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c-Myc Transactivation Domain-Associated Kinases: Questionable Role for Map Kinases in c-Myc Phosphorylation

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Abstract We have isolated and characterized cellular kinases which associate with the transactivation domain of c-Myc and phosphorylate Ser-62. We demonstrate that cellular Map kinases associate with c-Myc under stringent conditions and phosphorylate Ser-62. We also find that TPA stimulates the activity of the Myc-associated Map kinase to phosphorylate Ser-62. However, we do not observe an increase in Ser-62 phosphorylation in endogenous c-Myc after TPA treatment of cells. Since the regulation of the c-Myc-associated Map kinases does not correlate with the in vivo regulation of Ser-62 phosphorylation in c-Myc, we conclude that Map kinases are not the in vivo kinases for Ser-62. Although Ser-62 phosphorylation was not affected by TPA, phosphorylation at a different serine residue was significantly upregulated by TPA. J. Cell. Biochem. 72:483–491, 1999. © 1999 Wiley-Liss, Inc.

Key words: c-Myc; phosphorylation; kinases

Phosphorylation has been found to regulate the activities of many transcription factors and proto-oncogenes [Hunter and Karin, 1992; Meek and Street, 1992]. c-Myc has been shown to be highly phosphorylated, having at least five phosphorylation sites in the N-terminus transactivation domain, and five other sites in the C-terminus of the protein [Lutterbach and Hann, 1997]. Several studies have investigated the role of phosphorylation in c-Myc function. C-terminal casein kinase II sites in c-Myc are located adjacent to the DNA-binding domain [Luscher et al., 1989], but mutations at these sites did not alter the ability of c-Myc to co-transform Rat-1 cells with the *bcr-abl* oncogene [Street et al., 1990]. In contrast, alteration of phosphorylation in the N-terminus transactivation domain of c-Myc does have biological effects. Mutation at the Thr-58 phosphorylation site has been shown to enhance the transforming ability of c-Myc [Frykberg et al., 1987; Palmieri et al., 1983; Symonds et al., 1989; Chen et al., 1989]. The loss of Thr-58 is found in the majority of v-Myc proteins [Papas and Lautenberger, 1985] and in c-Myc proteins from Burkitt's lymphomas [Bhatia et al., 1993]. Although the in vivo Thr-58 kinase has not yet been identified, we have previously shown that a purified glycogen synthase kinase-3 α (GSK-3 α) specifically phosphorylates Thr-58 in vitro, but only if Ser-62 was prephosphorylated [Lutterbach and Hann, 1994]. Because the in vivo phosphorylation of Thr-58 is also dependent on a prior phosphorylation of Ser-62 [Lutterbach and Hann, 1994], we consider GSK-3 α a candidate physiological kinase for Thr-58.

The role of Ser-62 phosphorylation in c-Myc function remains controversial. Pulverer et al. [1994] has shown that a Ser-62 mutation severely reduces the ability of c-Myc to co-transform rat embryo fibroblasts with Ha-*ras*, while a Thr-58 mutation enhances transformation. In contrast, Henriksson et al. [1993] demonstrated that loss of either Thr-58 or Ser-62 enhances the ability of Rat 1A cells to grow in soft agar. In vitro, Ser-62 can be phosphorylated specifically by the mitogen activated protein (Map) kinases and the cyclin-dependent kinases (cdk) [Lutterbach and Hann, 1994; Seth et al., 1992] but the in vivo role of these kinases in the phosphorylation of Ser-62 has not been resolved. We have previously shown that phosphorylation of Ser-62

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in overexpressed c-Myc is stimulated by mitogens, suggesting that Map kinase is a candidate in vivo kinase for Ser-62 [Lutterbach and Hann, 1994]. Also in support of Map kinase involvement, Seth et al. [1992] have reported that overexpression of $p42^{mapk}$ enhanced Ser-62 phosphorylation on coexpressed c-Myc. However, we have found that coexpression of $p42^{mapk}$ and/or activated *ras* did not affect Ser-62 phosphorylation [Lutterbach and Hann, 1994], suggesting that Map kinases are not the in vivo Ser-62 kinase.

In this report we have isolated and characterized cellular kinases which associate with the N-terminal 100 amino acids of c-Myc. We demonstrate that Map kinases from cell extracts do bind to GST-Myc and phosphorylate Ser-62. Although TPA stimulates the activity of the Myc-associated Map kinase to phosphorylate Ser-62 on GST-Myc, TPA does not stimulate phosphorylation of Ser-62 on the endogenous c-Myc protein in vivo. This result thus questions the role for Map kinases in Ser-62 phosphorylation in vivo.

