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ELUTION OF THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE TYPE I ISOZYME DERIVED FROM EPIDIDYMAL FAT WITHIN THE TYPE II ISOZYME CHROMATOGRAPHIC PEAK

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No cAMP-dependent protein kinase activity is found upon DEAE-cellulose chromatography of mouse fat extracts at the low salt concentration characteristic of the Type I isozyme. The R_I detected in fat extracts by photoincorporation of the analog, 8-N_3[^{32}P]cAMP, elutes within the high salt Type II isozyme peak. The multiple charge variants of this photolabeled R_I which can be resolved by two-dimensional gel electrophoresis are similar to those of the histoptypically-related cultured cells, SV3T3 and 3T6, which do contain Type I kinase isozyme activity peaks. This high salt-eluting R_I may be part of a Type I holoenzyme whose elution properties are altered by interactions with other substances present in the extract.

cAMP-dependent protein kinase enzymes fall into two major isozymic classes, called Types I and II, based on the order of their elution from DEAE-cellulose columns (1). The isozymes have similar or identical catalytic (C) subunits and vary only in their regulatory (R) subunits (2). Although the respective in vivo roles of these isozymes have not been delineated, speculation on what these functions may be is largely based on alterations in the proportions of the isozymes during physiological changes (3). Such changes include development (4,5), cell cycle traverse (6), and neoplastic tranformation (7). These proportions are usually estimated by comparing the sizes of the two protein kinase activity peaks eluting from DEAE columns at low (Type I) and high salt (Type II) . An alternative method measures the relative amounts of the

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photoaffinity analog of cAMP, 8-N_3[^{32}P]cAMP (8), incorporated into each R-subunit. Although both assays often agree (9), R_I, which is detectable by photolabeling can also elute from DEAE columns outside of the Type I isozyme peak. R_I dissociated free from the C-subunit elutes between the Type I and Type II peaks (10), while R_I-subunits of an uncharacterized dissociation state elute at a variety of salt concentrations in addition to the Type I isozyme peak (11-13). The presence of Type I isozyme within the Type II kinase activity peak would certainly cloud the interpretation of those studies which rely solely on the proportion of these activity peaks to estimate relative isozyme concentrations. Herein we show that mouse epididymal fat extracts contain an R_I which elutes solely within the Type II isozyme kinase peak, and that no apparent structural differences exist between this R_I and those R_I-subunits contained in Type I isozyme peaks from related sources.

Materials and Methods:

Chromatography. Epididymal fat was removed from adult BALB/cByJ mice and homogenized in 10 mM potassium phosphate, pH 7.0, containing 1 mM EDTA (2 vol/gm wet wt.). The crude homogenate was passed through silk to remove excess lipid, and centrifuged at 27,000g for 35 min to obtain supernatant fractions. Chromatography on DE-52 cellulose (Whatman) equilibrated with this same buffer was done according to the method of Corbin et al. (1), as previously modified (14). Prior to chromatography, the extract was preincubated with ATP and $MgCl_2$ to final concentrations of 0.15 mM and 3.5 mM, respectively, to facilitate association of the Type I holoenzyme. After 30 min at 30°C the extract was chromatographed on a Sephadex G-25 column (30 X 0.9 cm) and eluted at 4°C with homogenization buffer at a flow rate of 30 ml/hr. Proteins eluting in the void volume, as determined by absorption at 280 nm, were pooled and 20-30 mg of protein was applied to the DEAE-cellulose column.

