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Pyruvate kinase type M_2 is phosphorylated at tyrosine residues in cells transformed by Rous sarcoma virus

Peter Presek, Manfred Reinacher* and Erich Eigenbrodt°

*Rudolf Buchheim-Institut für Pharmakologie, *Institut für Veterinär-Pathologie* and °*Institut für Biochemie und Endokrinologie, Justus Liebig-Universität Gießen, 63 Gießen, FRG*

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Chicken embryo cells (CECs) contain pyruvate kinase (PK) type M_2 (M_2-PK). Transformation of CECs by Rous sarcoma virus (RSV) leads to a reduction in the affinity of PK for the substrate phospho*enol*pyruvate. In vitro, M_2-PK can be phosphorylated at tyrosine residues by $pp60^{v\text{-}src}$, the transforming protein of RSV. To study tyrosine phosphorylation of M_2-PK in intact RSV-transformed cells, the protein was immunoprecipitated from ^{32}P-labeled normal and RSV-SR-A-transformed CECs. Phosphoamino acid analysis of immunoprecipitated M_2-PK revealed that M_2-PK of both normal and transformed CECs contained phosphoserine and small amounts of phosphothreonine. Only M_2-PK of transformed CECs contained phosphotyrosine in addition. For enzyme kinetic studies M_2-PK was partially purified by chromatography upon DEAE-Sephacel and hydroxyapatite. A decreased affinity for phospho*enol*pyruvate was observed 3 h after the onset of transformation using the temperature-sensitive mutant of RSV, ts-NY 68. The kinetic changes were correlated with tyrosine phosphorylation of M_2-PK, but there is no direct evidence that they are caused by post-translational modification of the enzyme.

Pyruvate kinase; Tyrosine phosphorylation; Transformation; (Rous sarcoma virus)

1. INTRODUCTION

Tyrosine-specific protein kinase activity is rare in normal fibroblasts [1]. Transformation by RSV causes an about tenfold increase in total cell phosphotyrosine and an increased tyrosine phosphorylation of a small list of cellular proteins (for review see [2,3]). It seems of particular interest that glycolytic enzymes, namely phosphoglyceromutase, enolase and lactate dehydrogenase have been described as candidate substrates of the transforming protein, $pp60^{v\text{-}src}$, in RSV-transformed chicken fibroblasts [4,5], because one of the most consistent biochemical characteristics of tumor cells including RSV-transformed cells, is an elevated rate of aerobic glycolysis [6,7].

Reversible phosphorylation of pyruvate kinase type L is an important mechanism of regulation of glycolysis in liver [8,9]. We reported previously that transformation of CECs by RSV leads to characteristic changes in the kinetic properties of pyruvate kinase (PK) including a lower affinity for the substrate phospho*enol*pyruvate [10]. In normal and in RSV-transformed CECs PK has been classified as type M_2 or K [9–11]. We suggested that the kinetic changes may be induced in part by an altered phosphorylation state of M_2-PK and that tyrosine phosphorylation of the enzyme by $pp60^{src}$ may be involved [10,12,13].

The purpose of this study was to demonstrate directly the tyrosine phosphorylation of pyruvate kinase in intact CECs transformed by RSV.

Correspondence address: P. Presek, Rudolf Buchheim-Institut für Pharmakologie, Frankfurter Str. 107, D-6300 Gießen, FRG

Abbreviations: CEC, chicken embryo cell; RSV, Rous sarcoma virus; SR-A, Schmidt-Ruppin strain of RSV, subgroup A; ts-NY 68, temperature-sensitive mutant of RSV, New York 68; PK, pyruvate kinase (EC 2.7.1.40); M_2-PK; PK type M_2; HPT, hydroxyapatite

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2. MATERIALS AND METHODS

2.1. *Materials*

DEAE-Sephacel and protein A-Sepharose were from Deutsche Pharmacia (Freiburg) and hydroxyapatite (DNA-grade) from Bio-Rad (Munich). *O*-Phosphoserine and *O*-phosphothreonine were obtained from Sigma (Munich). *O*-Phosphotyrosine was prepared in our laboratory as described previously [14]. Chemicals and enzymes for the pyruvate kinase test were obtained from Boehringer Mannheim (Mannheim). ^{32}P-orthophosphate, carrier free, [γ-^{32}P]ATP (3000 Ci/mmol) and [^{35}S]methionine (300–800 Ci/mmol) were purchased from Amersham Buchler (Braunschweig).

2.2. *Cells and viruses*

CECs were prepared and maintained as previously described [10]. RSV, Schmidt-Ruppin strain, subgroup A as a wildtype virus and the temperature-sensitive mutant of RSV, ts-NY 68 [15], were used to infect and transform CECs in culture. Nearly confluent cell cultures were labeled for 4 h with ^{32}P-orthophosphate (1 mCi/ml) in phosphate-free Dulbecco's modified Eagle's minimal essential medium with 2% newborn calf serum or with [^{35}S]methionine as described previously [10].

