DETECTION OF A NOVEL MAMMALIAN PROTEIN PHOSPHATASE WITH ACTIVITY FOR PHOSPHOTYROSINE

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Received 2 June 1981

1. Introduction

A novel protein kinase activity specifically phosphorylating tyrosine residues has recently been demonstrated [1,2], and several cellular proteins have been shown to contain phosphate on tyrosine [3]. At least two cellular regulatory systems, the EGF receptor and the $(Na^+ + K^+)$-ATPase, have now been reported to be phosphorylated at tyrosine residues [4–6]. Furthermore, cells transformed by Rous sarcoma virus contain up to 10-fold elevated levels of phosphotyrosine [7]. This has led to speculation that phosphorylation of tyrosine may be important in transformation by this virus [7].

In many cases where a metabolic pathway has been shown to be effected by phosphorylation, its regulation appears to be the sum of a kinase/phosphatase interaction [8,9]. With this in mind we have looked in cell extracts for activity capable of removing phosphate from tyrosine residues. Using IgG heavy-chain phosphorylated exclusively at tyrosine as a substrate, we have demonstrated a phosphatase activity capable of dephosphorylating phosphotyrosine. This phosphotyrosine phosphatase activity has properties which are significantly different from those of previously described mammalian phosphatases [9].

2. Materials and methods

IgG heavy chain ^{32}P-labelled in tyrosine was used as a substrate in our assays for phosphotyrosine phosphatase activity. Labelled IgG heavy chain was prepared as described by Collett and Erikson [1], making use of the phosphotyrosine kinase activity present in immunoprecipitates of Rous sarcoma virus-transformed cell extracts and tumor-bearing rabbit serum. Labelled heavy chain was resolved on 11% sodium dodecylsulfate (SDS)-polyacrylamide gels [10], located by exposing X-ray film (Kodak) to wet gels, excised, and simultaneously eluted and dialysed against 0.05 M NH_4HCO_3, pH 8.0 containing 0.1% SDS at 37°C for 20 h. Prior to lyophilization, the heavy chain was dialysed for 4 h at 37°C against 0.05 M NH_4HCO_3, pH 8.0. Acid hydrolysis and thin-layer electrophoresis of the phosphoamino acids [2] showed that the heavy chain prepared by the above procedure had better than 95% of the phosphate on tyrosine (fig.1), and that in the lyophilized form it was stable at room temperature for at least 3 weeks.

Rat muscle and liver extracts were used as a source of phosphotyrosine phosphatase (PTP) activity. Tissue was homogenized for 30 s in a Polytron (Brinkman) with 3 volumes of ice-cold buffer (10 mM Tris–HCl, pH 7.8 at 25°C, containing 4 mM EDTA, 30 mM 2-mercaptoethanol, 250 mM sucrose). Extracts were clarified by centrifugation at 6000 × g for 40 min at 4°C and desalted by gel filtration on Sephadex G-50 into 50 mM Tris–HCl, pH 7.0 at 25°C containing 30 mM 2-mercaptoethanol. The PTP assay, modified from a phosphorylase phosphatase assay [11], consisted of preincubating at 30°C 20 μl of diluted tissue extract with 20 μl of 50 mM Tris–HCl, pH 7.0 at 25°C containing the additions indicated. Five min later the reaction was initiated by the addition of 20 μl ^{32}P-labelled IgG heavy chain (4000 cpm) in 50 mM Tris–HCl, pH 7.0 at 25°C. The reaction was

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terminated after 20 min at 30°C by the addition of 100 μl 20% trichloroacetic acid. Protein-bound ^{32}P was quantitatively precipitated by adding 100 μl of a 10 mg/ml solution of bovine serum albumin, incubating at room temperature for 10 min, and microcentrifugation for 2 min. An aliquot of the trichloroacetic acid supernatant (150 μl) was counted in Liquiscint (National Diagnostics, Somerville, NJ). As an alternative to measuring trichloroacetic acid-soluble ^{32}P, the ^{32}P released was determined to be inorganic phosphate by the molybdate extraction procedure described by Antoniw and Cohen [12]. Briefly, 100 μl of the supernatant from the trichloroacetic acid-treated reaction mixture was mixed with 200 μl of 1.25 mM KH_2PO_4 in 0.5 M H_2SO_4, 500 μl of isobutanol:toluene (1:1) and 100 μl 5% ammonium molybdate. After vigorous shaking, the upper organic phase containing the inorganic ^{32}P was removed and counted in a scintillation counter.

^{32}P-labelled phosphorylase *a* was prepared and phosphorylase phosphatase activity was assayed as described by Foulkes and Cohen [11]. All other materials were reagent grade or better.

