Vol. 106, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1982 Pages 492-499

THE M1 AND M2 ISOZYMES OF PYRUVATE KINASE ARE THE PRODUCTS OF THE SAME GENE

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Received April 16,1982

The M1 and M2 isozymes of pyruvate kinase from several mammalian species were compared in their reactivity toward monoclonal anti-pyruvate kinase antibodies and by one and two-dimensional peptide mapping. Inter-species differences between the M2 isozymes were always accompanied by similar inter-species differences in the M1 isozymes, supporting the conclusion that these two isozymes are the products of the same gene.

INTRODUCTION

Muscle differentiation is accompanied by a switch from the expression of the M2 isozyme of pyruvate kinase to the M1 isozyme (1-3). Within a given species, the immunologic reactivity, the amino acid composition and the tryptic peptide fingerprint patterns of the two isozymes are very similar (1,4-7). However, the two isozymes can be distinguished by kinetic and electrophoretic properties. In addition, the molecular weight of the M2 isozyme is slightly greater than the M1 isozyme (1,6,7).

Recently it has been demonstrated that the differences in molecular weight and electrophoretic properties of the M1 and M2 isozymes result from differences in the mRNA coding for these two isozyme types (8). These studies indicate that the M1 and M2 isozymes are either the products of two closely related genes, or the product of a single gene from which two distinct mature mRNAs can be produced.

To determine whether the M1 and M2 isozymes are the products of one or two independent gene regions, we compared the M1 and M2 isozymes from several mammalian species in their reactivity toward anti-pyruvate kinase monoclonal antibodies and by one and two-dimensional peptide mapping studies. The one

0006-291X/82/100492-08$01.00/0

and two-dimensional maps of $^{[125]}$I labelled peptides from the human and rabbit M2 isozymes were very similar to the peptide maps of the M1 isozymes from the corresponding species. However, inter-species differences in immunologic reactivity and peptide maps noted between M2 isozymes from two species were always accompanied by similar inter-species differences in the respective M1 isozymes, supporting the conclusion that the two isozymes are the products of a single gene.

MATERIALS AND METHODS

Isolation of Pyruvate Kinases: Human, rat and mouse M1 isozymes were extracted from skeletal muscle in three volumes of 25 mM Tris-HCl (pH 7.4) containing 5 mM fructose 1,6-diphosphate and 10 mM dithiothreitol (Buffer A). The homogenate was centrifuged (20,000xg; 30 min) and pyruvate kinase isolated from the supernatant by ammonium sulfate precipitation between 55 and 70% saturation. The rabbit M1 isozyme was obtained from Sigma (St. Louis, Mo.). The M2 isozymes of pyruvate kinase were obtained from cells maintained in culture. Human WI-38 fibroblasts, Mouse 3T3 cells and rat L2 cells were obtained from the American Type Culture Collection (Rockville, Md.). Rabbit NB-6 cells were developed from a primary culture of newborn New Zealand white rabbit lung. Cells were trypsinized, centrifuged (800xg; 10 min), sonicated in Buffer A, centrifuged (20,000xg; 30 min) and the M2 isozyme in the supernatant used for radioimmunoassay. For peptide mapping experiments, human M2 isozyme was further concentrated by ammonium sulfate fractionation as described above and rabbit M2 isozyme was purified from spleen thru the CM-Sephadex chromatography step as described by Harkins et al. (9). Pyruvate kinase activity was determined by the technique of Valentine and Tanaka (10). The isozyme type present in each tissue and cell line was confirmed by the technique of Susor et al. (11).

Radioimmunoassay of Pyruvate Kinases: Anti-pyruvate kinase monoclonal antibodies were prepared as previously described (12,13). Pyruvate kinases were dialysed against Buffer A for 2 h at 4^{0}C, assayed for enzyme activity, diluted to 4 units/ml and serial 1:2 dilutions in Buffer A assayed by consumption radioimmunoassay as previously described (13).