2.3. *Enzyme assays*

Pyruvate kinase activity was determined as described by Eigenbrodt and Schoner [16]. pp60^{src} activity was measured in the solid phase assay using protein A-Sepharose as described previously [10]. The pp60^{src} kinase assays were performed with pp60$^{c\text{-}src}$ for 20 min at 30 °C and with pp60$^{v\text{-}src}$ for 5 min at 4°C.

2.4. *Preparation of extracts and procedure for partial purification of pyruvate kinase*

For cell lysis, 1 ml of ice-cold lysis buffer (10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100) was used for about 10^7 cells. All subsequent steps were carried out at 4 °C. Harvested cells were homogenized in a tightly fitting teflon homogenizer (12 strokes) and centrifuged at 50000 × *g* for 40 min. The resulting supernatant (extract) was used for further purification.

Extracts were passed over DEAE-Sephacel gels (column 1.5 × 10 cm; 3.5 ml of swollen gel), previously equilibrated with buffer A (= lysis buffer but lacking EDTA and Triton X-100). The gels were washed with buffer A and fractions of 2 ml were collected. Those containing pyruvate kinase activity were pooled and applied to hydroxyapatite (1 g) equilibrated with buffer A containing 5% (v/v) glycerol. The washing procedure was performed with the same buffer and bound material was eluted by a linear phosphate gradient (total volume 60 ml) up to 500 mM potassium phosphate. Fractions of 2 ml were collected.

2.5. *Antisera*

Sera of tumor-bearing rabbits (TBR-sera) for immunoprecipitating pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ were obtained as originally described [17]. Antisera against M_2-PK were produced as reported by Reinacher and Eigenbrodt [18].

2.6. *Immunoprecipitation of ^{35}S- and ^{32}P-labeled proteins of cell extracts*

Rabbit antiserum against M_2-PK, TBR-serum and control serum were adsorbed for 2 h at 4 °C onto protein A-Sepharose. 20 μl of antiserum were used for 5 mg (dry weight) protein-A Sepharose. Protein A-IgG complexes were washed 3 times with ice-cold buffer A (100 mM sodium phosphate buffer, pH 7.0; 10 mM EDTA; 10 mM NaF; 0.05% Triton X-100) and antigenic material was incubated together with the immobilized antibodies for 2 h at 4 °C. The immunocomplexes were extensively washed and blotted dry. After addition of 50 μl SDS-sample buffer, the samples were heated at 96 °C for 5 min and analysed on 11% SDS-PAGE according to Laemmli [19]. The ^{32}P-labeled proteins were visualised by autoradiography using intensifying screens (DuPont). Gels containing the ^{35}S-labeled samples were treated with dimethylsulfoxide/PPO (2,5-diphenyloxazol), dried and exposed to Kodak X-Omat film.

2.7. *Other methods*

Protein was measured according to Lowry et al. [20]. Two-dimensional analysis of ^{32}P-phosphoamino acids of ^{32}P-labeled pyruvate kinase was performed according to Hunter and Sefton [21].

3. RESULTS AND DISCUSSION

Direct protein targets of pp60$^{v\text{-}src}$ kinase in RSV-transformed cells are characterized by their phosphotyrosine content [2,3]. To prove this assumption for M_2-PK we analysed the phosphoamino acid composition of the enzyme in normal and RSV-transformed cells. CECs and RSV-SR-A-transformed CECs were labeled for 4 h with ^{32}P-orthophosphate and the clarified cell extracts passed over a DEAE-Sephacel gel. More than 90% of pyruvate kinase activity was found in the flow through accompanied with about 10% of pp60^{src} kinase activity calculated from extracts. Most of pp60$^{v\text{-}src}$ binds onto the DEAE-gel and can be eluted with a NaCl-gradient [22]. Partial purification of M_2-PK was performed to separate most of the pp60^{src} from M_2-PK because both proteins have similiar subunit molecular masses of 60 kDa (fig.1). pp60$^{v\text{-}src}$ seems to be slightly smaller than M_2-PK and in addition shows its typical degradation product of 54 kDa which was never observed in immunoprecipitates performed by M_2-PK antiserum.