Assays. Protein concentrations were determined according to Lowry et al. (15), with crystalline bovine serum albumin used as a standard. Protein kinase activity was assayed with a 250 µl reaction mixture containing 6.7 mM $MgCl_2$, 2.0 mM 2-(N-Morpholino) ethane-sulphonate (MES) buffer (pH 6.5), 233 µg Type II A mixed histone, 0.23 mM (γ-^{32}P]ATP (40 Ci/mmole), and a 25 µl aliquot from a DEAE-cellulose fraction, with or without 3.3 µM cAMP. After 30 min at 30°C, 100 µl aliquots were transferred onto filter paper and washed with trichloroacetic acid as described (16). The photoincorporation procedure used previously (17) was slightly modified. Twenty µl of a column fraction and 1 µl of MES buffer were added to a 5 µl aliquot of 8-N_3[^{32}P]cAMP to obtain a final analog concentration of 125 nM. These components were mixed with an airstream on a spot plate. The samples were incubated at 35°C for 30 min in the dark to promote exchange between the ligand and any endogenous cAMP already bound to regulatory subunits and were further incubated for 30 min at 4°C in the dark. Photolysis with UV (254 nM) was done at 4°C with a UVS-54 Mineralight handlamp held at 12 cm for 15 min. The reaction was terminated with 26 µl of an SDS-containing stop solution (18), and the photolabeled proteins separated on 8-12% linear gradient denaturing gels.

Gel Electrophoresis. Two-dimensional electrophoretic separation of crude extracts photolabeled with 8-N_3[^{32}P]cAMP was done as follows. Epididymal fat was homogenized in 0.32 M sucrose and post-mitochondrial fractions prepared as above. Roller bottles of BALB 3T3, SV3T3, and 3T6 cells were grown in Dulbecco's modified Eagle's medium, supplemented with serum. The cells were rinsed twice with cold 0.32 M sucrose, scraped and centrifuged at 900g. The pelleted cells were then sonicated for 10 sec at 1.5 watts on a Bronson sonicator, and frozen for later assay. Twenty µl of extract containing 20 µg of protein was photolabeled using an 8-N_3[^{32}P]cAMP concentration of 750 nM and the reaction terminated with 26 µl of lysis buffer (19). The O'Farrell (19) electrophoretic procedure was modified as described previously (17). First-dimension gels were run for 6400 volt-hrs. Samples were overlayed with 5% Nonidet P-40, 8 M urea, and 1% ampholines, composed of 0.8% of the pH 5 to 7 ampholine and 0.2% of the pH 3.5 to 10 ampholine, both from LKB Instruments, Inc. Second-dimension gels were 8 to 12% linear gradients. Dried gels were placed on Dupont X-ray film with Dupont Quanta-III intensifying screens for 1-2 days at -75°C for autoradiographic detection.

Results and Discussion:

Fig. 1A illustrates that only a single DEAE peak can be detected by protein kinase activity measurements upon chromatography of fat extracts; this activity elutes at the salt concentration range typical for the Type II holoenzyme (1). When individual column fractions derived from the fat extract are photolabeled, however (Fig. 1B), greater than one-third of the photolabeled material in this peak is R_I. Identification of this protein as R_I is based on its size (49 K) and pI range (5.4 - 5.6). The following considerations suggest that both the R_I and R_{II} subunits present in this Type II peak may be associated with C-subunits as holoenzymes: 1. The extract was preincubated with MgATP prior to chromatography to facilitate release of endogenously bound cAMP and encourage subunit reassociation (14,20); 2. Free R_I typically elutes from DEAE columns at salt concentrations intermediate to the Type I and Type II activity peaks (10); 3. No peak of C-subunit activity, which elutes at the void volume (14), was observed; 4. In mouse lung the R_I eluting in the Type II peak is a holoenzyme (M.S. Butley and A.M. Malkinson, unpublished results), although this has not yet been tested in other systems.

This high salt-eluting R_I was compared with R_I-subunits which elute at the more characteristic Type I isozyme position. Two-dimensional electrophoretic separation of photolabeled proteins allows qualitative comparisons of the various photolabeled R-subunit charge variants. Both fat tissue and BALB 3T3 cells (7,21,22) lack a Type I isozyme kinase activity peak, but con-

