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ECTOPROTEIN KINASE ACTIVITY OF THE ISOLATED RAT ADIPOCYTE

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SUMMARY

Intact adipocytes exhibit ectoprotein kinase activity as reflected by their ability to catalyze the transfer of the terminal phosphate of (γ-^{32}P) ATP to histone added to a cell suspension. This activity is substrate, time and cell number dependent. Lineweaver-Burk plots gave Km and Vmax values for ATP of 5 x 10^{-5} M and 7.14 pmoles/min/1.5 x 10^{5} cells. Cyclic AMP but not cyclic GMP in μM concentrations stimulates ectoprotein kinase activity. The controlled tryptic digestion of intact cells results in reduction of ectoprotein kinase activity. This activity is not due to leakage of intracellular protein kinases during the preparative procedure nor to penetration of histone into the cells. Additional phosphoproteins not accessible to endogenous protein kinase activity are also localized on the external surface of the intact fat cell.

INTRODUCTION

The plasma membranes of cells contain protein kinase activity as well as proteins which serve as substrates for kinase activity (1,2). In transformed and cultured cells, such as the Ehrlich ascites (3), glial (4), glioma (4,5), 3T3 and SV_{40} transformed 3T3 (6) cell, part of the protein kinase activity of the plasma membrane is exposed to the extracellular environment as determined by the ability of intact cells to transfer the terminal phosphate of ATP to exogenously added acceptor proteins. It is well-known that diverse and complex changes of the morphology, metabolism and behavior of cells result from viral or malignant transformation as well as from growth in an artificial

Abbreviations used: ATP, adenosine 5'-triphosphoric acid; cyclic AMP, adenosine 3', 5' cyclic monophosphoric acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; cyclic GMP, guanosine 3', 5' cyclic monophosphoric acid; KRB, Krebs Ringer bicarbonate buffer; LDH, lactate dehydrogenase.

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environment (7-10). It is not certain, therefore, whether the externally-directed protein kinase activity is an expression of transformed and cultured cells which is not shared by normal cells. De Pierre and Karnovsky refer to enzymes whose active sites are presumed to face the external medium as ectoenzymes (11).

We report the presence of ectoprotein kinase activity in a normal cell as determined by the catalytic transfer of the terminal phosphate of ATP onto a protein substrate added to a suspension of intact rat adipocytes and the use of other macromolecular probes to demonstrate the accessibility of this kinase activity to the extracellular environment. Further, we demonstrate the presence of additional proteins on the surface of the fat cell which serve as substrates for an exogenously added catalytic subunit of protein kinase but which are apparently inaccessible to endogenous kinase activity in the intact cell.

MATERIALS AND METHODS

Fat cells were isolated from epididymal fat pads of male Sprague-Dawley rats (120-200 grams) according to Rodbell's method (12) after animals were killed by cervical dislocation. C. histolyticum collagenase, Type 1 from Worthington Biochemical Corporation was used (5 mg/1 gram of fat). Cells were treated for 1 hour, 37°C in KRB, pH 7.4, made 4% with bovine serum albumin before passing through a nylon cloth of 200 µm pore size. This was followed by three washes in KRB without albumin before final suspension to an approximate concentration of 5×10^5 cells/ml. Cell number was determined in an inverted hemocytometer. All experiments were performed in polystyrene test tubes 75 x 15 mm, in a shaking water bath, 30°C, continuously flushed with 95% O_2, 5% CO_2.

Protein kinase activity was determined in KRB which contained 2.5 mM theophylline to inhibit phosphodiesterase hydrolysis of cAMP, 1 mM NaF to inhibit protein phosphatase activity, varying amounts of calf-thymus histone Type II A (Sigma, 50-200 µg), 2.5 to 50 µM ATP (γ-^{32}P) (specific radioactivity between 1,000-3,000 Ci/mmole with greater than 97% purity as obtained from New England Nuclear), and 1.5×10^5 cells in a total volume of 0.5 ml with and without µM amounts of cyclic nucleotides. Amount of label used for each tube was 0.5 µCi. The reaction was stopped by the addition of 0.5 ml 20% TCA. Carrier albumin (400 µg) was then added to the reaction mixture and the mixture was filtered through a millipore filter (0.45 µm pore size). The filter was washed extensively with 10% TCA. The radioactivity retained on the filter was determined in Bray's solution by scintillation spectrometry.

