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Phosphorylation of Endogenous Membrane Proteins by Endogenous Protein Kinase at the Outer Surface of Ehrlich Cells

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ABSTRACT

An endogenous protein kinase at the surface of Ehrlich cells has been studied. Using exogenous (γ^{32}P)ATP as a phosphoryl group donator, a transfer was demonstrated into endogenous acceptor protein(s) as well as to exogenous phosvitin. Seryl- and threonyl-residues isolated from the endogenous and exogenous acceptor protein were found to be labeled. The ratio between the labeled phosphorylserine and phosphorylthreonine was about 3.5:1 for both the endogenous acceptor of the intact cells and the exogenous acceptor. In similar experiments with a membrane preparation from Ehrlich cells, this ratio increased to about 7:1 provided the exogenous acceptor protein was absent. The results were independent of whether 1×10^{-5} M dibutyryl cyclic AMP was used or not with intact cells and a membrane fraction mainly consisting of vesicles. Whether the regulatory subunit of the membrane-associated protein kinase was in cis- or trans-disposition to the catalytic subunit no binding and dependence of the cyclic nucleotide was observed. Since the purified membrane fraction was considered free from endogenous cyclic AMP, it was concluded that the membrane-associated protein kinase of Ehrlich cells is not dependent on cyclic AMP. The critical role of arginine for the cyclic AMP dependence of the serine-containing residue in the catalytic subunit is discussed.

INTRODUCTION

A membrane-bound protein kinase which phosphorylates endogenous membrane proteins was first found in Ehrlich ascites tumor cells (3, 4, 35). Using exogenous (γ^{32}P)ATP as substrate it was apparent that the reaction took place at the outer surface of intact Ehrlich cells and resulted in phosphorylation of the protein-bound seryl- and threonyl-residues of the phosphate acceptor protein(s) (3, 4, 35, 36). Endogenous protein kinases effecting phosphorylation of endogenous membrane proteins have since then been found in several tissues including brain membrane fragments and synaptic preparations (22, 29, 45, 47, 49), human red cell membranes (6, 20, 39, 40, 41), rat liver plasma membranes (31, 44) and mitochondrial membranes (32), bovine anterior pituitary particulate fractions including plasma membranes (26, 27, 43), membrane preparations from bovine corpus luteum (7, 8) and hamster ovary cells (9), mouse mammary gland (30), renal medulla (15), cortex (17) and brush border (1), toad bladder (13, 14), canine heart (51), adrenal gland (48), myelin basic protein (10), HeLa cells (24, 33), muscle cells (5) and fat cells (11, 21).

The phosphorylation observed in these membrane systems is with few exceptions (32, 36, 59) cyclic AMP-dependent.

Most protein kinases have been shown to consist of two functional subunits, namely a catalytic subunit and a cyclic AMP binding subunit or regulatory subunit. Binding of cyclic AMP to the regulatory subunit activates the enzyme thereby releasing the fully active subunit which catalyses the phosphotransferase reaction (16, 18, 25, 34, 46).

The aim of the present study was to investigate further the cyclic AMP independent protein kinase associated with the outer surface of Ehrlich cells. The steric dispositions of the regulator to the catalytic subunit have been investigated by means of dibutyryl cyclic AMP. The reaction was also studied with exogenous acceptor proteins as well as with a purified membrane fraction.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade, (γ^{32}P)ATP was purchased from NEN chemicals, GmbH, Frankfurt am Main, Germany. Unlabeled ATP (sodium form), cyclic and dibutyryl cyclic AMP and phosvitin were all obtained from Sigma Chemical Company, St Louis, Mo., USA.

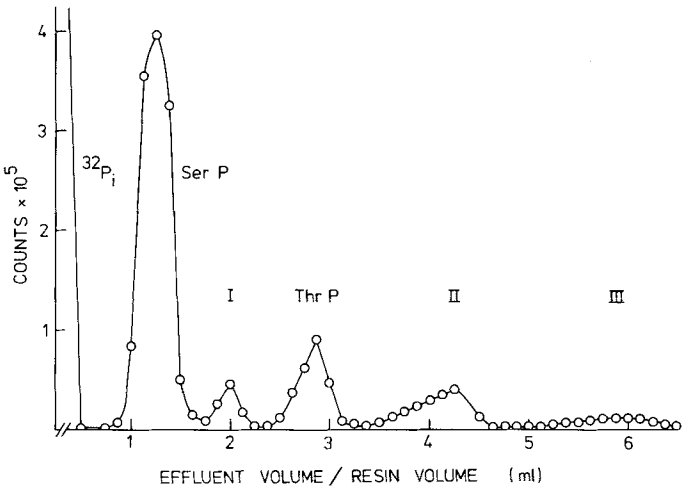

Fig. 1. Elution curve from a partial hydrolysate of the Schneider protein of exogenous phosvitin incubated with intact Ehrlich cells in the presence of 1×10^{-5} M dibutyryl cyclic AMP. Chromatography was performed on a 50 ml Dowex 50 (8% DVB) column with 0.01 M HCl as eluent.