MATERIALS AND METHODS

Cell Lines

NIH 3T3 and COS-7 cells were obtained from American Type Culture Collection (ATCC). COS cells and fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO Laboratories, Grand Island, NY) supplemented with 10% calf serum (defined-supplemented; Hyclone Laboratories, Logan, UT) and 1,000 U of 1:1 penicillin-streptomycin (GIBCO Laboratories) per ml, and were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Plasmids

Construction of GST-Myc50 and GST-Myc100 plasmids has been previously described [Lutterbach and Hann, 1994]. GST proteins were purified with glutathione agarose as described by Smith and Johnson [1988]. Single-stranded DNA form murine c-Myc in the pRcCMV vector (Invitrogen, La Jolla, CA) was used to create the A58 and A62 point mutations using *dut-ung- E-coli* as described [Kunkel, 1985]. Mutations were verified by sequence analysis, and the BamH1/PvuII fragment (expressing the N-terminal 100 amino acids of the A58 and A62 proteins) was then subcloned into pGex2T. The GST-Myc100 A58 and GST-Myc100 A62 proteins were purified as described [Smith and Johnson, 1988].

Cell Labeling

For mapping of the endogenous c-Myc protein, approximately 5×10^7 cells were labeled with 12.5 mCi of [^{32}P]-orthophosphate (ICN) for 3 h at 37°C in phosphate-free medium. For TPA stimulation, cells were then incubated with TPA (100 ng/ml) for 20 min.

Immunoprecipitation

Labeled cells were washed with 10 mM Tris-HCl, pH 8, 150 mM NaCl and solubilized in cold antibody lysis (Ab) buffer [Gaubatz et al., 1995] containing 10 mM iodoacetamide, 0.1 mM sodium vanadate, 15 mM sodium fluoride, 0.1 T.I.U. aprotinin, and 0.1 mM PMSF. The cellular lysate was then sonicated, precleared with *Staphylococcus aureus* membranes (Immuno-Precipitin; Bethesda Research Laboratories, Bethesda, MD), and immunoprecipitated with 5 μg of anti-mu-myc12C antibody and Immuno-precipitin as previously described [Hann et al., 1983].

Peptide Mapping and Phosphoamino Acid Analysis

[^{32}P]-orthophosphate-labeled c-Myc proteins were immunoprecipitated, separated by SDS-PAGE, transferred to nitrocellulose, and digested off the membrane with 10–20 μg of the indicated proteases, followed by performic acid oxidation (1 h at 0°C) and repeated lyophilization as previously described [Boyle et al., 1991]. Secondary digestion with proline endopeptidase (ICN) digestion was performed as previously described [Lutterbach and Hann, 1994]. The digested fragments were separated in the first dimension by electrophoresis using a Hunter thin layer electrophoresis chamber in pH 1.9 buffer (1.5 kV, 20 min), and then separated in the second dimension by ascending chromatography in phosphochromatography buffer [Boyle et al., 1991]. The origin of loading was located in the lower left corner of the thin-layer cellulose plate (Merck, Darmstadt, Germany) unless otherwise indicated.

Phosphoamino acid analysis was conducted on in vitro phosphorylated peptides or proteins and in vivo labeled proteins by acid hydrolysis at 110°C in 6 M HCl for 1 h. Samples were then lyophilized and analyzed first by electrophore-

sis at pH 1.9 for 20 min at 1.5 kV (with phosphoserine, phosphothreonine, and phosphotyrosine markers) and then by electrophoresis in the second dimension at pH 3.5 for 16 min at 1.6 kV. Markers were visualized with ninhydrin.

Myc-Associated Kinase Isolation

Cell extracts were prepared by scraping NIH 3T3 cells into lysis buffer (20 mM Hepes, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 1% Triton 100, 0.05% SDS, 0.5 mM DTT, 0.1 mM Na_3VO_4, 20 mM β-glycerophosphate, 100 μg/ml PMSF and 0.1 TIU aprotinin). After sonication, insoluble material was removed by centrifugation at 12,000g for 5 min at 4°C. The supernatant (300 μg of protein) was incubated with shaking at 4°C for 2 h with 1 μg of either GST, GST-Myc-100 or GST-Myc-50 protein (equalized by coomassie blue staining) immobilized on glutathione agarose beads. The beads were recovered by centrifugation at 4°C and then washed three times with 1 ml lysis buffer without SDS. The beads were then washed with 1 ml of kinase buffer (20 mM Hepes, pH 7.5, 15 mM MgAcetate) and resuspended in kinase buffer containing 30 μM ATP and 5 μCi [γ-^{32}P] ATP. The reaction was stopped with Laemmli buffer and analyzed by SDS-PAGE. Phosphopeptide mapping was performed as described above.