Homogenates of adipocytes were prepared by rapid freeze-thawing three times or by shearing according to Avruch and Wallach (13).

Total kinase activity was determined by the incorporation of label into the TCA precipitate from incubates including both cells and histone minus control incubates without cells. Endogenous kinase activity was determined by label incorporated into the TCA precipitate when cells were exposed to (γ-^{32}P) ATP without histone minus controls without cells. Ecto-protein kinase activity was designated as the difference between total and endogenous kinase activity. All samples were analyzed in triplicate. Quantitative values were derived from representative experiments. Each point is the average of triplicate or duplicate values.

To determine the specificity of phosphate incorporation as seryl and/or threonyl linkages, samples were hydrolyzed with 2N HCl under N_2 for 12 hours at 55°C, dried under vacuum, and chromatographed on thin-layer plates in n-butanol, acetic acid, water (120:30:50) with carrier unlabeled serine and threonine. Ninhydrin stain was used for detection. Total ^{32}P incorporated into thin layer plates at Rf's comparable to control serine and threonine samples was determined by scrapping the thin-layer spots and decolorization of ninhydrin with 0.1 N HCl before addition of Bray's solution for scintillation spectrometry.

Experiments were also conducted on aliquots suitable for SDS polyacrylamide gel electrophoresis of protein components. Reactions were stopped by the addition of equal volumes of sodium dodecylsulfate (SDS) sample buffer which were twice as concentrated as the "final sample buffer" described by Laemmli (14), SDS gel electrophoresis was performed according to Laemmli in slab gels of 1.5 mm thickness. The running gel was 10 cm long and consisted of a linear gradient of acrylamide from 7.5% to 17.5%. Proteins were visualized in gels by staining with Coomassie Brilliant Blue. Apparent molecular weights were estimated by comparison with the positions of proteins of known molecular weights which were electrophoresed on the same gel. Radioactive components were detected by auto-radiography of vacuum dried gels. Kodak X-Omat Film (X R-5) was used. Auto-radiographs were scanned on a Densicord recording electrophoresis densitometer (Photovolt Corp., N.Y.) and the scans were integrated using an Integraph model 49 automatic integrator (Photovolt Corp., N.Y.).

Trypsin and the trypsin inhibitor (α antitrypsin) were obtained from Sigma Co. The catalytic subunit of protein kinase used for these studies was also obtained from Sigma Co. All other chemicals used were reagent grade and were obtained from common suppliers.

RESULTS AND DISCUSSION

Addition of micromolar amounts of (γ-^{32}P)ATP to intact, isolated adipocytes results in the rapid incorporation of label into TCA precipitable proteins which is significantly increased by the addition of histone (Figure 1). This increase is due to labeling of histone itself as demonstrated by auto-radiography of parallel experiments subjected to SDS-PAGE (Figure 2). Cyclic AMP but not cyclic GMP enhances ectokinase activity (Figures 1 and 2). Ectokinase activity is also enhanced by increasing amounts of histone up to 150 μg/1.5 x 10^5 cells whereas

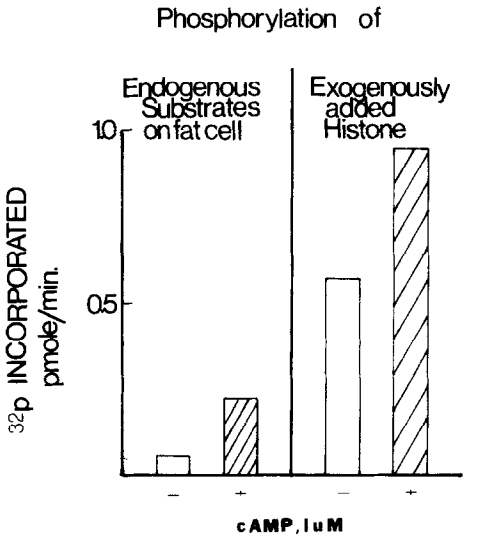

Figure 1 - Comparison of the recovery of ^{32}P in TCA precipitates of rat fat cells incubated with (γ-^{32}P)ATP with and without histone and cyclic AMP.

