Phosphorylation and activation of p40 tyrosine kinase by casein kinase-1

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Because examination of regulatory *trans*-phosphorylations can help elucidate the cellular functions of tyrosyl protein kinases, we have investigated the effects of phosphorylation by casein kinase-1 on the activity of the p40 tyrosyl protein kinase. We find that casein kinase-1 can phosphorylate the p40 tyrosyl kinase on serine and threonine residues, in part on a unique tryptic peptide. The phosphorylation induces a substantial increase in the tyrosyl protein kinase activity of p40, in contrast to most instances in which serine/threonine phosphorylation inhibits activity of tyrosyl protein kinases. These findings raise the possibility that p40 might be part of a protein phosphorylation network in which casein kinase-1 participates.

Protein kinase; Phosphotyrosine; Casein kinase-1; Protein

1. INTRODUCTION

Tyrosyl protein kinases were originally identified as the products of certain viral oncogenes [1] and in many cases have subsequently been shown to be transduced cellular regulatory enzymes (reviewed in [2]). For example, v-*erb*-B, the oncogene of avian erythroblastosis virus, is a mutated form of the receptor for epidermal growth factor, and v-*fms* is a mutated form of the receptor for colony stimulating factor-1 [3,4]. It is clear that these tyrosine kinases can be regulated not only by binding to their cognate ligand, but also by phosphorylation – either auto-phosphorylation, or *trans*-phosphorylation by other kinases. For example, the EGF and insulin receptors rapidly autophosphorylate on tyrosine in response to ligand binding, but also become phosphorylated on threonine by protein kinase C [5–15]. Both sorts of phosphorylation have been postulated to have regulatory significance, the auto-phosphorylation apparently activating and the threonine phosphorylation inhibiting receptor activity. Pp60^{src}, encoded by the *src* proto-oncogene, has no known regulatory ligand, but nonetheless is subject to regulatory phosphorylation. In the case of this tyrosyl protein kinase, phosphorylation at tyrosine 527 inhibits the enzyme's activity, whereas phosphorylation at serines 12 (protein kinase C) or 17 (protein kinase A) or tyrosine 416 (auto-phosphorylation) reportedly increases the tyrosine kinase activity of pp60^{src} under some circumstances [16–27]. In addition, pp60^{src} can be phosphorylated on serine and threonine by kinases regulated in conjunction with mitosis, apparently homologues of the yeast cell-cycle kinase cdc2 [28].

Identification of regulatory *trans*-phosphorylations can play an important part in elucidating the signalling systems and mechanisms of regulation utilized by tyrosine kinases of unknown cellular function. For example, the fact that pp60^{src} can be phosphorylated by cdc2, protein kinase A and protein kinase C suggests that this proto-oncogene product is in regulatory communication with these signalling systems and that it functions during the onset of mitosis. Consistent with this suggestion are findings that expression of constitutively activated forms of pp60^{src} greatly alters both adenylate cyclase activity and phosphatidylinositol turnover [29,30].

Because of the obvious regulatory significance of many tyrosine protein kinases, several such enzymes have been purified from normal tissue prior to determining their cellular functions [31,32]. One such kinase has been isolated from bovine thymus, and found to have a M_r of 40 kDa in its purified form [32,33]. The cellular functions of the p40 tyrosine kinase are currently unknown. To understand how the activity of this enzyme might be regulated, we have examined the ef-

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fects of *trans*-phosphorylation on enzyme activity. We report here that the serine/threonine protein kinase casein kinase-1 can phosphorylate p40 in vitro. The phosphorylation occurs in part on a unique tryptic peptide and induces a substantial increase in the tyrosine kinase activity of p40. These findings raise the possibility that p40 might be part of a protein phosphorylation network in which casein kinase-1 participates.

2. MATERIALS AND METHODS

2.1. *Enzyme purification and assay*

The protein tyrosine kinase (p40) was purified from bovine thymus as described by Zioncheck et al. [32]. Casein kinase-1 was obtained from rat liver cytosol as indicated in [34].

The phosphorylation of p40 was carried out at 30°C for 10 min. The reaction mixture contained 25 mM Tris pH 7.4, with 5 μCi of [^{32}P]γ-ATP (100 μM), 50 ng p40 10 mM $MgCl_2$, and 0.2 units of casein kinase-1 in a total volume of 200 μl. The reactions were stopped with the addition of SDS sample buffer and then subjected to electrophoresis on 12.5% SDS/polyacrylamide gels, as described by Laemmli [35]. The dried gels were then exposed to XAR-5 film (Kodak).

To examine the activation of the kinase activity of p40, incubations identical to those described above, but using nonradioactive ATP, were carried out. The p40 activity was subsequently measured by standard assay procedures according to [32].

