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Subunit-specific phosphorylation of pyruvate kinase in medullary thyroid carcinomas of the rat

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Pyruvate kinase from anaplastic medullary thyroid carcinomas contains predominantly K-type subunits, whereas pyruvate kinase from differentiated medullary thyroid carcinomas consist of M- and K-type subunits in about equal proportion. In order to analyse the incorporation of phosphate in the respective isozymes after endogenous phosphorylation of cytosolic extracts with [^{32}P]ATP, homotetrameric isozymes as well as heterotetrameric hybrids of differentiated tumors were resolved by affinity chromatography on Blue-Sepharose CL-6B and, if necessary, further purified by immunoprecipitation. SDS-polyacrylamide gel electrophoresis of purified isozymes and subsequent autoradiography showed the incorporation of phosphate in the K_4-type isozymes, but not in the other isozymes. The phosphorylation appeared to be cAMP-independent and occurred on a serine residue.

Pyruvate kinase; Isoenzyme; Purification; Phosphorylation; (Rat medullary thyroid carcinoma, Tumor)

1. INTRODUCTION

Reversible cAMP-dependent phosphorylation of pyruvate kinase (EC 2.7.1.40) from liver (L-type) is an important and well documented [1–4] mechanism for the hormonal regulation of glycolysis and gluconeogenesis in this organ. However, it is essentially unknown whether phosphorylation-dephosphorylation contributes to the regulation of K (kidney)-type and M (muscle)-type pyruvate kinases as well.

Recently, Fister et al. [5] were able to detect physiologically phosphorylated K-type pyruvate kinase in isolated chicken hepatocytes in which it is the predominant isozyme of pyruvate kinase. Phosphorylation was induced by a cAMP-independent protein kinase and occurred on a serine residue. However, the K-type from rat lung was not a substrate for this kinase [6]. Previously, Presek et al. [7] showed that pyruvate kinase from chicken embryo fibroblasts (K-type) became inactivated after infection of these cells with the Rous sarcoma virus, whereas Glossmann et al. [8] demonstrated that the transforming gene product of this virus, pp60$^{v\text{-}src}$, was able to phosphorylate purified K-type pyruvate kinase from chicken liver on a tyrosine residue. In contrast, K-type pyruvate kinase from unfertilized hen's egg could be activated in vitro by phosphorylation by protein kinase C from chicken oviduct [9]. Finally, mammalian K-type pyruvate kinase from pancreatic islet cells was shown to undergo calcium/calmodulin-dependent phosphorylation [10]. As K-type was the predominant isozyme in the tissues and cells used, no mention was made of a possible contribution of the M-type to the observed phosphorylation in any of the above studies.

Previous reports from our and other laboratories have described the expression and regulation of pyruvate kinase in various neoplastic tissues (reviewed in [3,11]). Recently, we described

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Abbreviations: AMTC, anaplastic (undifferentiated) medullary thyroid carcinoma; DMTC, differentiated medullary thyroid carcinoma; FDP, fructose-1,6-bisphosphate

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the isozyme pattern and kinetic properties of pyruvate kinase from differentiated and undifferentiated medullary thyroid carcinomas of the rat [12]. Pyruvate kinase from AMTC was characterised by the predominance of K-type isoenzyme and a loss of M-subunits with respect to DMTC. Here, we describe the K-type specific phosphorylation of pyruvate kinase in these tumors.

2. MATERIALS AND METHODS

2.1. *Carcinomas*

Well-differentiated, slowly growing medullary thyroid carcinomas have spontaneously developed in ageing rats of the Wag/Rij strain. Occasionally, poorly differentiated (anaplastic), rapidly growing carcinomas emerge from these tumors. Both variants have been maintained by subcutaneous transplantation. Tumors were obtained under ether anesthesia, quickly frozen in liquid nitrogen and stored at −70°C until use.

2.2. *Sample preparation*

Tumors were thawed, dissected and homogenised in 25 mM Tris-HCl (pH 7.3), containing 0.25 M sucrose, 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulfonyl fluoride. After removal of cell debris and nuclei (10 min, 1000 × *g*), the cytosol was obtained by ultracentrifugation (30 min, 150000 × *g*).

2.3. *Enzyme assay and isozyme distribution*

Pyruvate kinase activity was assayed as described before [12]. Cellulose acetate electrophoresis and subsequent activity staining of pyruvate kinase isozymes were performed according to previously described methods [12].