1.5 x 10^5 cells in 0.5 ml. Krebs-Ringer bicarbonate buffer, pH 7.4 containing 2.5 mM theophylline, 1mM NaF, 1μCi (γ-^{32}P)ATP at 5μM, ± histone 100 μg/ml, ± cyclic AMP 1μM. Incubation for 1 minute at 30°C. Reaction terminated by making 10% with TCA followed by filtration on millipore filter (HA, 0.45μM) and washing with 10% TCA. Radioactivity determined by scintillation spectrometry.

amounts in excess of this are inhibitory. Ectokinase activity increases linearly when cell number is raised from 0.3 to 6 x 10^5 cells per tube. When cell number is kept constant at 1.5 x 10^5 cells/tube ectokinase activity increases over ATP concentrations of 2.5 to 50 μM. The labeling of cell surface proteins, on the other hand, peaks at 2.5 to 5.0 μM ATP at constant cell number (Figure 3). Lineweaver-Burk plots of ATP concentrations versus velocity gave the following constants: for ecto-kinase activity, Km = 5 x 10^{-5} M and Vmax = 7.14 pmoles/min/1.5 x 10^5 cells while for cell surface kinase activity directed to endogenous substrates, Km = 3.1 x 10^{-6} M and Vmax = 0.625 pmoles/min/1.5 x 10^5 cells (Figure 3).

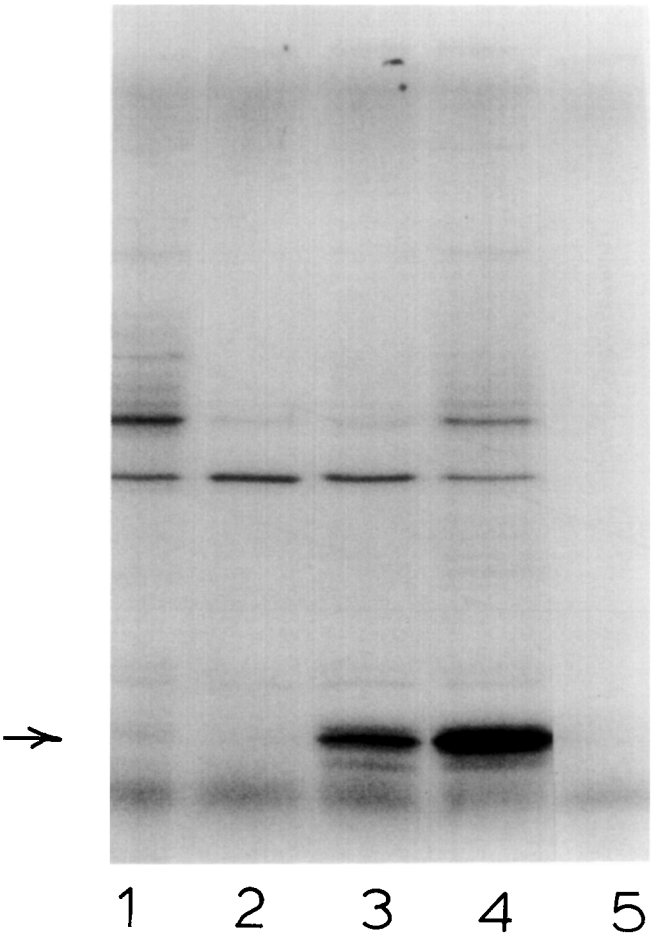

Figure 2 - Radioautographs of SDS-PAGE gel slabs of intact and homogenized rat fat cells incubated with (γ-^{32}P)ATP.

1.5×10^4 intact cells (for the homogenate the cells were frozen and thawed 3 x) in 0.05ml. Krebs-Ringer bicarbonate buffer, pH=7.4 containing 2.5mM theophylline, 1mM NaF, 10μCi (γ-^{32}P)ATP at 12.5 μM, cyclic AMP at 2μM, ± histone at 100μg/ml.

Lane 1, homogenate.
Lane 2, cells.
Lane 3, cells and histone.
Lane 4, homogenate and histone.
Lane 5, histone.
The arrow indicates the location of histone.

Since protein kinases released during the preparative procedure could catalyze the labeling of histone and the possibility of histone entry into cells could result in these findings, experiments were conducted to examine these possibilities.