2.2. *Phosphoamino acid analysis*

To determine the phosphoamino acid composition of phosphorylated proteins, phosphoproteins were eluted as described [36]. The sample was hydrolyzed in 6 N HCl for 1.5 h at 110°C under nitrogen atmosphere. Identification of phosphoamino acids was carried out by electrophoresis on cellulose thin-layer plates at pH 3.5 at 1000 V for 60 min. Phosphoserine, phosphothreonine and phosphotyrosine were used as markers and visualized by ninhydrin staining.

2.3. *Analysis of phosphopeptides by HPLC*

The phosphorylated p40 was excised from one-dimensional polyacrylamide gels and submitted to tryptic digestion. Trypsin digestion was carried out at 30°C with TPCK-trypsin (10 mg/ml added every 8 h) for 24 h. The material was then lyophilized and peptide mapping was carried out on HPLC using a Synchrom RP-P analytical column. The chromatography was developed using an acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. The gradient conditions were 0% (10 min), 0–25% acetonitrile/water (50 min), 25–75% (10 min) and 75% (5 min). The flow rate was 1 ml/min and fractions of 1.0 ml were collected and analyzed by Cerenkov counting.

3. RESULTS AND DISCUSSION

We first tested whether the p40 protein tyrosyl kinase could be phosphorylated by casein kinase-1. Both p40 and casein kinase-1 were capable of autophosphorylation (Fig. 1, lanes 1 and 2). When p40 was incubated with casein kinase-1 in the presence of Mg^{2+} and [γ-^{32}P]ATP, we found a marked increase in the phosphorylation of p40 tyrosine kinase (lane 3), relative to the autophosphorylation seen without preincubation (lane 1). The same result was obtained when p40 was immunoprecipitated with specific antibodies after phosphorylation with casein kinase-1 (data not shown).

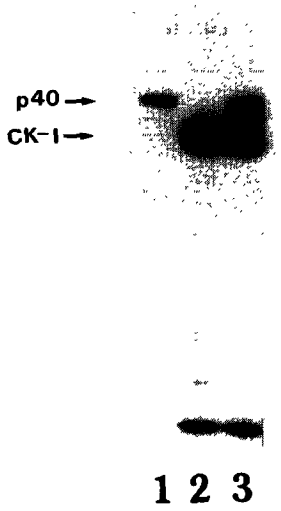

Fig. 1. Autophosphorylation and casein kinase-1 (CK-1)-mediated phosphorylation of p40 tyrosine kinase. Autoradiograph of autophosphorylated p40 tyrosine kinase (lane 1), autophoshorylated casein kinase-1 (lane 2) and p40 tyrosine kinase phosphorylated by casein kinase-1 (lane 3).

The phosphoamino acids in p40 phosphorylated by casein kinase-1 were analyzed by one-dimensional electrophoresis at pH 3.5. The autophosphorylation of p40 tyrosine kinase was shown to occur only at tyrosine residues, whereas phosphoserine and phosphothreonine were also evident after phosphorylation by casein kinase-1 (Fig. 2). The relatively small amount of phosphoserine and phosphothreonine could not account for the marked increase in the phosphorylation of p40 tyrosine kinase by casein kinase-1. Therefore, we suspected that the phosphorylation of p40 by casein kinase-1 enhanced the tyrosyl protein kinase activity of p40, which was then reflected in an increased autokinase activity.

To determine whether a change in the specific tyrosine kinase activity of p40 was induced by the casein kinase-1-catalyzed phosphorylation on serine and threonine residues, the effect of pre-incubation with casein kinase-1 on p40 activity was assayed by the ability of p40 to phosphorylate the peptide substrate angiotensin. (Casein kinase-1 cannot phosphorylate angiotensin, so the phosphorylation of this peptide is a measure solely of the kinase activity of p40.) Table I shows that a differential phosphorylation of angiotensin was consistently detected and was generally 1.5- to 2-fold greater in the p40 phosphorylated by casein kinase-1 than in p40 which had been pre-incubated under identical conditions without casein kinase-1. The enhancement of p40 activity was seen at various NaCl

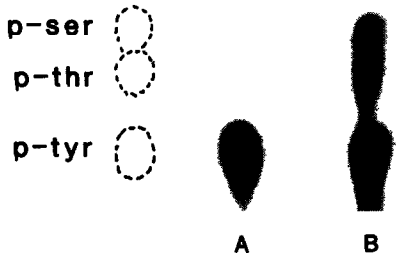

Fig. 2. Phosphoamino acid analysis of p40 tyrosine kinase. (A) Autophosphorylated p40 tyrosine kinase and (B) p40 phosphorylated by casein kinase-1 were extracted from a one-dimensional gel, hydrolyzed and subjected to thin-layer chromatography as indicated in section 2. P-ser, phosphoserine; p-thr, phosphothreonine; and p-tyr, phosphotyrosine.

concentrations, and thus the effect of casein kinase-1 appears to occur in addition to the previously described salt-dependent activation of p40 activity [32,33].