In Gel Renaturation Procedure

GST-Myc100 (5 μg) was incubated with cell extracts as described above, and associated proteins were removed by boiling in 1× sample buffer and resolved by 10% SDS-PAGE containing 60 μg/ml GST-Myc100. Renaturation and denaturation was similar to the procedure of Kameshita and Fujisawa [1989]. Briefly, after electrophoresis, the gel was washed in 20% (v/v) 2-propanol in 50 mM Tris/HCl, pH 8, and incubated for 1 h in 50 mM Tris/HCl containing 1 mM DTT. Proteins were denatured in 6 M guanidine HCl in 50 mM Tris/HCl containing 20 mM DTT and 2 mM EDTA and renatured by several washes in 50 mM Tris/HCl containing 0.04% (v/v) Tween 20, 1 mM DTT, and 2 mM EDTA. After washing in kinase buffer (40 mM HEPES, 0.1 mM EGTA, 1 mM DTT, 20 mM $MgCl_2$), the gel was incubated with 30 μM ATP and 100 μCi of [γ-^{32}P]ATP for 1 h at room temperature. After washing in 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate, the gel was dried and autoradiographed.

Immunoblot Analysis

Cell extracts were prepared in Ab buffer and proteins were separated by 10% SDS-PAGE. Proteins were then transferred to nitrocellulose and nonspecific sites were blocked with 5% milk in PBS (80 mM Na_2HPO_4, 20 mM Na_2HPO_4, 100 mM NaCl, pH 7.5) for 1 h and incubated with 1 μg/ml Map kinase antibody (R2, UBI, Lake Placid, NY) or 60 μl ascites fluid containing GSK-3 monoclonal antibody (obtained from J. Woodgett) overnight at 4°C. After three washes in PBST (PBS containing 0.05% [v/v] Tween 20), the nitrocellulose membrane was incubated with 100 μCi of [^{125}I]protein A. The membrane was then washed several times with PBST and exposed for autoradiography.

RESULTS

Affinity Isolation of Kinases That Phosphorylate c-Myc at Ser-62

We were interested in identifying and isolating the in vivo kinases that phosphorylate c-Myc at the major N-terminal phosphorylation sites. As a first approach to identify the in vivo kinases that phosphorylate c-Myc, we used an affinity procedure that has been used to successfully identify cellular kinases associated with c-Jun and ATF [Hibi et al., 1993; Gupta et al., 1995]. In theory, c-Myc associated kinase(s) are isolated by incubating a GST-Myc protein (immobilized on glutathione agarose) with cell extracts to allow binding, followed by washing and incubation with [γ-P^{32}] ATP to allow the associated kinase to phosphorylate GST-Myc. We used a GST-Myc fusion protein containing the N-terminal 100 amino acids of c-Myc (GST-Myc100) to isolate kinases that bind and phosphorylate the previously localized Thr-58, Ser-62, Ser-71, and Ser-82 phosphorylation sites [Lutterbach and Hann, 1994, 1997]. Figure 1A (lane 3) demonstrates that an associated kinase(s) from NIH 3T3 cell extracts was capable of phosphorylating the 43 kd GST-Myc-100 substrate. The 40 kd protein is related to the 43 kd GST-Myc100 protein as determined by Western blot analysis (data not shown). The GST (lane 1) and GST-Myc50 (lane 2) proteins did not recover a kinase activity that phosphorylated GST-Myc100. The 50 kd protein (Fig. 1A, indi-