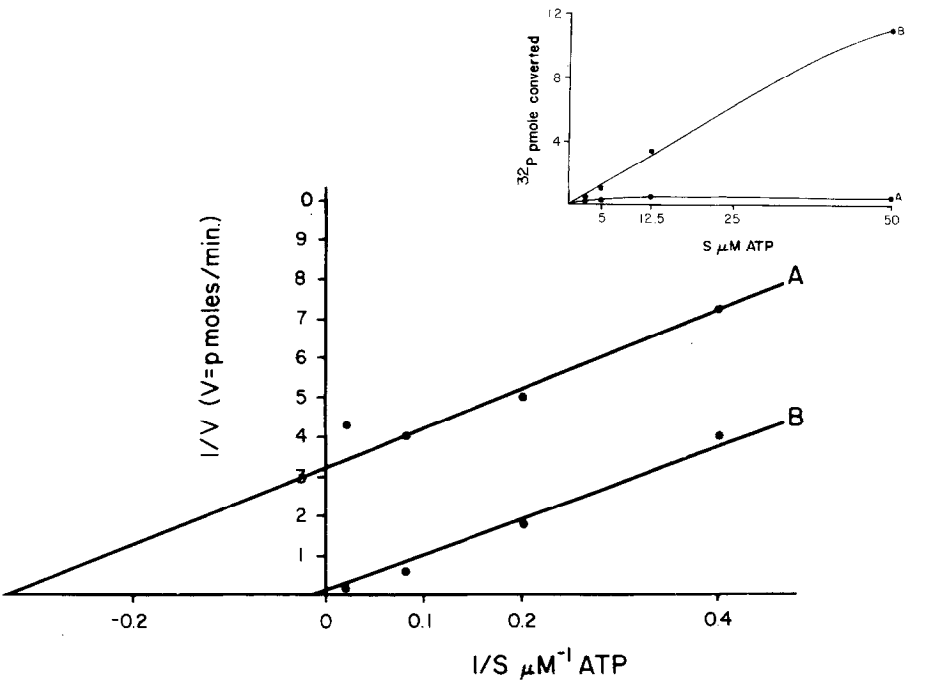

Figure 3 - Lineweaver-Burk plot of protein kinase activity of the intact rat fat cell.

A = endogenous phosphorylation as determined by the incorporation of label into cells.

B = ectokinase activity as determined by the incorporation of label into cells plus histone minus the incorporation of label into cells without added histone.

Conditions used were as described in the legend to Figure 1 except incubation was for 2 minutes with 12.5 μM ATP.

Infranatants from the final cell suspensions of several experiments were examined for protein kinase activity by recovery of label in histone in the TCA precipitate as well as by densitometric scans of auto-radiographs of SDS-PAGE gels. Less than 10% of the total ectoprotein kinase activity can be attributable to enzymes recovered in the infranatant by TCA precipitation studies. The density of the histone region of auto-radiographs of gels representing a cell suspension is 23.4 times that of the infranatant from an identical cell suspension.

The labeling of histone by a comparable homogenate is greater than that of an intact cell suspension (see Figure 2). This increase cannot be

accounted for by the contribution of endogenous histone as determined by the negligible increase in the histone region seen on the Coomassie stained gel. If the added histone were freely accessible to the intra and extracellular environment of intact cell preparations, no significant difference between the labeling of histone should be seen between the whole and broken cell preparations. Finally, by measurement of LDH activity in the infranatant as an index of cytoplasmic leakage, no more than 10% of total LDH activity can be recovered in the infranatant. Thus, approximately 90% of the label recovered in histone added to a suspension of intact cells must have been catalyzed by enzymes associated with unbroken cells with active sites exposed to the extracellular environment. In addition, this enzyme activity cannot be due to non-specific adsorption of protein kinase to the cell surface because 96.7% of kinase added to a cell suspension was recovered in the infranatant.

Prior trypsinization of intact cells results in reduction of the label incorporated by histone subsequently added to such cells (Figure 4, panel A). This indicates the catalytic site of the kinase involved in histone labeling is sensitive and accessible to trypsin. Since trypsin is too large to penetrate the intact cell, the kinase must be localized on the external surface of the fat cell. We have already reported the sensitivity of endogenous kinases to tryptic digestion (15) in the fat cell specifically, the enzyme that labels the 53,000 and 18,000 dalton components (see Figure 4, panel A). Whether the same or different kinase(s) is responsible for ecto and endogenous phosphorylation cannot be determined by these studies.