2.4. *Phosphorylation*

Phosphorylation of cytosolic extracts containing at least 5 units of pyruvate kinase activity in a total volume of 800 μl was performed in the above buffer after addition of 5 mM NaF and 30 μM Na-vanadate. Reaction mixtures were incubated for 5 min at 20°C before phosphorylation was initiated by the addition of 20 μM [^{32}P]ATP (15 Ci/mmol, New England Nuclear). After 10 min ^{32}P-incorporation was terminated by removing [^{32}P]ATP by gel filtration on Sephadex-G25 (PD10-column, Pharmacia, Sweden), equilibrated in 20 mM K-phosphate buffer, pH 7.1, containing 5% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM aminocaproic acid, 5 mM NaF, 30 μM Na-vanadate and 1 mM NAD. This buffer change was performed to create the proper conditions for the subsequent purification procedure.

2.5. *Purification procedures*

The pyruvate kinase isozymes of cytosolic extracts prepared and phosphorylated as described above were resolved by affinity chromatography according to Schering et al. [6] with some modifications. Briefly, the sample was loaded on a Blue-Sepharose CL-6B (Pharmacia, Sweden) column, equilibrated in K-phosphate buffer (see above). After a first wash with the same buffer the column was extensively washed with buffer from which NAD was omitted. Consecutively, the main fraction of pyruvate kinase activity was eluted by adding 2 mM FDP. The remaining enzyme activity was eluted by increasing the ionic strength with 1 M KCl. The peaks containing pyruvate kinase activity were concentrated by ultrafiltration (Centricon C30, Amicon, Danvers, MA).

If necessary, the isozymes were further purified by immunoprecipitation using a purified rabbit polyclonal antibody against human M-type pyruvate kinase. This antibody strongly crossreacts with M-type and K-type pyruvate kinases from rat. Prior to specific immunoprecipitation, non-specifically precipitating proteins were removed by incubation with non-immune rabbit-IgG coupled to protein A-Sepharose CL-4B (Pharmacia, Sweden) for 60 min at 4°C. Subsequently, the precleared fractions were incubated with protein A-Sepharose-coupled immune IgG for 60 min at 4°C. The protein A-immunocomplexes were collected by centrifugation over a prerun sucrose density gradient consisting of 0.5 ml of 1 M sucrose and 0.25 ml of 0.5 M sucrose in the presence of 200 mM KCl, 1% Nonidet P-40 and 1% Na-deoxycholate. The precipitates were washed again with the same solution lacking

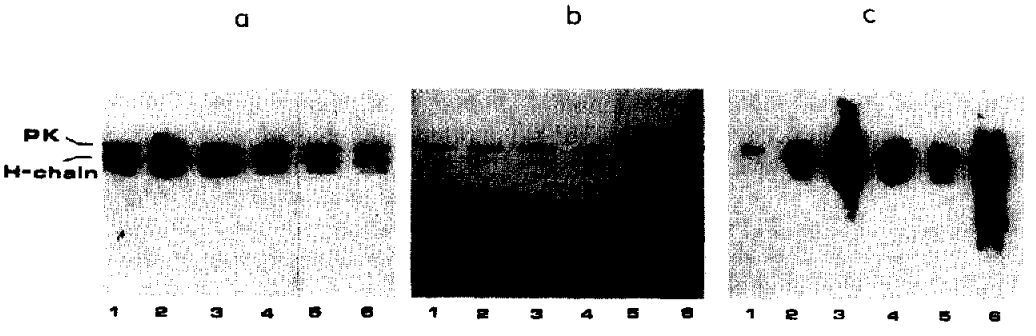


Fig.1. Immunoprecipitates from phosphorylated extracts of AMTC (lanes 1,2,5) and DMTC (lanes 3,4,6) analysed by SDS-PAGE (a), immunoblotting (b) and autoradiography (c). Phosphorylations were performed in the presence of 10 mM Mg^{2+} (lanes 1,3), 5 mM Mn^{2+} (lanes 2,4) and 10 mM Mg^{2+} + 1 μM cAMP (lanes 5,6).

sucrose and collected for SDS-polyacrylamide electrophoresis and phosphoamino acid analysis.

2.6. *Other procedures*

SDS-PAGE of immunoprecipitates and purified enzyme fractions on 8% gels was performed according to Laemmli [13]. Gels were stained with Coomassie brilliant blue and dried between cellophane membranes. Autoradiographs were obtained by exposure of dried gels against X-ray film (Kodak X-omat) using intensifying screens (Cronex-Dupont) at −70°C. Phosphoamino acid analysis was performed according to the method of Cooper et al. [14]. Immunoblotting of SDS-PAGE gels was performed according to standard procedures using a monoclonal antibody against K-type pyruvate kinase from human kidney, which crossreacts with the rat enzyme (unpublished).

3. RESULTS

In our initial experiments pyruvate kinase was immunoprecipitated from phosphorylated tumor extracts (AMTC and DMTC) and analysed by SDS-PAGE, immunoblotting and autoradiography (fig.1). It appeared that pyruvate kinase from both types of tumors was phosphorylated in the presence of either Mg^{2+} or Mn^{2+} in a cAMP-independent manner. Phosphoamino acid analysis of the immunoprecipitates revealed the presence of predominantly P-serine, although a minor spot of P-threonine could be detected as well (fig.2).