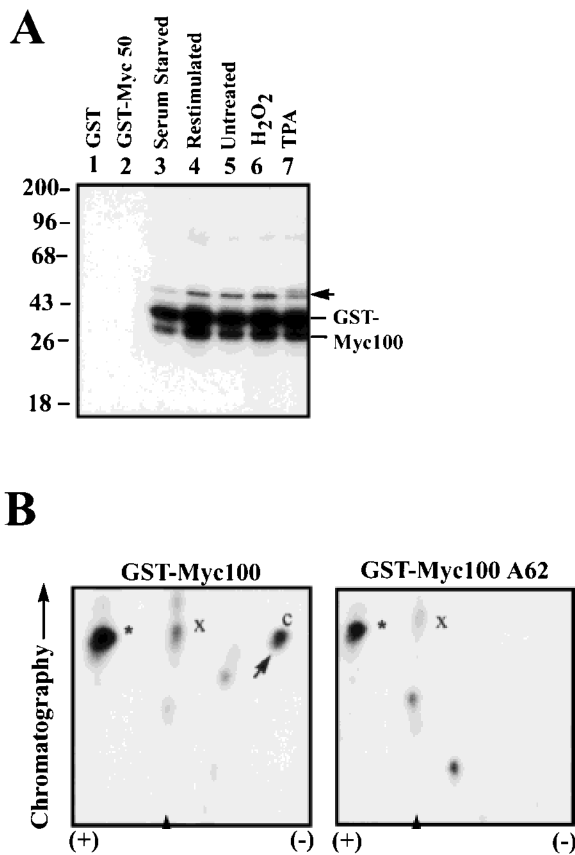


Fig. 1. Characterization of kinase activity that binds to the N-terminus of c-Myc. **A**: Isolation of Myc-associated kinase(s). Glutathione agarose-GST, GST-Myc50, or GST-Myc100 were incubated with whole cell extracts prepared from NIH 3T3 cells followed by washing and incubation with [γ-^{32}P]ATP as described in the Materials and Methods. The reactions were boiled in Laemmli buffer and analyzed by SDS-PAGE. An arrow indicates the position of an unknown c-Myc-associated protein. **B**: A Myc-associated kinase phosphorylates Ser-62. GST-Myc100 and GST-Myc100-A62 were incubated with cell extracts as described in A, followed by thermolytic mapping as described in the Materials and Methods. An arrow indicates the Ser-62 phosphopeptide c, an "×" indicates a peptide with similar mobility to phosphopeptide a [Lutterbach and Hann, 1994], an asterisk indicates the position of a serine-containing peptide observed in vivo. Arrowheads indicate the origin of loading.

cated with an arrow) was not reactive with Myc antibodies and either represents an autophosphorylated kinase or an associated protein that was phosphorylated by a Myc-associated kinase. Interestingly, this protein did not bind to GST or GST-Myc 50 (lanes 1 and 2) and we observed a slower migrating form of this 50 kd protein upon TPA treatment of cells (Fig. 1A, lane 7). It is important to note that we used stringent binding conditions in this procedure (1% Triton X-100, 200 mM NaCl, and 0.05% SDS) in order to decrease non-specific interactions.

Thermolysin digestion of GST-Myc100 phosphorylated by the associated kinase(s) from normally growing NIH 3T3 cells (Fig. 1A, lane 5) resulted in the phosphorylation pattern seen in the 2D maps shown in Figure 1B (GST-Myc100). We have previously established the location of Thr-58, Ser-62, Ser-71, and Ser-82 phosphopeptides in thermolytic analysis of in vivo phosphorylated c-Myc [Lutterbach and Hann, 1994, 1997]. We have established that Ser-62 phosphorylation is contained in a phosphopeptide designated peptide c [Lutterbach and Hann, 1994]. The absence of phosphopeptide c in GST-Myc100 Ala-62 confirmed that the associated kinase phosphorylated Ser-62 (Fig. 1B). We also observed a phosphopeptide (indicated with an "×" in Fig. 1B) with similar mobility to phosphopeptide a [Lutterbach and Hann, 1994] see Figure 4, corresponding to Ser-71 phosphorylation, but we have not confirmed that they are identical. The negative migrating phosphopeptide (designated with an asterisk) is not commonly seen in c-Myc phosphopeptide maps, but a similar phosphopeptide has been observed both in endogenous c-Myc from some cells (data not shown) and after phosphorylation of GST-Myc100 by GSK-3 β in vitro [Lutterbach and Hann, 1994].

Our previous results revealed that serum stimulation of quiescent cells enhanced Ser-62 phosphorylation several-fold in exogenously expressed c-Myc protein [Lutterbach and Hann, 1994]. To determine if the GST-Myc100 associated kinase was also capable of being stimulated by mitogens, we prepared cell extracts from serum-starved NIH-3T3 cells and from serum-starved cells that were restimulated with 20% serum. We also prepared extracts from growing cells that had been either heat shocked, treated with TPA, or treated with hydrogen peroxide. SDS-PAGE analysis of the GST-Myc100-associated kinase activity recovered from these cell extracts is shown in Figure 1A (lanes 3–7). We subjected these proteins to thermolysin digestion and 2D mapping and obtained the results seen in Figure 2. We observed a several-fold increase in Ser-62 phosphorylation relative to the other sites after serum stimulation of quiescent cells (Fig. 2, compare peptide c in restimulated versus serum starved). An increase in Ser-62 phosphorylation was also seen with TPA, heat shock, and hydrogen peroxide

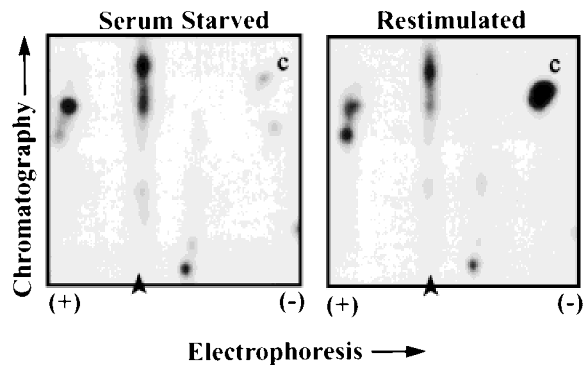


Fig. 2. The activity of the c-Myc-associated Ser-62 kinase is serum stimulated. GST-Myc100 was incubated with extracts from NIH 3T3 cells that had been placed in 0.05% serum for 24 h (serum-starved) or with extracts from serum starved NIH 3T3 cells restimulated with 20% serum for 20 min (restimulated) as described in A, followed by thermolytic mapping as described in the Materials and Methods. Arrowheads indicate the origin.

