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MAP kinase binds to the NH_2-terminal activation domain of c-Myc

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Abstract The transcription factor c-Myc is a substrate for phosphorylation by MAP kinases. Here we demonstrate that MAP kinase binds to c-Myc. The NH_2-terminal region (residues 1–100) is necessary and sufficient for this interaction. Binding to c-Myc is not dependent on the state of MAP kinase activation. However, the c-Myc/MAP kinase complex is disrupted by ATP. Together, these observations indicate that substrate binding interactions contribute to the specificity of phosphorylation by MAP kinases.

Key words: MAP kinase; c-Myc; Protein phosphorylation

1. Introduction

We have recently identified a novel family of human protein kinases, JNKs (also designated SAPKs [1]), that are activated by dual phosphorylation at Thr and Tyr within sub-domain VIII [2]. The mechanism of JNK activation by dual phosphorylation is similar to that reported for the ERK sub-family of MAP kinases [3,4]. However, the signal transduction pathways that lead to JNK and ERK activation are distinct [2]. Thus, there are at least two MAP kinase signal transduction pathways in mammalian cells.

A distinctive property of JNK protein kinases is the high level of substrate specificity of these enzymes. The only JNK substrate that has been identified is the transcription factor c-Jun [1,2,5,6]. This high level of substrate specificity is mediated by the binding of JNK to the δ sub-region of the transactivation domain of c-Jun [2,5,6]. Interestingly, the δ sub-region of v-Jun contains a deletion and it has been demonstrated that this mutation accounts for the failure of JNK protein kinases to bind and phosphorylate v-Jun [2,5,6]. Together, these data indicate that JNK protein kinases bind and phosphorylate specific substrates. ERKs, like JNKs, also exhibit a high level of substrate specificity [7–9]. It has been established that ERKs bind to NGF receptors [10,11], the protein kinase $p90^{rsk}$ [12,13], and the MAP kinase phosphatases 3CH134 and PAC-1 [14,15]. However, the role of these binding interactions is poorly understood.

The purpose of the studies described in this report was to investigate the interactions of ERK MAP kinases with substrates. We have previously demonstrated that ERK phosphorylates the transcription factor c-Myc at Ser-62 [7]. Here, we show that ERK binds to the NH_2-terminal activation domain of c-Myc. This binding interaction requires an extended region of c-Myc (residues 1–100) that includes the site of phosphorylation by ERK. Together, these data indicate that ERK, like JNK, can bind to substrates. This binding interaction provides a mechanism that may contribute to the substrate specificity of ERK and JNK MAP kinase isoforms.

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2. Materials and methods

2.1. Materials

[^{32}P]Phosphate was purchased from DuPont-NEN. [γ-^{32}P]ATP was prepared using a Gamma-Prep A kit (Promega Biotech) as described by the manufacturer. Thrombin was purchased from Sigma Chemical Co. Polyvinylidene difluoride membranes (Immobilon-P) were obtained from Millipore Corp. Purified $p34^{cdc2}$/cyclin B was obtained from Upstate Biotechnology Inc. Human ERK2 was purified from phorbol ester-treated cells as described [8,16]. Recombinant GST-fusion proteins were prepared by bacterial expression and glutathione agarose (Sigma Chemical Co.) affinity chromatography [17]. The recombinant c-Myc (residues 1–143) was purified after thrombin cleavage of GST-Myc [18].

2.2. Tissue culture

CHO cells were maintained in Ham's F12 medium supplemented with 5% (v/v) fetal bovine serum (Gibco-BRL). COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum (Gibco-BRL). Transient transfection of COS-1 cells was performed using 2 μg of plasmid DNA [19].