Immunoprecipitation experiments in solid phase were carried out with material of the extracts (not shown) and DEAE-Sephacel flow through using TBR-sera for detecting pp60^{src} and preadsorbed antisera against M_2-PK to identify PK. A comparison of normal CECs with RSV-transformed CECs revealed a nearly constant phosphorylation signal of immunoprecipitated M_2-PK after

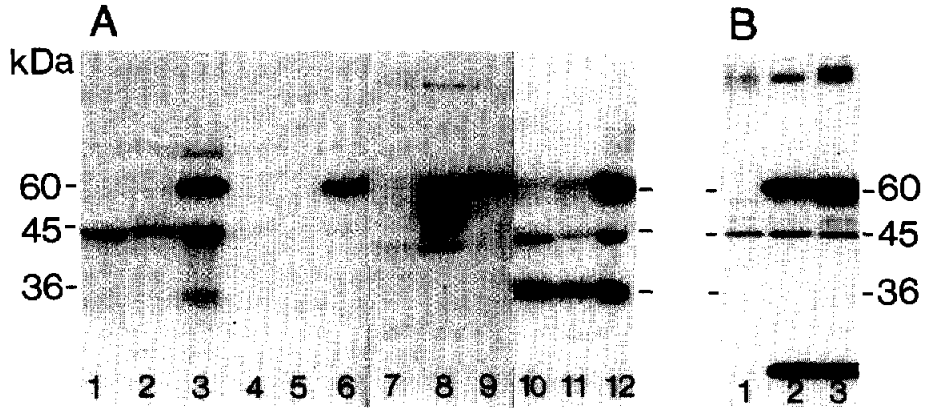

Fig.1. Immunoprecipitation of M_2-PK of normal and RSV-transformed CECs. Normal and RSV-SR-A-transformed CECs were labeled with ^{32}P-orthophosphate or [^{35}S]methionine as described. Extracts of the ^{32}P-labeled cells were chromatographed upon DEAE-Sephacel and subsequently upon hydroxyapatite (HPT). The peak fractions of M_2-PK activity of each column run were pooled and aliquots of 200 μl used for immunoprecipitation experiments (A). Immunoprecipitation of ^{35}S-labeled M_2-PK was performed with 400 μl of extracts (B). The immunoprecipitated labeled proteins were analysed on 11% SDS-PAGE and subsequently autoradiographed. The resulting autoradiograms (A) and the fluorogram (B) are shown. (A) ^{32}P-labeled CECs: lanes 1–3, DEAE flow through; lanes 4–6, HPT column run. ^{32}P-labeled RSV-CECs: lanes 7–9, DEAE flow through; lanes 10–12, HPT column run. Control serum: 1, 4, 7, 10; TBR-serum: 2, 5, 8, 11; M_2-PK antiserum: 3, 6, 9, 12. (B) ^{35}S-labeled cells: (lane 1) CECs, control serum; (lane 2) CECs, M_2-PK antiserum; (lane 3) RSV-CECs, M_2-PK antiserum.

transformation (fig.1A). No enhanced phosphorylation of precipitated M_2-PK could be detected in RSV-SR-A-transformed CECs. To quantitate the amounts of protein precipitated, we labeled the cells with [^{35}S]methionine. The analysis of the gels by fluorography revealed that M_2-PK of both non-transformed and RSV-transformed CECs was precipitated in nearly equal amounts (fig.1B).

Phosphoamino acid analysis of immunoprecipitated M_2-PK showed that both phosphoproteins contained phosphoserine, small amounts of phosphothreonine but only phosphorylated M_2-PK of RSV-SR-A-transformed CECs contained phosphotyrosine (fig.2). The amount of phosphotyrosine detected after acid hydrolysis is small, not more than 5% of total phosphoamino acids, but was distinctly found in four different experiments. Tyrosine phosphorylation of PK seems not to be generally associated with cellular transformation as has been demonstrated by Rijksen et al. [23]. M_2-PK (or K_4-type of PK) of medullary thyroid carcinomas of the rat contained phosphoserine and small amounts of phosphothreonine, but no phosphotyrosine was detectable.

In cellular extracts of RSV-SR-A-transformed CECs, M_2-PK showed a lower affinity for PEP and an increase in specific enzyme activity as compared to extracts of normal CECs [10]. These changes may be induced by a different metabolite composition of the crude extracts, therefore partially purified M_2-PK from the DEAE-Sephacel flow through was further chromatographed upon hydroxyapatite. Elution of M_2-PK was carried out by a linear phosphate gradient. Purification upon hydroxyapatite resulted in a M_2-PK preparation which was substantially free from pp60^{src} (fig.1A). The data of PEP affinity of normal and RSV-SR-A-transformed CECs are shown in table 1.