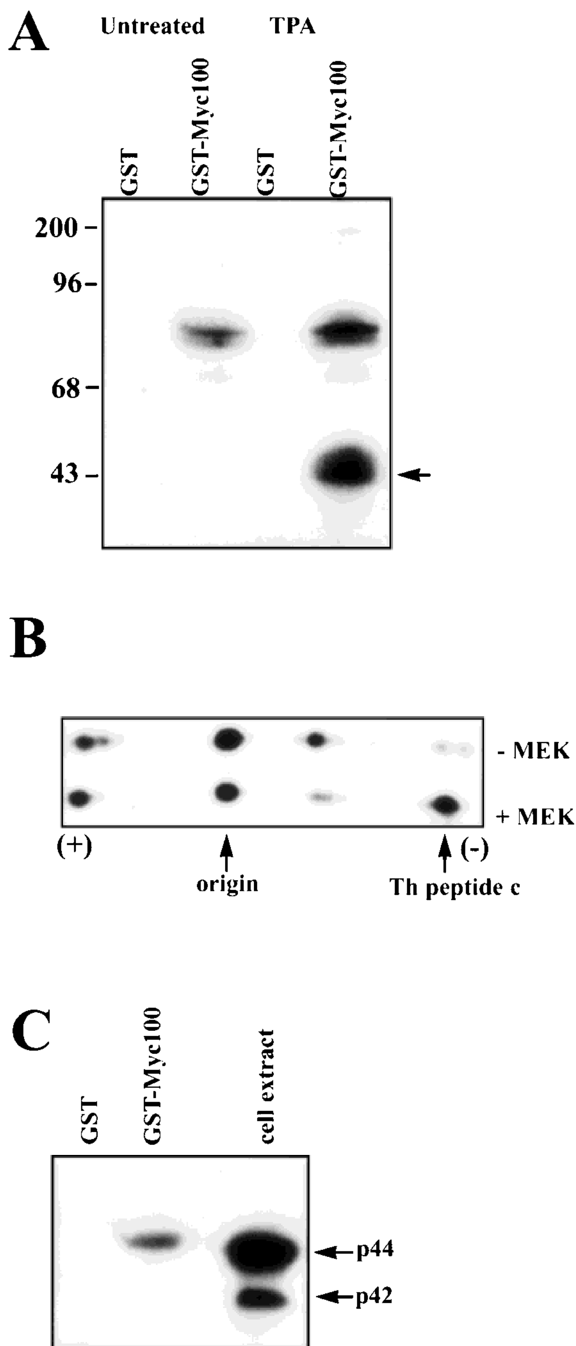
treatment of normally growing cells (data not shown). We also observed that the GST-Myc-100 associated kinase(s) strongly phosphorylated myelin basic protein but only weakly phosphorylated histone (data not shown). These results are consistent with a Map kinase involvement in Ser-62 phosphorylation. The GST-Myc100 associated kinase is not likely to be a stress-activated protein kinase since the activity of these kinases is not increased by TPA or mitogens [Kyriakis et al., 1994]. Cdk family members are also not likely candidates for the GST-Myc100 associated kinase since these kinases are not activated in quiescent cells after 20 min of serum stimulation [Sherr, 1994].

To further characterize the associated kinase(s) we subjected GST-Myc associated proteins to an in gel renaturation assay [Kameshita and Fujisawa, 1989]. GST-Myc100, but not GST alone, recovered kinase activities of approximately 43 kd and 70 kd (Fig. 3A). The activity of the 43 kd kinase was upregulated several-fold by TPA, while the 70 kd kinase was not significantly affected (Fig. 3A). A 43 kd TPA-stimulated kinase is characteristic of a Map kinase [Rossomando et al., 1989; Boulton et al., 1991], and thus we next determined if the Myc-associated kinase could be activated by a Map kinase activating kinase (MEK-1). As shown in Figure 3B, we found that addition of 100 ng of MEK-1 to GST-Myc100-associated proteins from serum-starved cells resulted in a strong enhancement of Ser-62 phosphorylation. Control experiments revealed that MEK-1 did not directly phosphorylate GST-Myc100 (data not shown) indicating that the increase in Ser-62 phosphorylation was the result of MEK-1 activation of a GST-Myc-associated Map kinase. This result also suggests that a Map kinase binds to GST-Myc100 independent of its activation state. As a final test for Map kinase association we performed Western blotting on GST-Myc100-associated proteins. After blotting GST-Myc100 associated proteins with Map kinase antiserum, we detected immunoreactivity with the 44 kd isoform present in cell extracts (Fig. 3C). A longer exposure also revealed immunoreactivity with the 42 kd isoform, and similar to results with the MEK assay, binding of Map kinases to GST-Myc100 was independent of kinase activity (data not shown). We therefore conclude that cellular Map kinase isoforms can bind to GST-Myc100 and phosphorylate Ser-62 under these conditions.