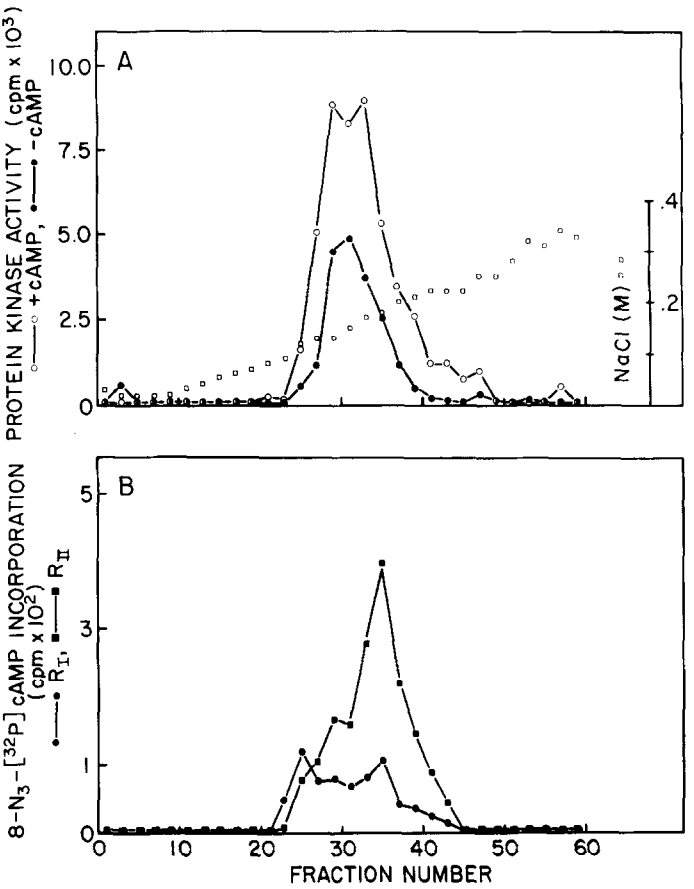

Figure 1 (A) Protein kinase activity in the presence (○——○) or absence (●——●) of cAMP, and (B) 8-N_3[^{32}P]cAMP incorporation into R_I (●——●) and R_{II} (■ —— ■) in DEAE-cellulose fractions collected after chromatographic elution of extracts from epididymal fat. The NaCl concentrations were estimated by conductivity measurements of each fraction and extrapolation from a standard curve.

tain R_I-subunits detectable by 8-N_3[^{32}P]cAMP photolabeling (Fig. 1B; 22). This is interesting since adipocytes are one of the potential differentiated products that these multipotent mesenchymal precursor cells can give rise to (23). The SV3T3 viral transformant and 3T6, a spontaneous transformant which has an anchorage dependence intermediate between BALB 3T3 and SV3T3, each contain a demonstrable Type I kinase isozyme activity peak (22). Fig. 2 is an autoradiogram prepared from a two-dimensional gel of photolabeled proteins obtained from extracts of BALB 3T3, 3T6, SV3T3, and epididymal fat. These electrophoretic patterns are similar to each other, and to those described for rat ovarian follicles (24), mouse lung (17), and mammalian brain (25). When large