Peptide Mapping of Purified $^{[125]}$I Labelled Pyruvate Kinases: One hundred micrograms of partially purified pyruvate kinases were iodinated with 400 µCi of [125]I (14) and the labelled proteins eluted from a Sephadex G-25 column in phosphate buffered saline containing 1% bovine serum albumin and 5 mM fructose 1,6-diphosphate. The [125]I labelled pyruvate kinase was isolated by the combination of immunoadsorption to plastic microtiter plates coated with anti-pyruvate kinase monoclonal antibody and subsequent sodium dodecyl sulfate-acrylamide gel electrophoresis of the bound proteins as previously described (13). One-dimensional peptide mapping was performed by the technique of Cleveland (15). Bands containing purified labelled pyruvate kinases were removed from the first gel, digested with 1.25 µg Staphylococcus aureus V8 protease (Miles Laboratories, Elkhart, In.) and peptides electrophoresed into 12-20% acrylamide gradient gels. Autoradiograms were prepared from the dried gels and scanned at 600 nm. For two-dimensional peptide mapping, bands containing pyruvate kinases were cut from the gel, dried overnight under high vacuum and digested with 100 µg trypsin (chymotrypsin free, Calbiochem, La Jolla, Ca.) in 0.5 ml of 0.1 M NH_4HCO_3 (37^{0}C; overnight). Peptides in the supernatant were lyophilized and redigested in 20 µl of 0.05 M sodium

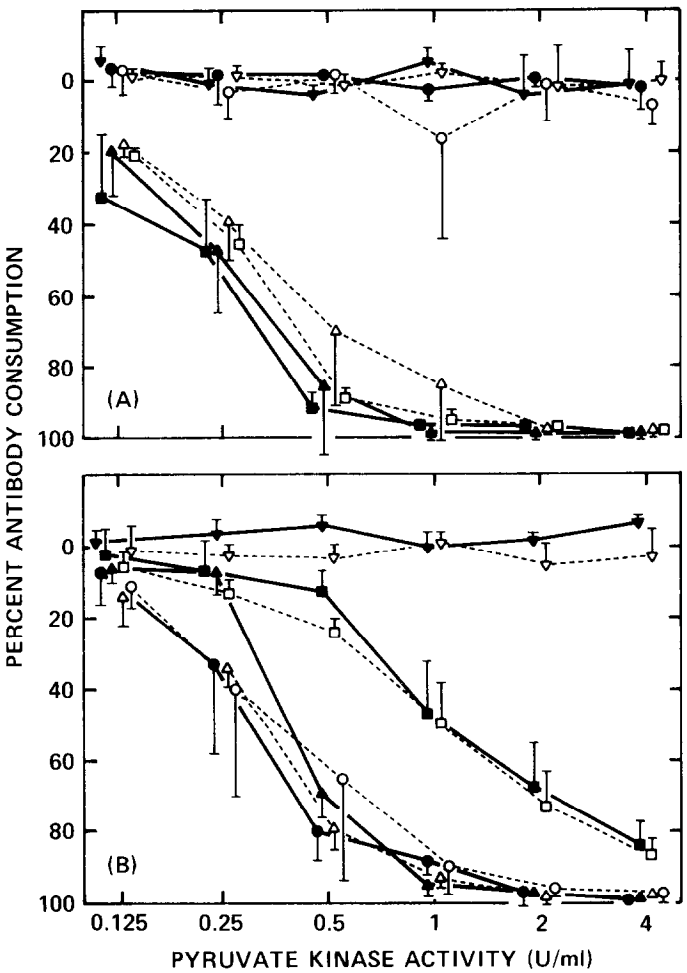

Figure 1. Consumption of anti-pyruvate kinase monoclonal antibodies by the M1 and M2 isozymes of pyruvate kinase from several mammalian species. Consumption of antibody 5C-C2 (panel A) and antibody 6A-C7 (panel B) by rabbit M1 (▲) and M2 (△) isozymes, human M1 (■) and M2 (□) isozymes, rat M1 (●) and M2 (○) isozymes and mouse M1 (▼) and M2 (▽) isozymes are shown. Data represent the mean and standard deviation of triplicate experiments.

borate (pH 8.5) containing 100 µg trypsin. One to five µl of digest were spotted on cellulose thin-layer plates (0.1mm thickness, Brinkmann) and [125]I labelled peptides separated in the first dimension by electrophoresis in 8% acetic acid and 2% formic acid (pH 2.0) at 450 volts for two hours. Plates were dried and peptides separated in the second dimension by ascending chromatography in butanol:pyridine:acetic acid:water (15:10:3:9 volume). Plates were again dried, autoradiograms prepared and photographed.