TPA Stimulates Phosphorylation of Endogenous c-Myc But Not at Ser-62

We next attempted to correlate the regulation of the associated kinase activity with the in vivo regulation of phosphorylation of the endogenous c-Myc protein. Serum stimulation of Ser-62 phosphorylation by the GST-Myc100-associated kinase was consistent with our previous observation that exogenous c-Myc phosphorylation at Ser-62 was serum stimulated in NIH 3T3 cells [Lutterbach and Hann, 1994]. However, we have found that phosphorylation at Ser-62 and Thr-58 can also be upregulated by overexpression of c-Myc, and we therefore interpret results using overexpressed c-Myc with caution [Lutterbach and Hann, 1997]. We were thus interested in examining phosphorylation of endogenous c-Myc. We cannot determine if serum restimulation affects Ser-62 phosphorylation of the endogenous c-Myc protein from NIH 3T3 cells because of the low protein levels in serum-starved cells. Therefore, we examined the effects of TPA stimulation in NIH 3T3 cells since the associated Map kinase was stimulated several-fold by TPA. Cells were labeled for 3 h and stimulated with TPA for 20 min. The endogenous c-Myc was immunoprecipitated, separated by SDS-PAGE, and digested by thermolysin as described in the Materials and Methods. An aliquot of the labeled cells was also subjected to the GST-Myc100 binding procedure and TPA stimulation of Ser-62 phosphorylation by the associated Map kinase was con-

firmed (data not shown). Figure 4 demonstrates that Ser-62 phosphorylation in the endogenous c-Myc was not stimulated by 20 min of TPA treatment. We also did not observe changes in phosphorylation at Ser-62 in vivo after 4 h of TPA treatment (data not shown). Thus, the lack of TPA stimulation of Ser-62 phosphorylation in the endogenous c-Myc protein suggests that the Map kinases that associate with GST-Myc100 in vitro are not the in vivo kinases for Ser-62.



Although Ser-62 phosphorylation was not affected by TPA, we did observe stimulation of a phosphopeptide designated by the arrows in Figure 4A. Interestingly, in a separate experiment when the cells were at a higher density, this otherwise minor phosphopeptide became a major phosphopeptide upon TPA stimulation (Fig. 4B). This TPA-stimulated phosphopeptide contains phosphoserine (data not shown).

DISCUSSION

Our previous attempts to characterize the kinases that phosphorylate c-Myc in vivo have utilized both in vivo and in vitro studies. Our previous observations demonstrated that both $p42^{mapk}$ and cdk family members specifically phosphorylate GST-Myc100 at Ser-62 in vitro [Lutterbach and Hann, 1994]. Consistent with potential Map kinase involvement in c-Myc phosphorylation, we observed mitogenic stimulation of Ser-62 phosphorylation when c-Myc was overexpressed in NIH 3T3 cells [Lutterbach and Hann, 1994]. However, we did not observe alterations in the phosphorylation pattern of c-Myc upon coexpression of $p42^{mapk}$ with c-Myc [Lutterbach and Hann, 1994]. In addition, expression of activated ras with $p42^{mapk}$ and c-Myc resulted in a three-fold increase in Map kinase activity without altering c-Myc phosphorylation [Lutterbach and Hann, 1994].

Fig. 3. An associated kinase that phosphorylates Ser-62 is a Map kinase. **A:** In gel renaturation analysis of GST-Myc100 associated proteins. NIH 3T3 cell extract (300 µg protein) was incubated with 5 µg of GST or GST-Myc100. After washing, the complex was boiled in Laemmli buffer and analyzed by SDS-PAGE in a 10% polyacrylamide gel polymerized in the presence of 60 µg/ml GST-Myc100. Removal of SDS, renaturation of proteins, and the in-gel kinase assay was performed as described in the Materials and Methods. An arrow marks the position of a TPA-stimulated kinase. Arrowheads mark the origin. **B:** Activation of c-Myc associated Ser-62 kinase by MEK-1. GST-Myc100 was incubated with extracts from serum-starved NIH 3T3 cells (0.05% serum for 24 h) as described in A. Half of the GST-Myc100 protein was incubated with [γ-^{32}P]ATP alone while the other half was incubated with [γ-^{32}P]ATP and100 ng of activated baculovirus-produced MEK-1 for 10 min at room temperature. The samples were then subjected to thermolytic mapping and electrophoresis at pH 1.9 as described in the Materials and Methods. Arrows indicate the origin and the position of Ser-62 phosphopeptide c. **C:** Direct interaction of Map kinases with GST-Myc100. GST and GST-Myc100-associated proteins were isolated as in A, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a Map kinase antibody as described in the Materials and Methods. Fifty µg of NIH 3T3 cell extract was used as a control. Arrows indicate the positions of the $p42^{mapk}$ and $p44^{mapk}$ isoforms.

