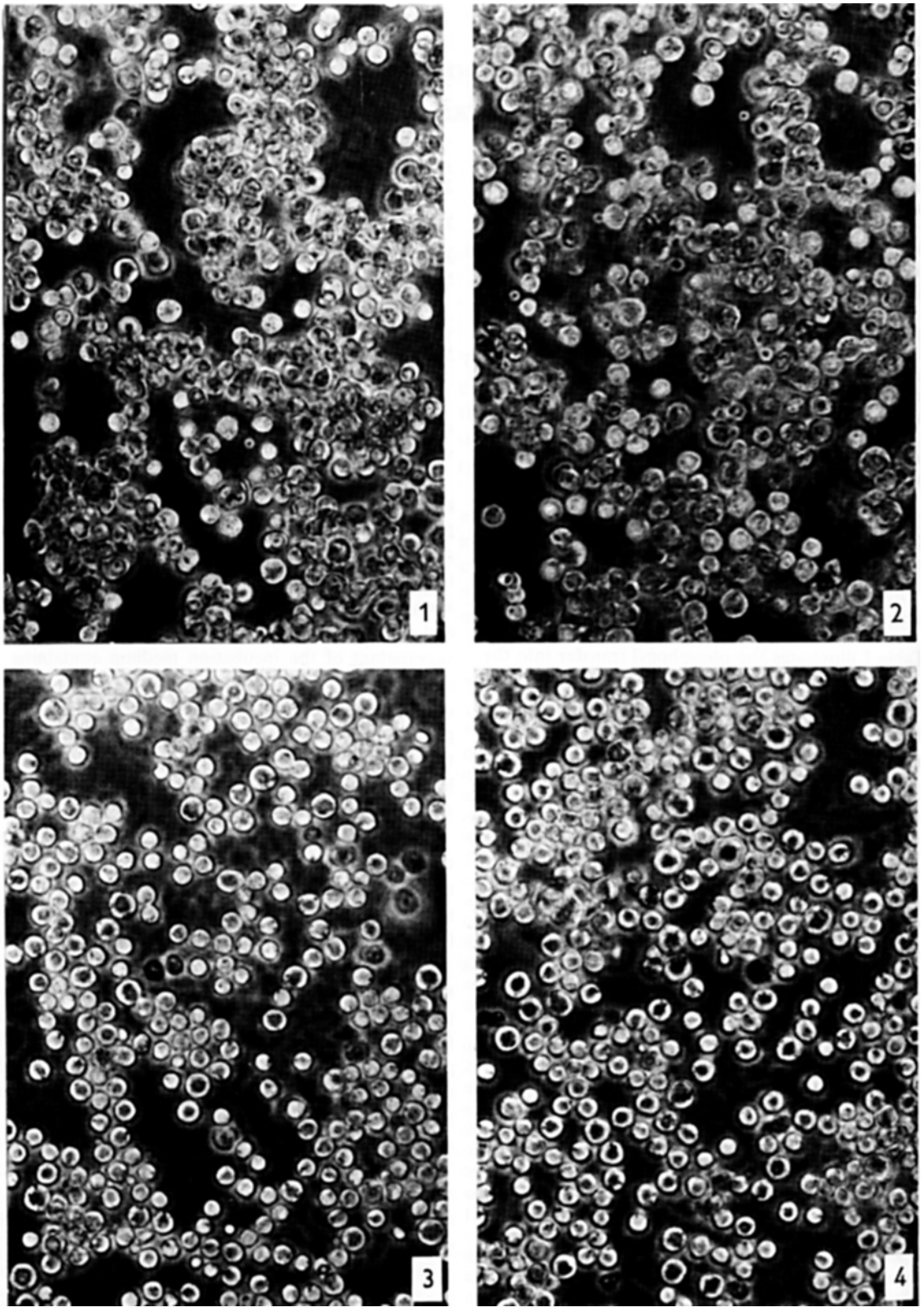
The figures denote (^{32}P) phosphoryl transfer in pmol/5×10^{8} cells/min at 37°C.

As is seen in the table, the terminal phosphoryl group could be transferred to both types of acceptor proteins but no activity was found in control II, indicating absence of contaminating protein kinase in the commercial phosvitin preparation. It is also seen that phosphorylserine, phosphorylthreonine and phosphopeptides could be isolated from the supernatant of the incubation medium containing only tumour cells and (γ^{32}P)ATP with no acceptor protein (control I). Histone seems to be a poor acceptor protein, the activity of which is lower than that of the control I. This inhibiting effect of histone alone can be abolished by adding cyclic AMP to the incubation medium.

Contrary to histone, phosvitin is an excellent acceptor protein, being about 20 times as effective as histone in the absence of cyclic AMP. The latter substance does not have any significant effect on the phosphoryl transfer into phosvitin.

As is seen in Table II no corresponding difference exists as regards phosphoryl transfer into proteins of the different cell pellets. There is a slight tendency of higher values of cell pellets from experiments with histone as acceptor proteins than the control I experiment and the experiments with phosvitin. Thus, no significant amounts of the added histone have been trapped in the cell pellet. This assumption is also confirmed by the dry weight determinations of the supernatant proteins (figures not given in Tables) which all are about the same.

