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Regulation of a rat lung protein tyrosine kinase activity by reversible phosphorylation/dephosphorylation

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Incubation of a partially purified protein tyrosine kinase from rat lung with Mg^{2+} and ATP resulted in about 10–15-fold activation of the enzyme activity as judged by the phosphorylation of poly(Glu:Tyr,4:1), an exogenous substrate. The activation was time dependent and was associated with the phosphorylation of a single protein band of 50 kDa. Phosphoamino acid analysis of the phosphorylated protein indicated that tyrosine was the amino acid being phosphorylated. Upon gel filtration on a Sephacryl S-200 column, the phosphorylated protein co-eluted with protein tyrosine kinase and ATP-binding activities, suggesting that all three activities are part of the same protein. In addition, pretreatment of the partially purified protein tyrosine kinase with alkaline phosphatase inhibited its enzyme activity which could be restored by reincubation with Mg^{2+} and ATP. These data suggest that a temporal relationship exists between the phosphorylation and the activation states of rat lung protein tyrosine kinase, and that the phospho- and dephospho- forms represent the active and inactive (or less active) forms, respectively, of the enzyme.

Protein tyrosine kinase; Autophosphorylation; Dephosphorylation; Activation; Inactivation

1. INTRODUCTION

Protein tyrosine kinases are enzymes which catalyze the phosphorylation of proteins in tyrosine residues ([1] and for a review see [2]). These protein kinases have been shown to be intrinsic to the transforming gene products of several retroviruses [3–5] and to the receptors of several mitogenic polypeptide growth factors and hormones including epidermal growth factor [6], platelet-derived growth factor [7], insulin [8], insulin-like growth factor-I [9] and colony-stimulating factor [10]. In addition, protein tyrosine kinase activities have also been detected in normal and even in non-proliferative non-nucleated cells [11–13]. It has recently been demonstrated that intrinsic protein tyrosine kinase activity of certain hormone receptors and oncogenes is necessary for the expression of their biological activities [14–16].

Although the activities of hormone receptor protein tyrosine kinase have been shown to be regulated by receptor occupancy, the possible regulator(s) or modulator(s) of retroviral or non-viral, non-receptor protein tyrosine kinase is (are) unknown. However, one common feature shared by several protein tyrosine kinases is their ability to undergo an autophosphorylation reaction [2]. The autophosphorylation has been shown to cause an activation [17–21], inactivation [22] or no effect [23] on various protein tyrosine kinases.

The present studies were undertaken therefore, to examine the effect of autophosphorylation and dephosphorylation on the protein kinase activity

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol

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of a partially purified protein tyrosine kinase from rat lung particulate fractions.

2. MATERIALS AND METHODS

2.1. *Materials*

Poly(Glu:Tyr, 4:1), L-tyrosine-agarose, insoluble alkaline phosphatase and Nonidet P-40 were purchased from Sigma. Electrophoresis chemicals were obtained from Bio-Rad Laboratories. [γ-^{32}P]ATP was obtained from Amersham. All other chemicals were purchased from commercial sources.

2.2. *Methods*

2.2.1. Partial purification of tyrosine protein kinase from rat lung

Tyrosine protein kinase utilized in these studies was partially purified from rat lungs as described earlier [24] except that it was further purified on an L-tyrosine-agarose column as described by Fukami and Lipmann [25]. The specific activity of the enzyme at this stage was about 1 nmol/min per mg protein using poly(Glu:Tyr, 4:1) as exogenous substrate.

2.2.2. Assay of protein tyrosine kinase activity

Protein tyrosine kinase activity was determined by the phosphorylation of exogenous substrate [poly(Glu:Tyr, 4:1)] as described earlier [24].

2.2.3. Autophosphorylation

Autophosphorylation of the purified enzyme preparation was performed in a medium containing 50 mM Tris-Cl, pH 7.5, 30 mM $MgCl_2$, 100 μM sodium orthovanadate, 10 μM leupeptin, 10 μM pepstatin A, 100 μM [γ-^{32}P]ATP (spec. act. 2000–4000 cpm/pmol), and an aliquot of the enzyme preparation. The incubation was performed at 30°C and the reaction was terminated by adding an equal volume of a stop mixture containing 5% SDS (w/v) and 50% (v/v) β-mercaptoethanol. The labeled proteins were analyzed by subjecting the aliquots to SDS-PAGE followed by autoradiography as described earlier [26].

In experiments where the effect of autophosphorylation on protein tyrosine kinase activity was studied, the autophosphorylation conditions were the same as above except that 100 μM unlabeled ATP was used and the reaction was terminated by adding an excess of EDTA.