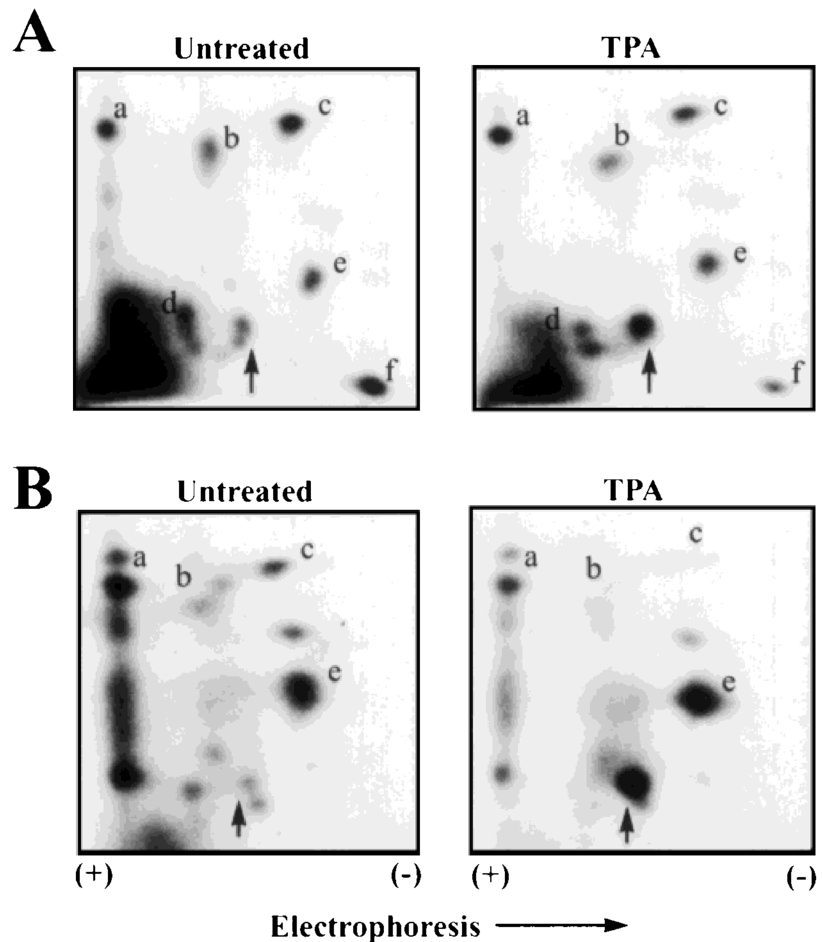


Fig. 4. Mapping analysis of c-Myc from TPA-stimulated NIH 3T3 cells. **A:** Subconfluent NIH 3T3 cells were labeled with [^{32}P]-orthophosphate for 3 h and TPA (200 ng/ml) was then added for 20 min. c-Myc was immunoprecipitated and processed for thermolytic mapping as described in the Materials and Methods. Arrows indicate the position of a TPA-stimulated phosphopeptide. **B:** Confluent NIH 3T3 cells were treated with TPA and processed as in A.

Using a GST-Myc affinity approach, we have obtained substantial evidence that cellular Map kinase isoforms can associate with GST-Myc100 under stringent conditions and phosphorylate Ser-62. While this work was in progress, another group reported that Map kinases could bind to and phosphorylate a GST-Myc fusion protein, but the sites of phosphorylation were not mapped, and no correlation with endogenous c-Myc phosphorylation was attempted [Gupta and Davis, 1994]. Based on these experiments and previous in vitro experiments [Seth et al., 1992], Map kinases have been considered to be physiological kinases for c-Myc. However, it is critical to show that the associated kinase activity is regulated in the same way as the in vivo phosphorylation. Our comparison of the GST-Myc 100-associated Map kinase activity with in vivo regulation of Ser-62 phosphorylation in endogenous c-Myc strongly suggests that the p42/p44 Map kinases are not the in vivo Ser-62 kinases. We found that TPA strongly stimulated the activity of the GST-Myc100-associated Map kinase to phosphorylate Ser-62. In contrast, the phosphorylation of Ser-62 in endogenous c-Myc was not stimulated by TPA at early (20 min) or later times (4 h) after TPA stimulation. Thus the in vitro interaction between GST-Myc100 and Map kinases may not represent a physiological association, which supports the results of our previous Map kinase/c-Myc co-expression experiments. Interestingly, TPA enhanced the phosphorylation of c-Myc at a different serine residue. We also observed similar upregulation of this phosphorylation site in cells treated with TNF- α [Lutterbach and Hann, unpublished observations]. This TPA/TNF-stimulated phosphorylation is potentially interesting, since functionally significant residues in c-Jun have been shown to be regulated by TPA [Smeal et al., 1992]. This site was not observed after in vitro phosphorylation of full length GST-Myc by p42^{mapk} or protein kinase C (α and β isoforms), suggesting that an alternate TPA/TNF stimulated kinase is in-

volved [Lutterbach and Hann, unpublished results].