RESULTS

Immunologic Studies: Four anti-pyruvate kinase monoclonal antibodies were developed which had similar affinity toward the rabbit M1 and M2 isozymes. The reactivity of these antibodies toward the M1 and M2 isozymes from several other mammalian species was compared. As shown in Figure 1a, the human M1

and M2 isozymes both reacted toward antibody 5C-C2 with an affinity similar to that seen toward the rabbit isozymes. In contrast, neither the M1 nor the M2 isozymes from the rat or mouse reacted with this antibody, indicating that both isozymes from each species had lost the epitope detected by this antibody. The pattern of reactivity of the M1 and M2 isozymes from the four species toward antibody 5A-A10 was similar to that seen toward antibody 5C-C2.

The reactivity of the M1 and M2 isozymes toward antibody 6A-C7 is shown in Figure 1b. In this case, the rat M1 and M2 isozymes both reacted with this antibody with an affinity similar to that seen toward the rabbit isozymes. Neither the M1 nor the M2 isozyme from the mouse reacted with this antibody, indicating that both isozymes had lost the epitope detected by this antibody. The human M1 and M2 isozymes reacted toward this antibody with a similar affinity. However, the reactivity of the human isozymes was significantly reduced compared to the reactivity of the rabbit and rat isozymes, indicating the occurance of a similar change in the epitope detected by this antibody in both human isozymes. The pattern of reactivity of the M1 and M2 isozymes from the four species toward antibody 6B-H12 was similar to that seen toward antibody 6A-C7.

Peptide Mapping of Human and Rabbit M1 and M2 Isozymes of Pyruvate Kinase:

$^{[125]}$I labelled rabbit and human M1 and M2 isozymes of pyruvate kinase were purified and one-dimensional peptide mapping performed by the method of Cleveland, et al. (15). The peptide maps of the human M1 and M2 isozymes were very similar (Figure 2a). In contrast, the peptide map of the rabbit M1 isozyme (Figure 2b) was clearly distinguishable from the maps of the human isozymes. The peptide map of the rabbit M2 isozyme was similar to that of the rabbit M1 isozyme (Figure 2b).

Two-dimensional analysis of complete tryptic digests of $^{[125]}$I labelled rabbit and human M1 and M2 isozymes was also performed. Thirty-eight peptides were identified in photographs of the tryptic peptide map of the human M2 isozyme (Figure 3). Each of these peptides was also present in the human M1 isozyme. Similarly, thirty-two peptides were identified in the tryptic peptide

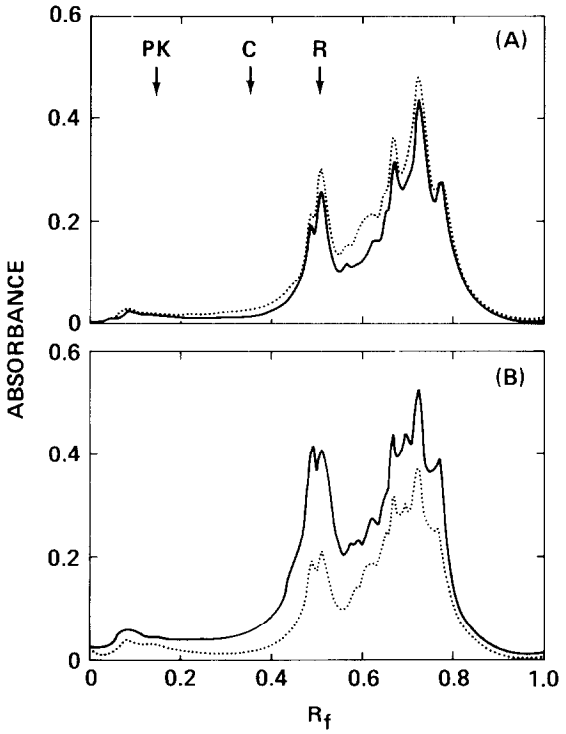

Figure 2. One-dimensional peptide maps of human and rabbit pyruvate kinases. Upper panel (A), peptide maps of human M1 (solid line) and M2 (dotted line) isozymes. Lower panel (B), peptide maps of rabbit M1 (solid line) and M2 (dotted line) isozymes. Data shown are densitometric tracings (600 nm) of four adjacent lanes in a single autoradiogram. The migration of standard proteins is shown (PK, rabbit M1 isozyme of pyruvate kinase; C, chymotrypsin; R, ribonuclease A).