2.3. Plasmids

The plasmids encoding GST-fusion proteins with c-Myc residues 1–439 and 98–439 [20], residues 1–262 [7], and derivatives of residues 1–204 [21] have been described. Other GST-Myc constructs were prepared by sub-cloning PCR fragments of the c-Myc gene into the vector pGEX-3X (Pharmacia-LKB Biotechnology Inc.). The structure of the plasmids constructed was confirmed by dideoxy sequencing. The plasmids encoding GST fusion proteins with c-Jun residues 1–79 [5], Rb residues 379–928 [22], EGF-R residues 647–688 [23] have been described. The GST-GAP-SH2 consists of residues 178–277 of human GAP cloned into pGEX-2T [24]. The human ERK2 expression vector pCMV-$p41^{mapk}$ and pCMV-(Ala-54/55)$p41^{mapk}$ were described previously [16,19].

2.4. In vitro protein kinase assays

Phosphorylation assays were performed in 50 μl of kinase buffer (10 mM $MgCl_2$, 25 mM HEPES (pH 7.4) with 50 μM [γ-^{32}P]ATP (10 μCi/nmol). The phosphorylation reactions were terminated by the addition of Laemmli sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. In-situ protein kinase assays using the substrate polymerized into the gel were performed after SDS-PAGE by renaturation and incubation with [γ-^{32}P]ATP as described [25].

2.5. Binding assays

Cells were lysed in binding buffer (1 mM EDTA, 0.1% Nonidet P-40, 150 mM KCl, 25 mM β-glycerophosphate, 1 mM Na-orthovanadate, 10 μg/ml leupeptin, 1 mM PMSF, 25 mM HEPES pH 7.4) and a soluble extract was prepared by centrifugation in a microfuge for 30 min at 4°C. The cell extract (200 μg) was then incubated with recombinant

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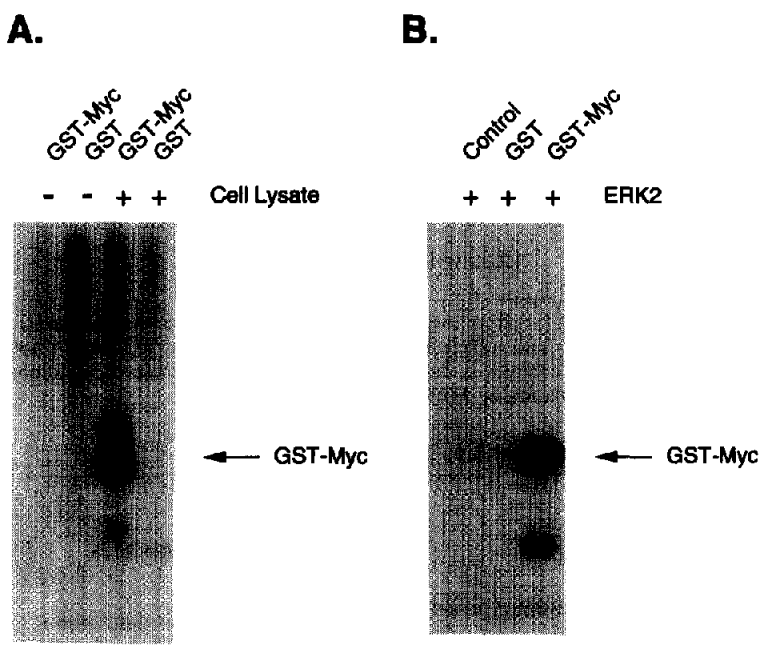

Fig. 1. Identification of a protein kinase activity that binds to c-Myc. Cell lysates (A) or purified ERK2 (B) were incubated with GST or GST-Myc (residues 1–143) pre-bound to glutathione-agarose. The agarose was incubated for 90 min at 4°C and then washed extensively. The agarose beads were resuspended in kinase buffer with [γ-^{32}P]ATP and incubated for 10 min at 22°C. The phosphorylation reaction was terminated by the addition of Laemmli sample buffer. The phosphorylated proteins were examined by SDS-PAGE and autoradiography.

GST fusion proteins (30 μg) pre-bound to 40 μl of GSH-agarose in 0.5 ml binding buffer. After incubation for 90 min at 4°C, the agarose beads were washed extensively with binding buffer. The bound proteins were eluted with Laemmli sample buffer.