3. RESULTS

3.1. *Effect of preincubation with Mg-ATP on protein tyrosine kinase activity*

Fig.1 shows that the preincubation of a partially purified preparation of protein tyrosine kinase with Mg-ATP activated the kinase activity as judged by the phosphorylation of an exogenous substrate, poly(Glu:Tyr, 4:1). The activation was rapid and time dependent. About 3–6-fold activation was observed as early as 15 min after preincubation, reaching near maximum activation

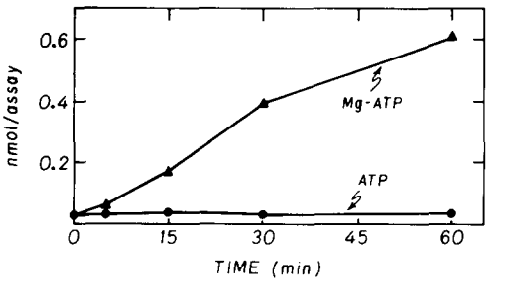

Fig.1. Effect of preincubation with Mg-ATP on protein tyrosine kinase activity. Purified rat lung protein tyrosine kinase preparations were incubated in a medium containing 50 mM Tris-Cl, pH 7.5, 100 μM sodium orthovanadate, 10 μM leupeptin, 10 μm pepstatin A, 30 mM $MgCl_2$ and 100 μM unlabeled ATP (Mg-ATP) (▲) or in the same medium but without Mg (ATP alone) (●). The reaction was done at 30°C and at indicated times, the reaction was stopped by adding 35 mM EDTA (final conc.) in Mg-ATP (▲) tubes and 35 mM EDTA plus 30 mM $MgCl_2$ (final conc.) in ATP (●) tubes and by transferring the tubes to an ice bath. After diluting, both sets of tubes were assayed for their protein tyrosine kinase activity by using poly(Glu:Tyr, 4:1) as described in section 2.

(about 15-fold) after 60 min. Incubation of the kinase preparations with ATP alone (fig.1) or with Mg^{2+} alone (not shown) did not alter the enzyme activity, suggesting that the phosphorylating conditions (in the presence of both Mg^{2+} and ATP) are essential for the enzyme activation.

3.2. *Effect of preincubation with Mg-[γ-^{32}P]ATP on the phosphorylation of lung protein tyrosine kinase preparation*

Since phosphorylating conditions resulted in the activation of the lung tyrosine kinase activity, it was of interest to determine whether these conditions alter the phosphorylation of protein(s) present in the partially purified preparations of rat lung protein tyrosine kinase. The data in fig.2 show that incubation of the partially purified preparation with Mg^{2+} and [γ-^{32}P]ATP resulted in the phosphorylation of single 50 kDa protein band as determined by SDS-PAGE and autoradiography. The phosphorylation of this band was time dependent. The phosphoamino acid analysis of this band indicated that tyrosine was the only amino acid being phosphorylated (not shown).

3.3. *Co-elution of autophosphorylatable protein, kinase and ATP-binding activity*

To determine if the 50 kDa phosphoprotein cor-

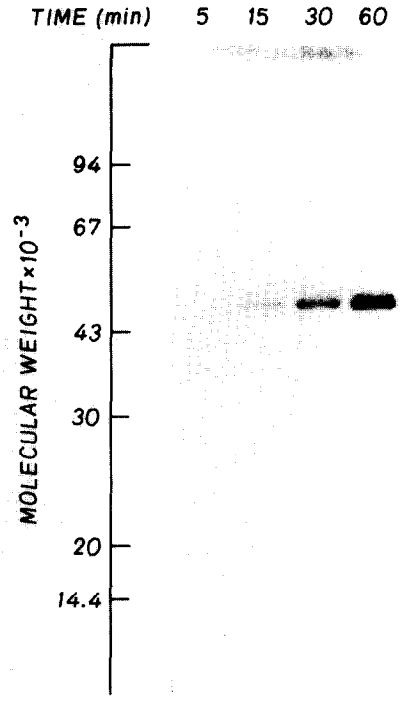

Fig.2. Time course for the autophosphorylation of rat lung protein tyrosine kinase. The purified protein tyrosine kinase preparation was subjected to autophosphorylation as described in section 2 and labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

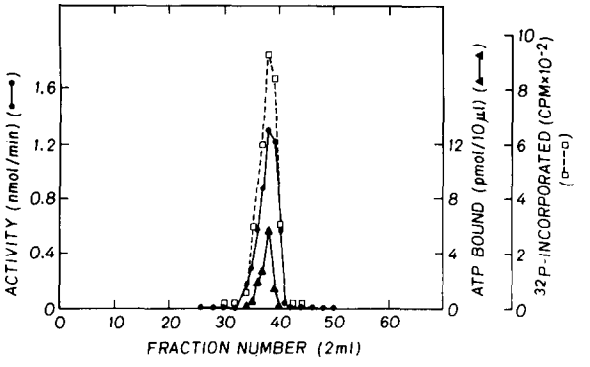

Fig.3. Co-elution of autophosphorylatable protein, protein tyrosine kinase and ATP-binding activity from a Sephacryl S-200 column. A partially purified protein tyrosine kinase preparation from rat lung was subjected to gel filtration on a Sephacryl S-200 column (1 × 85 cm) equilibrated in 25 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 0.1% Nonidet P-40, 5% glycerol and 100 mM KCl. 2 ml fractions were collected at a flow rate of 8 ml/h. Aliquots from each fraction were assayed for autophosphorylation, protein tyrosine kinase activity using exogenous substrate poly(Glu:Tyr, 4:1) and [α-^{32}P]ATP-binding activity as described in section 2.