Preparation of tumor cells. The Ehrlich ascites tumor cells were grown for 7–8 days in five-week-old male Swiss albino mice obtained from the Anticimex Breeding Farm, Norrviken, Stockholm. The tumor cells were separated by centrifugation of the ascitic fluid, which had been diluted without delay 10-fold with ice-cold Krebs-Ringer bicarbonate medium, in order to diminish the tendency of cell agglutination. The cells were then washed twice in the Krebs-Ringer bicarbonate medium. Final washing was performed with a medium of 130 mM NaCl and 25 mM KCl for experiments with intact cells. For the preparation of a membrane fraction the cells were finally washed in a sucrose-(0.250 M)-tris-glycyl-glycine (0.016 M) buffer, pH 8.0, also containing 2 mM $CaCl_2$, followed by centrifugation. This was carried out in the International Refrigerated Centrifuge by acceleration for 10–15 sec up to at most 5 700 rpm followed by retardation for about 2 min. All preparatory steps were carried out at 4°C.

Preparation of plasma membrane fraction. The procedure described by Ronquist and Christensen (38) was followed exactly. The final membrane fraction was suspended in 130 mM NaCl and 25 mM KCl in a concentration corresponding to about 20 mg membrane protein per ml.

Incubation procedure. Incubations were performed directly after the preparation of the cells and the membrane fraction. About 1×10^8 cells or a membrane fraction corresponding to 20 mg membrane protein were suspended in an incubation volume of 9.5 ml consisting of 130 mM NaCl and 25 mM KCl. It contained in addition 350 μmoles of Tris-acetic-acid buffer, pH 7.5, 20 μmoles of $MgCl_2$, 10 μmoles of Na_2HPO_4 (unlabeled), 5 μmoles of glutathione (reduced form) and 0.5 μmoles of (γ^{32}P)ATP. In some experiments, 2.5 μmoles of phosvitin were also included, either with or without dibutyryl cyclic AMP (final concentration 1×10^{-5} M). The commercial phosvitin preparation was free from protein kinase activity (37). Incubation was performed at 37°C and terminated after 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 trichloro-acetic acid (10% final concentration). In order to completely precipitate the low molecular weight acceptor protein, 50 mg of bovine albumin were added to the precipitating agent and the cell pellets were precipitated with trichloroacetic acid. The precipitation was allowed to proceed for at least 24 h. The precipitates were washed once with 10% trichloroacetic acid containing 7 μmoles of unlabeled orthophosphate and 2 μmoles of ATP.

In order to remove nucleic acid and phospholipids, the precipitate was treated according to Schneider (42). Partial hydrolysis of the Schneider protein was performed using the method of Lipmann (28). Labeled phosphorylserine and phosphorylthreonine and phosphopeptides were isolated by ion exchange chromatography as described previously (4).

A radiometric determination of the different fractions from column chromatography was carried out in a Nuclear Chicago Scintillation Counter by measuring the Cerenkov radiation.

RESULTS

Figure 1 illustrates a typical elution chromatogram. Besides the peaks for phosphorylserine (SerP) and phosphorylthreonine (ThrP), three other peaks containing labeled phosphopeptides are seen. Table I shows that the transfer of a phosphoryl group to an exogenous acceptor protein as well as to the endogenous acceptor protein(s) is not influenced by the presence of 1×10^{-5} M dibutyryl-cyclic AMP. The ratio between labeled phosphorylserine and phosphorylthreonine was about 3.5 : 1. The aliphatic cyclic nucleotide is assumed to penetrate the plasma membrane. Thus, providing the cyclic AMP-binding subunit is in *trans*-position to the catalytic

Table I. *Incorporation of the terminal phosphoryl group of (γ^{32}P)ATP into phosphorylserine (SerP), phosphorylthreonine (ThrP) and different phosphopeptides (peaks I, II and III, respectively, see Fig. 1) of either extrinsic or endogenous acceptor protein of intact Ehrlich cells*

Comparison of incorporation with and without 1×10^{-5} M dibutyryl-3',5'-AMP

	SerP	ThrP	Peak 1	Peak 2	Peak 3
(*a*) Phosvitin as exogenous phosphoryl group acceptor	0.515[a]	0.135	0.096	0.073	0.012
(*b*) As (*a*) plus 1×10^{-5} M dibutyryl-3',5'-AMP	0.488	0.107	0.089	0.065	0.011
(*c*) Endogenous membrane protein as phosphoryl group acceptor	0.134	0.031	0.011	0.021	–
(*d*) As (*c*) plus 1×10^{-5} M dibutyryl-3',5'-AMP	0.132	0.026	0.015	0.009	–

[a] The figures denote the phosphoryl group transfer in nmol/1×10^{8} cells per min at 37°C into acceptor protein.

subunit, it would nevertheless be possible for the added cyclic nucleotide to react.