The isozyme compositions of the two tumor types were different. Cellulose acetate electrophoresis showed almost exclusively K_4-isozyme in AMTC (fig.3b, lane 5), whereas in DMTC the complete set of M- and K-containing tetramers was observed with a roughly equal proportion of M- and K-subunits (fig.3b, lane 1). We therefore wondered which isozymes and which subunits were

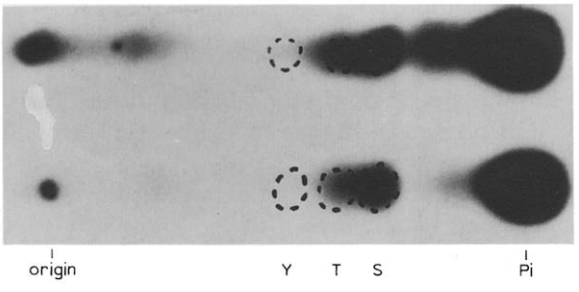
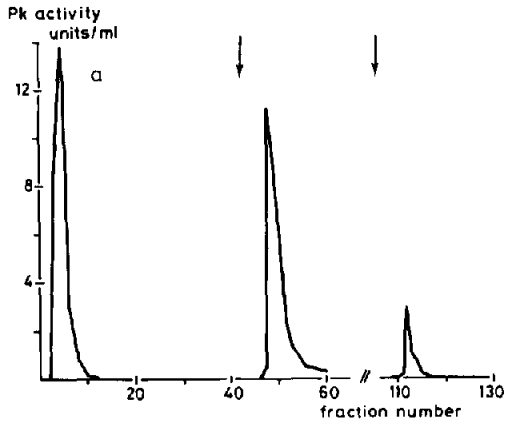


Fig.2. Phosphoamino acid analysis of immunoprecipitates from phosphorylated extracts of AMTC (upper track) and DMTC (lower track). The positions of P-tyrosine (Y), P-threonine (T) and P-serine (S) were detected by ninhydrin staining of added standards (circled areas).



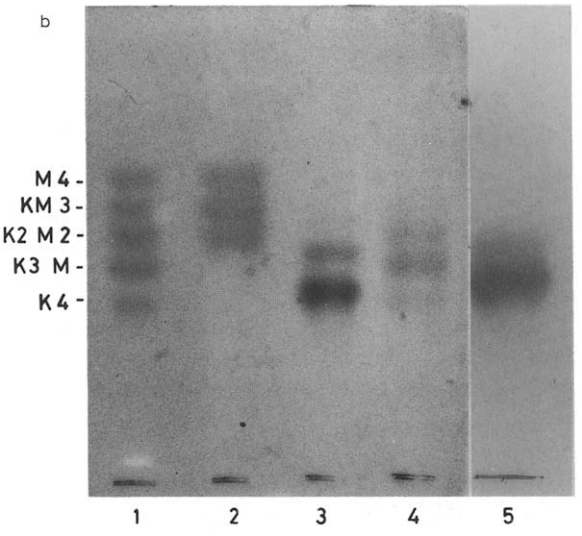


Fig.3. (a) Blue-Sepharose chromatography of pyruvate kinase from DMTC. The arrows indicate the start of the elution with 2 mM FDP and 2 mM FDP + 1 M KCl, respectively. (b) Cellulose acetate electrophoretic patterns of pyruvate kinase from whole cytosolic tumor extracts and chromatographic fractions from a. Lanes: 1, DMTC, whole cytosolic extract; 2, first elution peak from a; 3, peak two; 4, peak three; 5, AMTC, whole cytosolic extract.

involved in the phosphorylation of pyruvate kinase in this tumor. As the molecular masses of M- and K-subunits are very similar [15], our initial approach of immunoprecipitation followed by SDS-PAGE and autoradiography was not satisfactory in this respect. We therefore decided to separate isozymes from phosphorylated DMTC by affinity chromatography on Blue-Sepharose (fig.3a). By this method K_4 pyruvate kinase could be specifical-

ly eluted, whereas M_4 and M_3K appeared in the flow-through. K_3M and K_2M_2 were eluted after specific elution of K_4 by increasing the ionic strength. Consecutively, the K_4 fraction could be analysed without any further purification. Only one major non-phosphoprotein contaminant was present (fig.4a) together with some minor contaminants of which one appeared to be heavily phosphorylated (fig.4b). The fractions containing the other isozymes were further purified by immunoprecipitation prior to analysis by SDS-PAGE and autoradiography. It appeared that type K_4-pyruvate kinase was exclusively phosphorylated, whereas M_4 and M-containing hybrids were not (fig.4b).