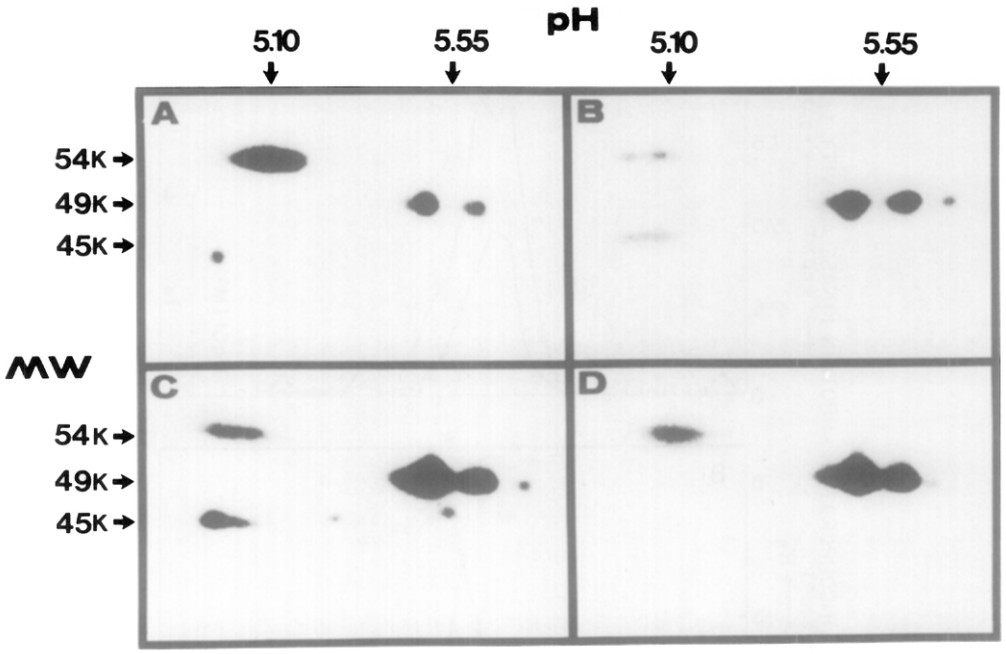

Figure 2 Autoradiogram of proteins from (A) BALB 3T3, (B) 3T6, (C) SV3T3 and (D) epididymal fat extracts which were labeled with 750 nM 8-N_3[^{32}P]cAMP and fractionated by two-dimensional gel electrophoresis.

amounts of 8-N_3[^{32}P]cAMP are incorporated into R_I, four discrete charge variants ranging in pI from 5.4 to 5.6 are observed in all samples, with the two central spots incorporating the most label. With less labeling of R_I, only two or three of these charge species are detected.

R_{II} migrates as a spot elongated along the pI dimension, suggesting the presence of two R_{II} proteins of similar but non-identical charge. Labeled charge variants at 37K and at 42-44K most likely represent proteolytic fragments of the R-subunits (26,27). These fragments are probably endogenously present in these samples since homogenization in the presence of protease inhibitors does not reduce the amount of labeling of these proteins. Multiple 42-44K charge variants are found in labeled extracts from the cultured cell lines and are most prominent in the SV3T3 extracts, but only a single 37K fragment is detected in the labeled fat extract. The main proteolytic fragment derived from 8-N_3[^{32}P]cAMP-labeled R-subunits in mouse lung (17) and brain (25) is also a single 37K spot.

The affinities of the R_I subunits for binding 8-N_3[^{32}P]cAMP are also similar for the fat and cultured cell samples (data not shown). Neither the affinities nor the amounts of ligand bound/mg protein were affected when the cell density was varied during growth or by varying the protein concentration from 0.4-2.5 mg/ml in the photolabeling assay.

Interactions between R_I and other cellular substances, possibly substrates for the protein kinase enzyme, may influence its conformation and net charge, and thereby its elution position from DEAE columns. This explanation for the atypical ion-exchange properties of R_I is suggested by the similarity of the R_I charge variants eluting at the low and high salt concentrations as resolved by two-dimensional electrophoresis. Estimates of the relative proportions of the protein kinase isozymes based solely on the sizes of the cAMP-dependent kinase activity peaks may not be valid since the type II activity peak may contain the Type I isozyme. On the other hand, the relative quantitative incorporation of 8-N_3[^{32}P]cAMP into each R-subunit can be modified by changes in ionic strength (28) and in the endogenous concentrations of MgATP (29), and MgGTP and adenosine (30), all of which can affect the binding capacity of R-subunits for the analog. A lack of stoichiometry in the binding of 8-N_3[^{32}P]cAMP even to purified R-subunits has been described (31). This analog is perhaps most useful for characterizing such properties of the cAMP-dependent kinases as subcellular distribution and modifications of R-subunit structure (30). DEAE chromatography and quantitative photolabeling studies, along with immunochemical titration with antisera prepared against R-subunits, may be required to accurately describe the concentrations and functional status of R-subunits within the cell.

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