2.6. Western blot analysis

Proteins resolved by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes (Immibilon-P) and analyzed by Western blotting. The membranes were probed with a monoclonal anti-ERK2 antibody (Zymed Inc.) or a monoclonal anti-p34^{cdc2} antibody (Transduction Laboratories Inc.). Immune complexes were visualized using the enhanced chemiluminescence procedure (Amersham International PLC).

3. Results

3.1. Association of protein kinase activity with the transcription factor c-Myc

The binding of protein kinases was investigated by incubation of c-Myc with extracts prepared from cultured cells. The immobilized c-Myc was washed extensively and the bound protein kinases were detected by addition of [γ-^{32}P]ATP. These experiments demonstrated that one or more protein kinases bind to c-Myc (Fig. 1A). We investigated the bound protein kinases by SDS-PAGE using an in-gel protein kinase assay with the substrate (c-Myc) polymerized in the gel. The major activity detected corresponded to a 42-kDa protein kinase (data not shown). In previous studies, we have demonstrated that the 42-kDa protein kinase ERK2 phosphorylates c-Myc within the NH_2-terminal activation domain at Ser-62 [7]. We therefore tested the hypothesis that ERK2 may account, in part, for the protein kinase activity present in cell extracts that binds to c-Myc. It was observed that purified ERK2 bound to immobilized c-Myc (Fig. 1B). Together, these data demonstrate that the MAP kinase ERK2 binds and phosphorylates the transcription factor c-Myc.

3.2. ERK2 forms a complex with c-Myc

To establish the specificity of the interaction between ERK2 and c-Myc, we examined whether ERK2 binds to other proteins. Recombinant GST-fusion proteins corresponding to both substrates and non-substrates were prepared (Fig. 2A). These proteins were incubated with purified ERK2 and [γ-^{32}P]ATP. GST-Myc [7], GST-EGFR [25] and GST-Jun [7] were phosphorylated by ERK2 (Fig. 2B). However, GST, GST-Rb, and GST-GAP-SH2 were not detectably phosphorylated (Fig. 2B). The binding of ERK2 was examined by incubation of each GST-fusion protein with cell extracts. The complexes were collected on GSH-agarose beads and washed extensively. ERK2 bound to the GST fusion proteins was detected by Western blot analysis. Fig. 2C demonstrates that the binding of ERK2 was only observed in experiments using GST-Myc. This observation demonstrates that c-Myc associates with ERK2.

Previous studies have demonstrated that c-Myc is phosphorylated in vitro at Ser-62 by p34^{cdc2} [26,27]. The phosphorylation of the same site by p34^{cdc2} and ERK2 suggests that p34^{cdc2}, like ERK2, may bind to c-Myc. To examine this possible interaction, we incubated cell lysates with immobilized c-Myc, and detected the bound kinase by Western blot analysis. Fig. 3 shows that p34^{cdc2} binding to c-Myc was not detected. Together, these data provide evidence for a specific binding interaction between c-Myc and ERK2.