3. Results

We have used the heavy chain of IgG specifically radiolabelled with ^{32}P at tyrosine (fig.1) as a substrate for phosphotyrosine phosphatase (PTP) activity. Tissue extracts prepared from rat muscle and liver yielded a phosphatase activity capable of dephosphorylating the heavy chain. A typical result is shown in fig.2. Both the liver and muscle extracts catalysed the release of ^{32}P in a time-dependent manner, and the specific activity of this reaction was similar for the two tissues. Zinc was capable of significantly inhibiting the reaction rate when either the muscle (fig.2) or the liver (data not shown) extracts were used. The release of ^{32}P was biphasic with respect to time. This was not due to enzyme inactivation since, if fresh enzyme was added to the reaction mixture at 20 min, there was no second burst of activity during a subsequent 5-min incubation. Furthermore, additional experiments demonstrated that both the initial and later rates of dephosphorylation were Zn^{2+} sensitive and F^- insensitive (data not shown).

Table 1 shows the effect of various agents on the phosphotyrosine phosphatase activity demonstrable in rat muscle extracts. The effect of these reagents

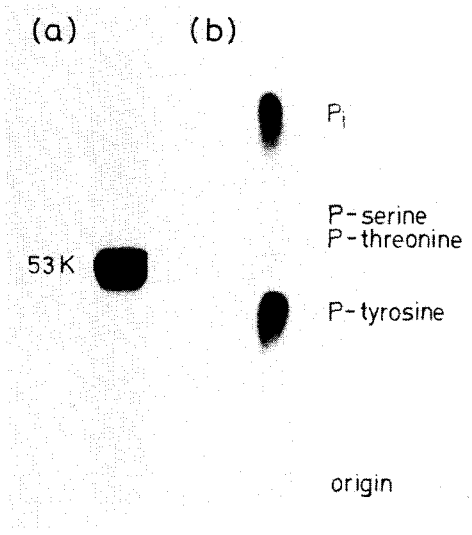

Fig.1. SDS-polyacrylamide gel electrophoretic resolution of ^{32}P-labelled IgG heavy chain (a) and its phosphoaminoacid analysis (b). (a) ^{32}P-labelled heavy chain prepared as described in Materials and methods was re-electrophoresed on 11% SDS-polyacrylamide gels in order to confirm that after lyophilization the ^{32}P remained covalently bound to the heavy chain (53 K). (b) The positions of the marker phosphoaminoacids phosphothreonine, phosphoserine and phosphotyrosine are indicated.

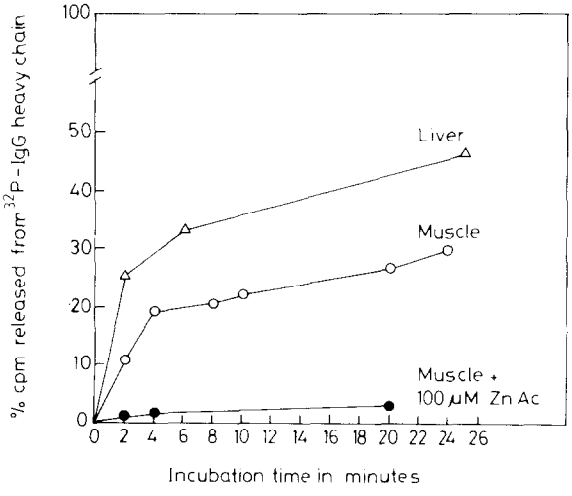

Fig.2. Time course of the phosphotyrosine phosphatase reaction. Tissue extracts and ^{32}P-labelled IgG heavy chain were prepared and the PTP activity assayed as described in Materials and methods. The liver and muscle extracts were diluted to 2.4 mg protein ml^{-1} and 1.3 mg protein ml^{-1}, respectively, in 50 mM Tris–HCl, pH 7.0, at 25°C, containing 30 mM 2-mercaptoethanol. The reaction mixture contained approx. 4000 cpm per reaction tube.

Table 1
Effect of various agents on phosphotyrosine phosphatase activity as a percentage of control values

Additions	Phosphotyrosine phosphatase activity	Phosphorylase phosphatase activity
None	100	100
+ 1 mM EDTA	92	93
+ 50 units phosphatase inhibitor-2 + EDTA	94	33[a]
+ 1 mM $MnCl_2$	78	90
+ 1 mM Mg acetate	85	104
+ 100 μM $CaCl_2$	97	103
+ 100 μM Zn acetate	15	107
+ 150 mM KCl	30	38
+ 50 mM F^- + 1 mM EDTA	95[b]	0–0.2
+ 95°C, 4-min treated extract	0	0