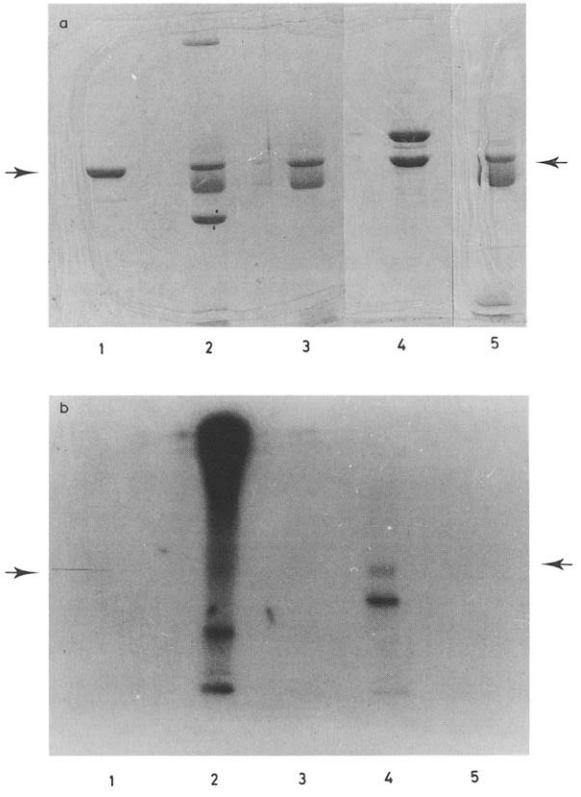


Fig.4. Isozyme-specific phosphorylation of pyruvate kinase from DMTC analysed by SDS-PAGE (a) and autoradiography (b). Pyruvate kinase was either directly immunoprecipitated from a phosphorylated extract (lane 2), or purified by Blue-Sepharose chromatography as shown in fig.3a. Peak one (isozymes M_4 and M_3K) and peak three (K_3M and K_2M_2) were further purified by immunoprecipitation (lanes 3 and 5, respectively). Peak two (type K_4) was electrophoresed directly (lane 4). Rabbit muscle pyruvate kinase (Boehringer, FRG) was used as a referent (lane 1). The arrows indicate the position of pyruvate kinase.

4. DISCUSSION

The expression of K-type pyruvate kinase in tumors and cancer cells is often shown to be linked to their growth rate and differentiation state [3,11]. Eigenbrodt et al. [16] have proposed that this isozymic shift towards the expression of the K-type would enable phosphorylation and hence, inhibition of the enzyme in order to provide adequate amounts of precursors upstream in glycolysis for purine and pyrimidine synthesis. Indeed, cAMP-independent phosphorylation of K-type pyruvate kinase on a tyrosine residue in transformed chicken embryonic fibroblasts was shown to result in a decreased catalytic activity of the enzyme [7]. In contrast, Ca/calmodulin-dependent phosphorylation of the K-type from mammalian pancreatic islet cells apparently did not influence pyruvate kinase activity [10]. The latter studies, however, were not conclusive in this respect, as no data were given on the stoichiometry of the incorporation of phosphate.

In the present and previous studies [12] we showed that the expression of K-type pyruvate kinase is favored in correlation with the dedifferentiation of medullary thyroid carcinomas in accordance with observations on many other tumors [3,11]. We now show that in these tumors K_4-type pyruvate kinase can be phosphorylated by a cAMP-independent protein kinase on a serine residue, whereas the M-subunit containing homo- and heterotetramers are not. This finding fits well in the afore mentioned hypothesis of Eigenbrodt et al. [16], although we were not able yet to show an effect on the catalytic activity or kinetic properties of the enzyme. Conclusions about an influence of phosphorylation on the activity of pyruvate kinase and its physiological relevance, however, can only be drawn after the separate isolation and characterization of phospho- and dephosphoenzymes and determination of the phosphorylation state of the enzyme in in vivo systems.

Pyruvate kinase K- and M-subunits are produced from the same gene by differential RNA splicing [17,18] and differ only in a region of 45 amino acids [17]. This region is supposed to form part of the domain, which is responsible for inter-

subunit contact and for mediating the allosteric properties of the enzyme [17]. As the phosphorylation experiments of the present study are conducted in whole tissue homogenates, the subunit specificity of the phosphorylation is not due to intratumoral heterogeneity of cell types or isozyme compositions, but results from differences in susceptibility of pyruvate kinase isoenzymes to phosphorylation-dephosphorylation reactions. The phosphorylation site of the K_4-type is therefore either situated on the K-type specific region of the K-subunit or is exposed elsewhere on the surface of the enzyme molecule by a specific conformation induced only by the tetramerization of K-subunits.

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