3.3. ERK2 binds to the NH_2-terminal activation domain of c-Myc

To define the site of interaction of ERK2 with c-Myc, we

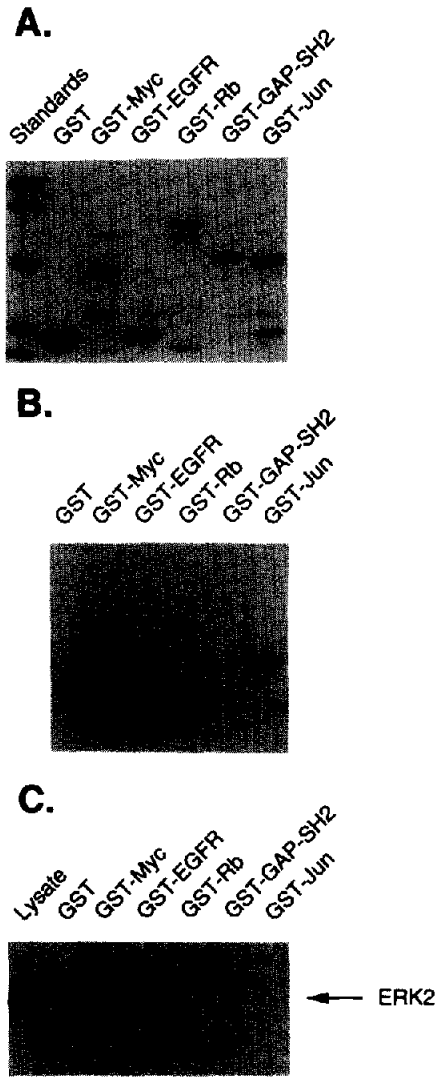

Fig. 2. Demonstration of complex formation between ERK2 and c-Myc. (A) Purified GST-fusion proteins were resolved by SDS-PAGE and stained with Coomassie blue. The protein standards are 106, 80, 49.5, 32.5 and 27.5 kDa. (B) The GST-fusion proteins were incubated with purified ERK2 and [γ-^{32}P]ATP for 10 min at 22°C. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. (C) The binding of ERK2 to the GST-fusion proteins was examined. Cell extracts were incubated with GST-fusion proteins pre-bound to glutathione-agarose. After incubation for 90 min at 4°C, the agarose was washed extensively. The bound fraction was subjected to Western blot analysis with an anti-ERK2 monoclonal antibody. Binding of ERK2 to GST-Myc (residues 1–143) was observed. However, no binding of ERK2 to GST, GST-Rb, GST-EGFR, GST-GAP-SH2, or GST-Jun was detected.

prepared a series of recombinant proteins consisting of different domains of c-Myc. Fig. 4 shows that ERK2 binds to the full-length c-Myc protein and to NH_2-terminal fragments of c-Myc. Deletion analysis demonstrates that c-Myc residues 1–100 were required and sufficient for binding to ERK2. This region contains the site of c-Myc phosphorylation by ERK2 (Ser-62).

3.4. ATP disrupts the c-Myc/ERK2 complex

We investigated the role of ERK2 protein kinase activity in the binding interaction with c-Myc. It was found that ERK2 activation resulted in no significant change in binding to c-Myc (Fig. 5). This observation suggests that ERK2 protein kinase activity is not required for interaction with c-Myc. To test this hypothesis, we investigated the properties of a catalytically inactive mutated ERK2 (Lys^{54}/Lys^{55} replaced with Ala^{54}/Ala^{55}). Fig. 5 shows that there was no marked difference in binding to c-Myc in experiments using wild-type and catalytically inactive forms of ERK2.

To further examine the role of ERK2 catalytic activity, we investigated the effect of substrate phosphorylation on ERK2 binding to c-Myc. We incubated immobilized GST-Myc bound to ERK2 in the presence and absence of ATP. Fig. 6 shows that bound ERK2 dissociates slowly from the complex with c-Myc. However, addition of ATP resulted in the dissociation of ERK2.

4. Discussion

It has been established that there is a critical role for protein–protein interactions in MAP kinase signal transduction pathways. For example, in the yeast *Saccharomyces cerevisiae*, genetic and biochemical studies have demonstrated that the MAP kinase Fus3 is activated within a protein complex consisting of the Ste5 protein bound to the MAPKK Ste7 and the MAPKKK Ste11 [28]. An Ste5-like protein has not been identified in mammalian cells. However, additional protein–protein interactions in the mammalian MAP kinase pathway have been described. Thus, the MAPKKK c-Raf-1 functions as a large