To define phosphorylation site(s) affected specifically by casein kinase-1, phosphorylated samples of p40 were subjected to extensive proteolysis using trypsin, and the ^{32}P-labeled peptides were separated by reverse-phase chromatography (Fig. 3). $^{32}PO_4$ incorporated into p40 by autophosphorylation was recovered in four peaks, with most of the radioactivity present in peak 4. Five peaks were observed in p40 phosphorylated by casein kinase-1, four of which coincided with the phosphopeptides labeled by autophosphorylation. However, the percent of the phosphorylation found in peak 4 was higher in p40 which had been phosphorylated by casein kinase-1 than it was in the autophosphorylated p40. When the radioactive peptides were analyzed to identify phosphoamino acids, it was found that peptides 1, 2, 3 and 4 contained phosphotyrosine and peptide 5 contained similar amounts of phosphotyrosine, phosphothreonine and phosphoserine (data not shown). These results support the conclusion that the p40 tyrosine kinase activity is stimulated by phosphorylation with casein kinase-1.

The role of p40 in cellular metabolism is not known, but like many other intracellular protein kinases, it

Table I

p40 Tyrosine kinase activity

NaCl (mM)	p40 phosphorylated by[a]		Activation ratio
	Auto-phosphorylation	CK-1	
12[b]	100	180 ± 19	1.8
200[c]	144 ± 3.4	294 ± 16	2.0
1000[d]	255	529	2.0

[a] % of autophosphorylation of p40 at 12 mM. Results represent the means ± SEM of 4 independent experiments for [b] and [c] and 1 experiment for [d]

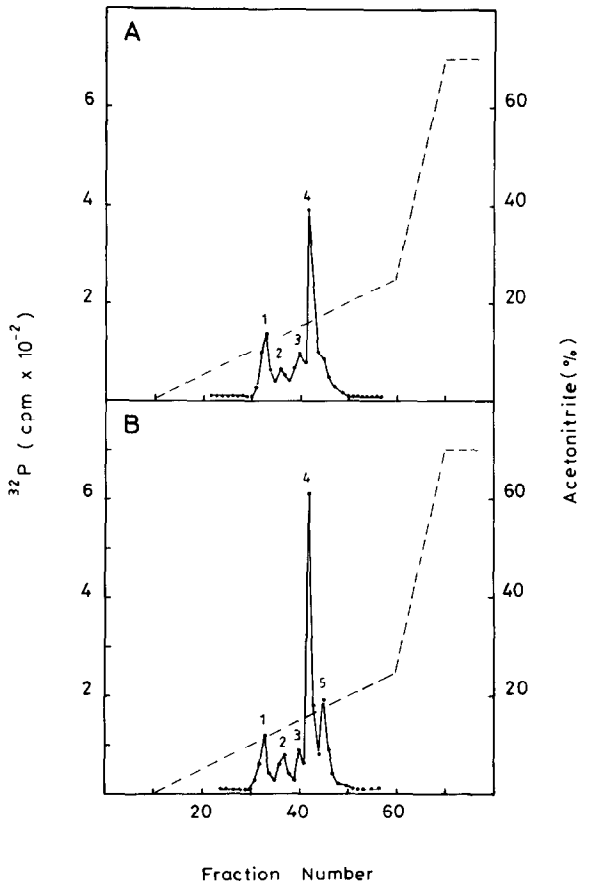

Fig. 3. HPLC chromatograms of tryptic ^{32}P-peptides from p40 tyrosine kinase. (A) p40 tyrosine kinase autophosphorylated and (B) p40 phosphorylated by casein kinase-1 were eluted from one dimensional SDS polyacrylamide gels and subjected to proteolysis with trypsin. Phosphopeptide fragments were resolved by chromatography on HPLC (see section 2).

might have a role in the growth response of cells to external signals. Interestingly, the p40 kinase has recently been found to be a fragment of a larger protein [33]. Although it is not known what significance this missing portion of p40 might have for the regulation and function of the enzyme, it is clear that at least some regulatory aspects of the kinase are maintained in the p40 portion, namely the strong sensitivity to salt and its regulation by *trans*-phosphorylation (Table I).

In summary, the phosphorylation of p40 protein tyrosine kinase by casein kinase-1 at serine and threonine residues has been studied, and found to result in an increase in its tyrosine-specific protein kinase activity. Moreover, at least two specific phosphorylation sites affected by casein kinase-1 have been shown. Most serine/threonine phosphorylations of tyrosyl protein kinases inhibit the activity of the tyrosyl kinase. The results presented here represent only the second case in which a serine/threonine phosphorylation is associated with a tyrosyl kinase activation, the other case being $pp60^{src}$ [19,27,28]. Elucidation of the physiological significance of these results will depend on future infor-

mation on the in vivo functions of the p40 kinase and of casein kinase-1. However, our findings indicate that p40 might be part of a kinase network which also involves casein kinase-1 and perhaps other serine/threonine kinases.

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