map of the rabbit M2 isozyme (Figure 3); each of these peptides was also present in the rabbit M1 isozyme. The rabbit M2 isozyme could be distinguished from the human M2 isozyme by the presence of several peptides unique to each species (Figure 3). Each of these peptides which distinguished the rabbit and human M2 isozymes were also present in the peptide maps of the M1 isozyme from that species.

Within a given species, $^{[125]}$I labelled peptides present in the M2 isozyme but not present in the M1 isozyme could not be identified by these peptide mapping techniques. $^{[125]}$I labelled peptides present in the M1 isozymes from both species but not present in the M2 isozymes were not identified in the one-dimensional peptide maps. One such peptide was present in the two-dimensional peptide maps of the human and rabbit isozymes (data not shown). Two-

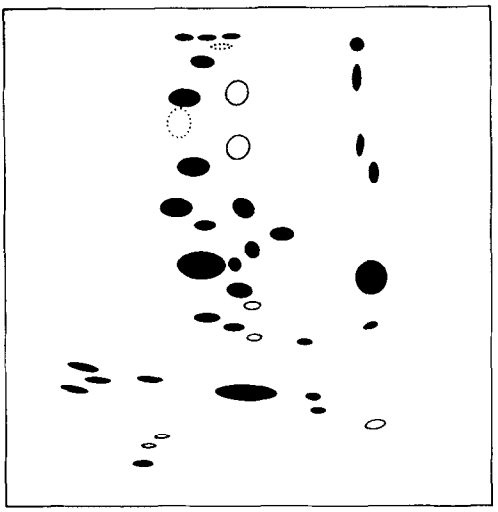

Figure 3. Composite drawing of two-dimensional peptide maps of complete tryptic digests of [125]I labelled human and rabbit M2 isozymes of pyruvate kinase. Electrophoretic separation is represented in the horizontal direction. The origin (anode) is the right lower corner. Peptides common to both rabbit and human M2 isozymes (shaded), peptides found in the human M2 isozyme but not in the rabbit M2 isozyme (solid outline, unfilled) and peptides found in the rabbit M2 isozyme but not in the human M2 isozyme (dotted outline, unfilled) are shown.

dimensional analysis of arginine-containing peptides from mouse isozymes demonstrated two of thirty-two peptides unique to the M2 isozyme and a single peptide unique to the M1 isozyme (7).

DISCUSSION

It has recently been demonstrated that the differences between the M1 and M2 isozymes of pyruvate kinase result from differences in the mRNA coding for these two isozyme types (8). Because different mature mRNAs can be produced from a single gene (16-22), it is possible that the M1 and M2 isozymes are the products of a single gene or two closely related genes. If the M1 and M2 isozymes are the products of two independent genes, inter-species differences could occur in one isozyme type, but not be found in the other isozyme. However, if the two isozymes are the product of the same gene, inter-species differences in the portion of the enzyme common to both isozyme types would result in a similar change in the structure of both the M1 and M2 isozymes.

We compared the immunologic reactivity and peptide maps of the M1 and M2 isozymes of pyruvate kinase from several mammalian species. Inter-species differences were demonstrated by both techniques. In each case, differences in immunologic reactivity of the M2 isozyme toward anti-pyruvate kinase monoclonal antibodies and differences in the enzyme structure of the M2 isozyme demonstrated between two species by peptide mapping were always accompanied by similar differences between the M1 isozymes from the two species. These findings strongly support the conclusion that the M1 and M2 isozymes are the products of a single gene. Presumably reorganization of the gene during development, changes in the portions of the gene which are transcribed and/or changes in the processing of the transcripts into mature mRNA explain the switch from the expression of the M2 isozyme to the expression of the M1 isozyme which accompanies muscle development.

ACKNOWLEDGEMENT

This work was supported by grant GM 28428 from the National Institutes of Health.

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