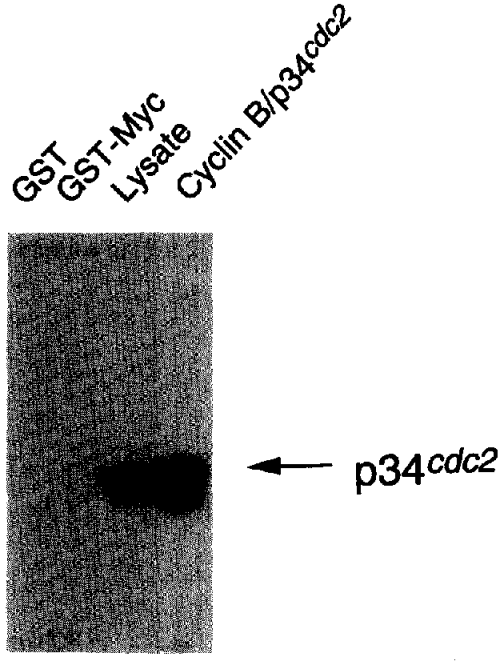

Fig. 3. c-Myc does not bind to the $p34^{cdc2}$ protein kinase. CHO cells were arrested in mitosis using nocodazole. The cells were lyzed in binding buffer and a soluble extract was prepared by centrifugation at 100,000 × *g* for 15 min. The extract was incubated for 90 min at 4°C with GST or GST-Myc (residues 1–143) pre-bound to glutathione-agarose. The agarose was washed extensively and the bound proteins were analysed by immunoblotting with a monoclonal anti-$p34^{cdc2}$ antibody. The antibody specificity was confirmed by immunoblot analysis of the cell lysate and purified $p34^{cdc2}$/cyclin B.

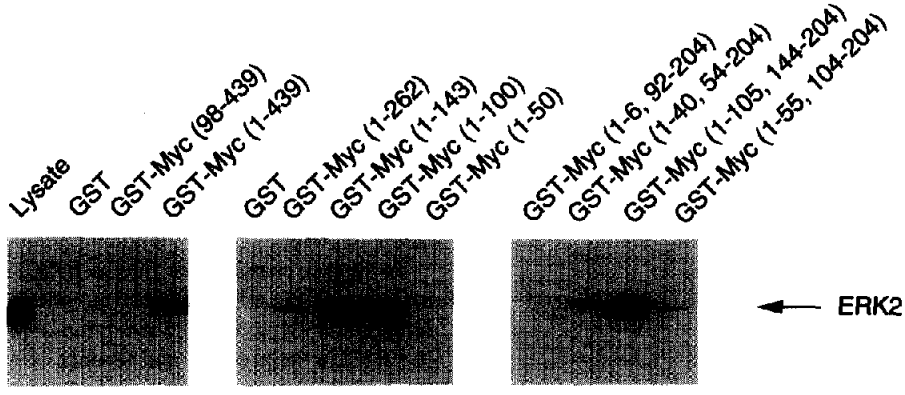

Fig. 4. ERK2 binds to an NH_2-terminal region of the c-Myc transactivation domain. The binding of ERK2 to a panel of GST-Myc fusion proteins was examined. Cell extracts were incubated with GST and GST-fusion proteins pre-bound to glutathione agarose. After incubation for 90 min at 4°C, the agarose was washed extensively and the bound fraction was subjected to Western blot analysis with an anti-ERK2 monoclonal antibody. The region required for binding to ERK2 was identified as the c-Myc NH_2-terminus (residues 1–100).

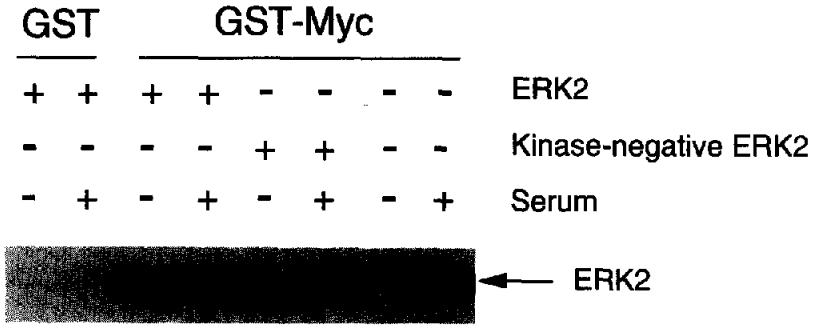

Fig. 5. Enzymatically active and inactive forms of ERK2 bind to c-Myc. COS cells were mock-transfected or transfected with an ERK2 expression vector. The COS cells were serum-starved for 18 h and activated with 10% (v/v) fetal calf serum for 30 min. The cells were then harvested and soluble extracts were prepared. These extracts were incubated with GST and GST-Myc (residues 1–143) pre-bound to glutathione-agarose. After incubation for 90 min at 4°C, the agarose was washed extensively and the bound fraction was subjected to Western blot analysis with an anti-ERK2 monoclonal antibody.