To study in detail the time course of these phenomena, we used CECs infected with the transformation-defective, temperature-sensitive mutant of RSV, NY 68. M_2-PK was partially purified as described above. We found an about 3-fold decrease in the PEP affinity 3 h after the onset of transformation at the permissive temperature of 36 °C. We can correlate the kinetic changes of M_2-PK with tyrosine phosphorylation of the enzyme but we have no direct evidence that this modification is responsible for the changed kinetic behaviour. The modest degree of tyrosine phosphorylation in M_2-PK (2–5% of total phosphoamino acids) may be too low to explain the metabolic effects observed after transformation. On the other hand, the affinity of enolase for

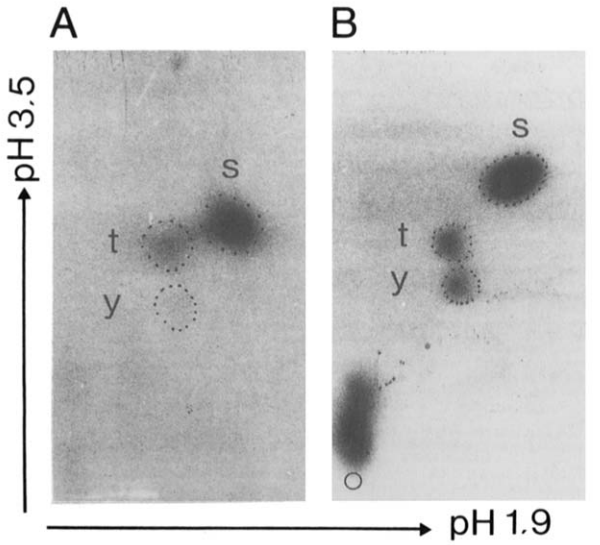

Fig.2. Phosphoamino acid composition of M_2-PK of normal and RSV-transformed CECs. Immunoprecipitated M_2-PK of the DEAE flow through (^{32}P-labeled 60 kDa bands) were cut out of the dried SDS-gels and two-dimensional analysis of phosphoamino acids was performed according to Hunter and Sefton [11] hydrolysing the eluted ^{32}P-M_2-PK with 6 N HCl at 110 °C for 2 h. (A) M_2-PK of CECs; (B) M_2-PK of RSV-SR-A-transformed CECs. The positions of the standard phosphoamino acids, identified by ninhydrin, are shown by dotted lines on the autoradiogram: y, phosphotyrosine; t, phosphothreonin; s, phosphoserine.

its substrates is not affected by tyrosine phosphorylation even though the enzyme becomes phosphorylated at tyrosine residues up to 30–50% of total phosphoamino acids [5]. Recently, Kamps et al. [24] demonstrated that tyrosine phosphorylation of most of the identified protein substrates in RSV-transformed cells including enolase and lactate dehydrogenase does not seem to be essential for inducing transformation. However, this was not investigated with respect to pyruvate kinase.

It has been pointed out that partitioning of glycolytic enzymes may have consequences for their activity in vivo [25]. Changes in partitioning may be a mechanism of metabolic control and may also be regulated by the phosphorylation state of an enzyme. Recently, Low et al. [26] reported that tyrosine phosphorylation of band 3 protein altered binding and activity of some glycolytic enzymes to that protein and it seems that glycolysis may be modulated by this mechanism in vivo. Another report by Pierce and Philipson [27] demonstrated that binding of glycolytic enzymes to sarcolemmal

Table 1

Influence of transformation on phospho*enol*pyruvate (PEP) affinity of partially purified pyruvate kinase type M_2

	$K_{0.5}$ PEP (mM)	Phosphotyrosine
(A) Uninfected CECs	0.53	not detectable (n = 4)[a]
RSV-SR-A-transformed CECs	1.53	detectable (n = 4)[a]
(B) RSV-ts-NY 68		
infected CECs 42 °C	0.66	not detectable[b]
30 min 36 °C	0.54	not performed
3 h	2.00	detectable[b]
12 h	1.48	detectable[b]

[a] Phosphoamino acids analysed from immunoprecipitated M_2-PK

[b] Phosphoamino acids analysed from partially purified M_2-PK (HPT-pool)

and sarcoplasmatic reticular membranes is reversible, charge-dependent and inhibitory. One may speculate that changes in distribution of glycolytic enzymes are also varied by phosphorylation/dephosphorylation altering the actual charge of that protein. It has also been suggested that phosphorylation of pyruvate kinase may serve as a signal for proteolytic degradation [9] but the importance of proteolytic modification of the enzyme to physiological regulation remains to be elucidated.

The complexity of metabolic pathways and the different levels of control make it unlikely that only one enzyme would cause metabolic disregulation producing the transformed phenotype. On the other hand, changes in pyruvate kinase activity, which is the key enzyme in the lower part of the glycolytic pathway, may influence glycolytic disregulation more drastically than phosphoglyceromutase, enolase and lactate dehydrogenase.

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NOTE ADDED IN PROOF

Recently, it has been shown that a larger spectrum of proteins than mentioned in section 1 is phosporylated at tyrosine residues in RSV-transformed cells [Kamps, M.P. and Sefton, B.M. (1988) Oncogene 2, 305–315].