It is conceivable that the cellular cyclic AMP had already reacted with the receptor site of the regulatory subunit on the inner surface of the intact membrane. In this case, additional cyclic AMP would have no effect. Therefore, experiments were performed on purified membrane vesicles instead of the intact cells. It is evident from Table II that no stimulatory effect by cyclic AMP could be observed with the membrane vesicles with or without exogenous acceptor protein. In fact, the cyclic nucleotide had a slight inhibitory effect. The ratio between labeled phosphorylserine and phosphorylthreonine was about 3.5 : 1 as in the case of the intact cells provided that the exogenous acceptor protein was present. In the absence of this acceptor protein, the ratio increased to about 7 : 1.

DISCUSSION

It is suggested that the protein kinase of the plasma membrane of Ehrlich cells is not cyclic AMP-dependent. Assuming that the catalytic and regulatory subunits are *trans*-disposed, then it follows that the ATP-substrate must be *cis*-disposed to the catalytic subunit whereas the cyclic AMP reacting with the regulatory subunit must be *trans*-disposed to the substrate. This hypothetical situation may be studied in the present experimental systems which involve the outer surface of intact cells and membrane vesicles, respectively. The ATP substrate cannot penetrate the membranes (2, 19) while the aliphatic dibutyryl cyclic AMP can. However, no effect could be observed neither with intact cells nor with membrane vesicles regardless of the presence of exogenous acceptor protein. Protein kinases associated with membranous structures are usually stimulated by cyclic AMP. However, recent reports deal with membrane-bound protein kinase apparently independent of cyclic AMP (5, 9, 32). No explanation has so far been given.

The immediate amino acyl sequence of the sites of phosphorylation by cyclic AMP dependent protein kinases of several different substances has been summarized by Daile et al. (12). Provided the phosphoryl transfer reaction is cyclic AMP-stimulated, the non-globular acceptor as well as the

Table II. *Incorporation of the terminal phosphoryl group of (γ^{32}P)ATP into phosphorylserine (SerP), phosphorylthreonine (ThrP) and different phosphopeptides (peaks I, II and III, respectively, see Fig. 1) of either extrinsic or endogenous acceptor protein from membrane vesicles prepared from Ehrlich cells*

Incubations were performed with or without 1×10^{-5} M dibutyryl-3',5'-AMP in the medium

	SerP	ThrP	Peak 1	Peak 2	Peak 3
(*a*) Phosvitin as exogenous phosphoryl group acceptor	0.358[a]	0.116	0.040	0.050	0.024
(*b*) As (*a*) plus 1×10^{-5} M dibutyryl-3',5'-AMP	0.293	0.099	0.027	0.043	0.014
(*c*) Endogenous membrane protein as phosphoryl group acceptor	0.176	0.025	0.020	0.012	0.007
(*d*) As (*c*) plus 1×10^{-5} M dibutyryl 3',5'-AMP	0.159	0.025	0.014	0.012	0.006

[a] The figures denote the phosphoryl group transfer in nmol/mg Schneider protein of membrane vesicles per min at 37°C into acceptor protein. For comparison with Table 1, 1×10^{8} Ehrlich cells correspond to about 15 mg Schneider protein from membrane vesicles prepared from Ehrlich cells.

globular one contain at least one arginyl residue in close vicinity to the active seryl residue. However, since the amino acid sequence adjacent to the phosphorylserine position has not been determined for the endogenous acceptor protein(s) of Ehrlich cells nor for the exogenous acceptor, the phosphorylserine-containing peptide and other phosphopeptides isolated from the endogenous acceptor of Ehrlich cells were quantitatively analyzed for amino acids (36). This study showed that the arginine content of the serine-rich phosphopeptide was less than 2% of the serine. This result differs strikingly from that of Daile et al. (12), and also from recent results by Kemp et al. (23). The former indicated an arginine/serine ratio of at least 1 : 1.

The relatively constant ratio between labeled phosphorylserine and phosphorylthreonine of about 3.5 : 1 increased markedly to about 7 : 1 in experiments with membrane vesicles without any exogenous phosphoryl group acceptor. This may be attributable to the elution of part of the endogenous acceptor proteins of the membrane during the preparation of the membrane vesicles. We have shown previously that membrane-bound proteins can be eluted (50). Furthermore, the results of such an elution are highly dependent upon the composition of the elution medium. However, other possibilities may exist, e.g. a conformational change during preparation of the acceptor protein resulting in another labeling pattern of the protein.

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