[a] The phosphorylase phosphatase activity of rat muscle extracts is due to two different protein phosphatases termed protein phosphatase-1 and protein phosphatase-2b (J. G. Foulkes, unpublished observation). Protein phosphatase-1, the major activity, is inhibited by inhibitor-2 whereas protein phosphatase-2b is not [9]

[b] Activity is calculated relative to 50 mM KCl; the high ionic strength alone was slightly inhibitory

Rat muscle extracts were incubated with either ^{32}P-labelled IgG heavy chain (phosphotyrosine phosphatase activity) or ^{32}P-labelled phosphorylase *a* (phosphorylase phosphatase activity) as described in the Materials and methods. Enzyme activity (20 min reaction at 30°C) in the presence of the various reagents is given as a percentage of the activity in the absence of additions, each the average of duplicates for two determinations.

on the phosphorylase phosphatase activity present in the same extracts is shown for comparison. It is significant that inhibitor-2, a specific inhibitor of the broad specificity enzyme known as protein phosphatase-1 [11], and sodium fluoride have little effect on the PTP activity while rendering the phosphorylase phosphatase activity of protein phosphatase-1 practically inactive. On the other hand, Zn^{2+} greatly reduced the PTP activity while having no effect on protein phosphatase-1 activity. This was quite specific for Zn^{2+} since the other divalent cations tested (Mn^{2+}, Mg^{2+}, Ca^{2+}) were largely without effect. Similar observations, i.e., the F^-/EDTA and phosphatase inhibitor-2 insensitivity of the PTP activity, were also made with the rat liver extracts.

Measurement of the trichloroacetic acid-soluble ^{32}P in the standard PTP assay does not preclude the possibility that the solubilized label was produced by a proteolytic activity in the crude tissue extract. Therefore, a molybdate-organic solvent extraction technique was used which quantitatively separates inorganic phosphate from ^{32}P-radiolabelled peptides. This method demonstrated that 100% of the trichloroacetic acid-soluble counts removed from the ^{32}P-labelled IgG heavy chain by the tissue extract was recovered as inorganic ^{32}P (results not shown). This inorganic ^{32}P was not the result of H_2SO_4 hydrolysis during the molybdate-organic solvent fractionation since no inorganic ^{32}P was recovered when tissue extract was omitted from the reaction with ^{32}P-labelled IgG heavy chain prior to the solvent fractionation.

4. Discussion

Using a protein probe radiolabelled specifically at tyrosine, we have been able to demonstrate a phosphotyrosine phosphatase activity in rat muscle and liver tissues. This activity is distinct from the broad specificity enzyme termed protein phosphatase-1 not only in its substrate specificity, but also in its sensitivity to Zn^{2+} and its insensitivity to F^-/EDTA and phosphatase inhibitor-2 [9,11]. (As these studies were nearing completion, Dr D. Brautigan, Dept. Biochemistry, Univ. of Washington at Seattle, USA, reported at the EMBO-Meeting on Protein Phosphorylation in Villejuif, March 1981, that he had identified a phosphotyrosine phosphatase with similar properties.) The biphasic nature of the PTP reaction with ^{32}P-labelled IgG heavy chain may indicate that there are several sites for tyrosine phosphorylation on the IgG heavy chain, some of which are especially susceptible to dephosphorylation. It is also significant that the PTP activity was not restricted to a single cell type, but was observed in extracts of both rat liver and muscle, suggesting the ubiquity of this enzymatic activity. (The Zn^{2+}-sensitive, sodium fluoride-insensitive activity is also present in extracts of uninfected and Rous sarcoma virus infected chicken embryo fibroblasts. R. F. Howard and A. Ziemiecki, unpublished observations.)

It follows that, since cells possess an endogenous phosphotyrosine protein phosphatase activity as well as tyrosine-specific protein kinases [3–7], cells can probably dynamically regulate their levels of phosphotyrosine. It is as yet unclear whether the elevated

levels of phosphotyrosine found in cells transformed by Rous sarcoma virus [7] are due solely to increased protein kinase activity or to reduced protein phosphatase activity, or to both. Nevertheless, the demonstration of both phosphorylating and dephosphorylating activities suggests that transformation is associated with an imbalance in the kinase/phosphatase regulation of tyrosine phosphorylation of certain proteins. It will be meaningful to determine whether correction of this imbalance can restore the metabolic regulation typical of cells prior to transformation.

Acknowledgements

The authors wish to thank Drs Philip Cohen, Robert R. Friis and L. S. Jefferson for helpful discussions and for use of their laboratory facilities during the course of these studies. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47, Virologie) and the British Diabetic Association (to Philip Cohen). R. F. Howard was a recipient of a U.S. National Institutes of Health – General Medical Sciences Postdoctoral Fellowship (GM 06868).

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