Figures 1–5 illustrate the tumour cell behaviour in the incubation media containing different acceptor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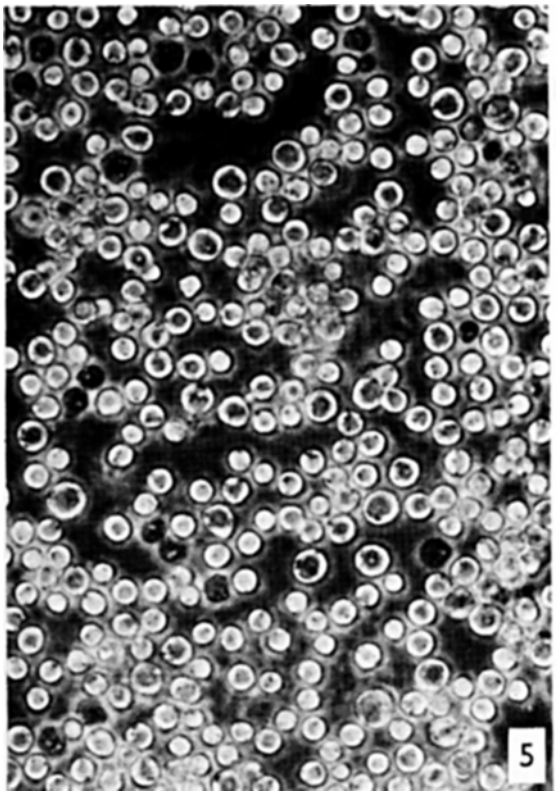

Fig. 1. Phase-contrast microscopy of Ehrlich cells incubated in a medium with histone as extrinsic acceptor protein. Photograph taken after 4 minutes incubation at 37°C. Microagglutinates are present. ×800.

Fig. 2. Cells as in Fig. 1 but 1×10^{-5} M cyclic AMP is included in the incubation medium. Microagglutinates are present. ×800.

Fig. 3. Ehrlich cells incubated with phosvitin as extrinsic acceptor protein. Photograph taken after 4 minutes incubation at 37°C. No microagglutinates are seen. ×800.

Fig. 4. Cells as in Fig. 3 but 1×10^{-5} M cyclic AMP is included in the incubation medium. No microagglutinates are observed. ×800.

Fig. 5. Ehrlich cells incubated in a medium lacking any extrinsic acceptor protein. Photograph taken after 4 minutes incubation at 37°C. No microagglutinates are seen. ×800.

proteins with or without cyclic AMP but otherwise under similar conditions. It is clear that the histone protein facilitates cell micro-agglutination contrary to phosvitin. It should be pointed out that the tendency of cell sedimentation in the experiment with histone was not obviously changed, compared with phosvitin experiments during the short incubation times used.

DISCUSSION

Substantiation of a biochemical reaction at the cell membrane of an intact cell is a delicate problem. It is necessary to prove that the enzymatic activity is due neither to irrelevant components of the suspending medium, e.g. an enzymatic contamination of the acceptor protein, nor to a non-specific leakage of the enzyme molecules from the interior of the cell. This cannot be accomplished with conventional membrane isolation procedures, but requires precisely controlled cellular techniques. Our procedure is based upon the addition of (γ^{32}P)ATP and the acceptor protein to the exterior of the suspended cells in the isotonic medium under conditions which do not appreciably impair cell viability which remains unaltered throughout the incubation procedure.

All protein kinases known so far have molecular weights in the range of 150 000–250 000. Non-specific leakage of intracellular protein kinase seems therefore unlikely (cf. references 1, 2, 10, 14).

Instead, we have reason to believe that the reactions observed are catalysed by membrane-bound protein kinase and that at least part of it is facing the external medium, thus enabling the reaction described to occur. A protein kinase has been claimed to be associated with the plasma membrane of the anterior pituitory gland in a recent paper (8). However, in this report no information is given as regards the "sideness" of the prepared membrane fraction. Therefore no conclusion can be drawn about the localization of the enzyme activity within the plasma membrane.

It is of considerable interest to register the presence of protein kinase at the outer surface of the Ehrlich cell. Furthermore, in an earlier publication (2) we have reported about the formation of ATP at the cell membrane of the tumour cell. Phosvitin with its netto-negative charge was evidently much more effective as an acceptor protein than the positively charged histone protein.

It is well known that practically all animal cells have a netto-negative surface charge. Our results suggest a model for cell to cell interaction where the protein kinase at the surface of one cell may

catalyse the phosphorylation of the endogenous acceptor protein of the surface membrane of another cell using the ATP-generating capacity of the outer part of the membrane (2) as phosphoryl group donor. This type of phosphorylation might well represent a type of regulatory function (cf. ref. 5) where two cells in a reciprocal way can influence each other. We may discern another superior principle for the overall metabolic regulation of cells constituted in a multicellular organism. This principle could be exerted at the cell membrane primarily rather than at the genome level in the nucleus of each single cell concerned.

The endogenous acceptor protein seems to be eluted to a certain degree because of the activity found in the supernatant of control I (see Table I). Elution of proteins from the plasma membrane has been observed to occur under certain conditions (12, 14).

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