Other proteins can be identified on the cell surface which appear to be phosphoproteins which are restricted from endogenous surface kinase enzymes. The addition of the catalytic subunit of beef heart cyclic AMP-dependent protein kinase to intact cells results in the labeling of components not visualized when intact cells are incubated with ATP alone and

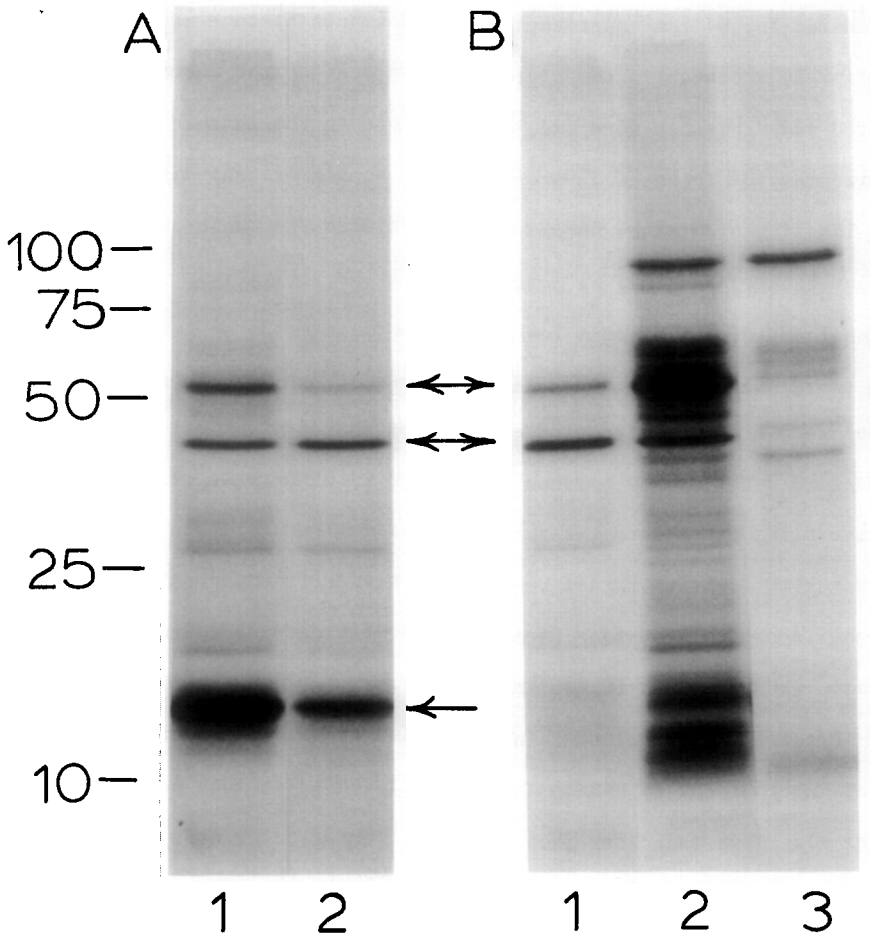

Figure 4 - Radioautographs of SDS-PAGE gel slabs of trypsin treated intact rat fat cells incubated with (γ-^{32}P)ATP.

Trypsinized cells were incubated with 50 µg/ml trypsin and 25 µg/ml DNAase for 15 min. at 30°C and then a 5-fold excess of trypsin inhibitor was added. The buffer and cell controls had trypsin and trypsin inhibitor added simultaneously. In some cases, after trypsin treatment the catalytic subunit of beef heart cyclic AMP dependent protein kinase was added at 2 nanomolar units/ml. Labeling was after trypsin treatment using the same conditions as in Figure 2.

Panel A
Lane 1, Control cells plus histone.
Lane 2, Trypsinized cells plus histone.

Panel B
Lane 1, Control cells.
Lane 2, Control cells plus added protein kinase.
Lane 3, Buffer control plus added protein kinase.

The arrows indicate the position of the 53,000 and the 18,000 dalton components and of histone. The scale on the left shows molecular weights (in daltons) x 10^{-3}.

the labeling of the added enzyme is taken under consideration (see Figure 4, panel B). Components on the cell surface which serve as phosphate acceptors but which are not accessible to endogenous kinase activity in the intact cell include one component slightly greater than 53,000 daltons and seven others ranging between 18,000 to 42,000 daltons when compared to coelectrophoresed molecular weight standards.

These observations demonstrate the topographic localization of the catalytic subunit of a cyclic AMP-dependent protein kinase on the surface of the intact fat cell which will interact with substrates which are not part of the cell surface. Further, the intact fat cell possesses other protein acceptors on its surface which are not available to endogenous enzymes located within the plasma membrane but which can be phosphorylated by exogenous enzymes. These topographical characteristics would allow for the bidirectional interaction of fat cells with their environment via the protein kinase system.

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