membrane-bound complex [29] that associates with Ras and the MAPKK Mek [29–34]. Protein–protein interactions have also been implicated in the mechanism of substrate phosphorylation by the JNK group of MAP kinases [2,5,6]. In contrast, a role for protein–protein interactions in the substrate specificity of the ERK group of MAP kinases has not been established.

Substrate recognition by ERK is dependent on the primary sequence surrounding the site of phosphorylation [4,7–9]. Although the optimal motif is Pro-Xaa-Ser/Thr-Pro, the minimal primary sequence required for phosphorylation is Ser/Thr-Pro [4]. This primary sequence motif (Ser/Thr-Pro) is, however, insufficient to account for the high level of ERK substrate specificity [4]. Substrate selection may therefore be guided by properties of the substrate in addition to the primary sequence at the site of phosphorylation. Substrate binding represents one

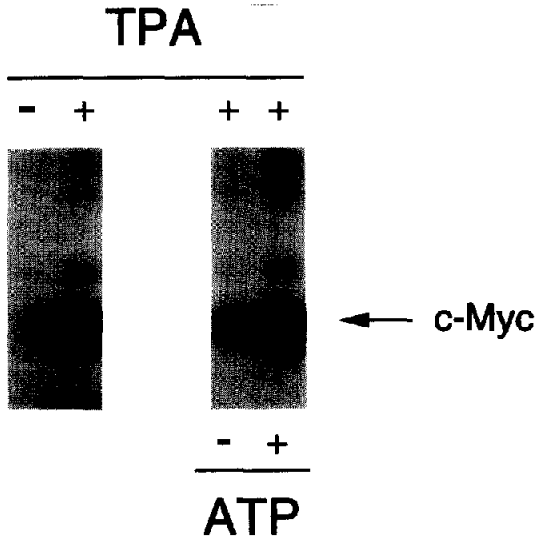

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Fig. 6. The ERK2 complex with c-Myc is disrupted by ATP. COS cells were transfected with an ERK2 expression vector. The cells were treated with and without 100 nM TPA for 30 min prior to harvesting. The soluble extracts obtained were incubated with GST-Myc (residues 1–143) pre-bound to glutathione-agarose. After incubation for 90 min at 4°C, the agarose was washed extensively. The bound proteins were eluted at 22°C for 20 min using kinase buffer supplemented with and without 50 μM ATP. The ERK2 activity bound to the column prior to elution (left) and in the eluate (right) was measured using the c-Myc NH_2-terminal domain and [γ-^{32}P]ATP as substrates. The c-Myc NH_2-terminal domain substrate was prepared by purification of the c-Myc fragment obtained after thrombin cleavage of GST-Myc (residues 1–143).

mechanism that may contribute to the specificity of substrate phosphorylation by ERK.

In this report we describe the binding of ERK to the substrate c-Myc. Previous studies have described the functional interaction of c-Myc with several proteins, including Max [35,36], the p107 Rb-related protein [37], TFII-I [38], and the TATA-binding protein [39,40]. Complex formation with ERK therefore represents an additional interaction mediated by c-Myc.

The binding interaction with c-Myc does not require ERK2 protein kinase activity. However, the formation of the c-Myc/ERK2 complex is disrupted by ATP. This effect of ATP suggests that the c-Myc/ERK2 complex represents an intermediate in the phosphorylation reaction rather than a non-productive enzyme/substrate complex. This binding interaction therefore provides a mechanism that may contribute to the specificity of substrate phosphorylation by ERK.

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