responded to the protein tyrosine kinase activity, the partially purified preparation of rat lung tyrosine kinase was subjected to gel filtration chromatography and the fractions were analyzed for autophosphorylation, tyrosine kinase and ATP-binding activities. The data presented in fig.3 show that all the 3 activities co-eluted in the same fraction and indicate that these activities are the function of the same protein. An analysis of the peak fraction after autophosphorylation followed by SDS-PAGE and autoradiography revealed the labeling of the 50 kDa band (not shown), suggesting that this band represents the protein tyrosine kinase.

3.4. *Effect of dephosphorylation on protein tyrosine kinase activity*

Since the phosphorylation resulted in the activation of the kinase activity, the effect of dephosphorylation on the kinase activity was studied. For these experiments the partially purified rat lung protein tyrosine kinase was dephosphorylated by treatment with insoluble alkaline phosphatase coupled to beaded agarose.

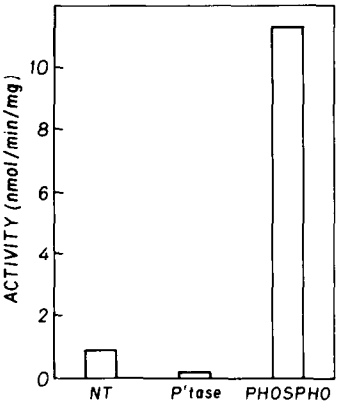

Fig.4. Effect of dephosphorylation and rephosphorylation on protein tyrosine kinase activity. A partially purified preparation from rat lung was incubated with insoluble alkaline phosphatase coupled to beaded agarose for 15 min at 24°C and then centrifuged to remove the phosphatase. The clear supernatant was divided into two fractions: one of the fractions (P'tase) was used as such for assaying its protein tyrosine kinase activity, and the other fraction was incubated with 30 mM Mg and 100 μM unlabeled ATP in an autophosphorylation medium for 60 min before assaying its protein tyrosine kinase activity (PHOSPHO). NT represents the activity in a control sample which was not treated with alkaline phosphatase.

The data presented in fig.4 show that the phosphatase treatment resulted in about 80% inactivation of the kinase activity as compared to untreated control. Furthermore, when the phosphatase-treated preparation was incubated with Mg-ATP, in order to rephosphorylate the protein, the kinase activity was not only restored to its original value, but was also stimulated by about 10-fold. These data indicate that the phosphorylation and dephosphorylation of rat lung protein kinase are fairly reversible processes and that they lead to the activation and inactivation, respectively, of the enzyme activity.

4. DISCUSSION

In this study, we have shown that a partially purified protein tyrosine kinase from rat lung is activated by autophosphorylation of tyrosine residues. Although autophosphorylation-mediated activation of insulin receptor protein tyrosine kinase [17] is well established, the autophosphorylation-mediated activation of EGF-receptor protein tyrosine kinase remains controversial: Bertics and Gill [20] showed its activation, whereas Downward et al. [23] were unable to demonstrate any effect. Furthermore, the protein tyrosine kinase activity of the gene products of Rous sarcoma virus (RSV), pp60Src [18], and Fujinami sarcoma virus, p130 gas-bps [19], are stimulated by phosphorylation, but the activity of a corresponding proto oncogene product of RSV, pp60 c-Src, has been shown to be inhibited by phosphorylation [22]. Similarly, a non-viral, and non-receptor associated normal cellular protein tyrosine kinase from rat spleen is also activated by autophosphorylation [21]. In addition, autophosphorylation of protein tyrosine kinases purified from soluble and particulate fractions of bovine thymus [27] and spleen [28] has also been demonstrated, but the effect of autophosphorylation on the kinase activity was not studied in these cases. Thus, it would appear that autophosphorylation is a property shared by most, if not all the protein tyrosine kinases.

The fact that alkaline phosphatase treatment, which selectively dephosphorylates the phosphotyrosine residue of protein [29], leads to the inactivation and that its rephosphorylation, by Mg-ATP treatment, causes an activation of the protein tyrosine kinase activity provides direct evidence for the reversibility of the autophosphorylation reaction. In addition, these data demonstrate, for the first time, that a temporal relationship exists between the phosphorylation and activation states of the kinase and that the phospho- and dephospho- forms represent the active and inactive (or less active) forms, respectively, of the lung protein tyrosine kinase.

Autophosphorylation of protein tyrosine kinase might serve as an important regulatory mechanism to control its catalytic activity and thereby the phosphorylation of its endogenous substrates. At present, however, the nature of factor(s) controlling the phosphorylation state of the kinase is (are) not known. It is also not known if altered physiological states would alter the in vivo phosphorylation levels of the enzyme. Further work is needed to clarify these points.

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