The general approach we have used to isolate Myc-associated kinases has been successful when applied to isolating physiological kinases for other transcription factors. For example, GST- c-Jun [Hibi et al., 1993] and GST-ATF [Gupta et al., 1995] were found to interact with associated kinases under less stringent conditions (200 mM NaCl, 0.05–1% Triton X-100) than used in our GST-Myc100-associated kinase procedure (200 mM NaCl, 1% Triton X-100, 0.05% SDS). Despite our stringent conditions, it appears that the Map kinases, which are abundant in fibroblasts [Kazlauskas and Cooper, 1988; Ely et al., 1990], can spuriously associate with Myc in cell extracts. Since the in vivo Ser-62 kinase does not appear to be TPA-stimulated, another possibility is that the 70 kd Myc-associated kinase detected in the in gel renaturation assay could be the kinase that phosphorylates Ser-62. The activity of this kinase was not stimulated by TPA, which does correlate with in vivo findings. In addition, a 50 kd associated protein is also recovered in this procedure but we have yet to determine whether this Myc-associated protein is a kinase. It is also possible that the physiological Ser-62 kinase is not recovered in this procedure. For example, it is possible that he kinase is either not efficiently extracted under these conditions or its kinase activity is not preserved during the procedure. In addition, a protein complex may be involved in the in vivo phosphorylation of c-Myc that may not be recovered by this procedure. Interestingly, it has been shown that a p107/cyclin A complex can phosphorylate c-Myc at Ser-62 in vitro [Hoang et al., 1995].

Although the identity of the in vivo Ser-62 kinase remains unresolved at this point, certain kinases can be excluded. We have previously examined a range of kinases for their ability to phosphorylate the transactivation domain of c-Myc in vitro [Lutterbach and Hann, 1994]. Of the kinases tested (which included cAMP- and cGMP- dependent kinases, calcium calmodulin kinase II, pim kinase, protein kinase C, double stranded DNA-dependent kinase, GSK-3 α and GSK-3 β, p42 map kinase, and cdk-1) only Map kinase and cdk-1 were found to specifically phosphorylate Ser-62 in c-Myc. However, as described above, Map kinases do not appear to be involved in the in vivo phosphorylation of Ser-62 in c-Myc. The stress-activated kinases, which can be stimulated by TNF- α [Kyriakis et al., 1994], are also not likely candidates for in vivo phosphorylation of Ser-62 since TNF- α did not alter Ser-62 phosphorylation. We are currently examining cdk family members as candidate kinases for Ser-62 phosphorylation in vivo. A cdk/p107/cyclin A complex has been shown to phosphorylate Ser-62 in vitro, and a role for this complex in Ser-62 in vivo phosphorylation in endogenous c-Myc is under investigation.

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REFERENCES

Bhatia K, Huppi K, Spangler G, Siwarski D, Iyer R, Magrath I. 1993. Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytoma. Nature Gen 5:56–61.

Boulton TG, Nye DJ, Robbins NY, Ip E, Radziewjewska SD, Morganbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. 1991. ERKs: A family of protein-serine/threonin kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663–675.

Boyle WJ, van der Greer P, Hunter T. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol 201:110–149.

Chen C, Biegalke BJ, Eisenman RN, Linial ML. 1989. FH3, a v-myc avian retrovirus with limited transforming ability. J Virol 63:5092–5100.

Ely CM, Oddie KM, Litz JS, Rossomaondo A, Kanner SB, Strugill TW, Parsons SJ. 1990. A 42 kd tyrosine kinase substrate linked to chromaffin cell sectetion exhibits an associated MAP kinase activity and is highly related to a 42 kd mitogen stimulated protein in fibroblasts. J Cell Biol 110:731–742.

Frykberg L, Graf T, Vennstrom B. 1987. The transforming activity of the chicken c-Myc gene can be potentiated by mutations. Oncogene 1:415–421.

Gaubatz S, Imhof A, Dosch R, Werner O, Mitchell P, Buettner R, Eilers M. 1995. Transcriptional activation by Myc is under negative control by the transcription factor AP-2. EMBO J 14:1508–1519.

Gupta S, Campbell D, Derijard B, Davis RJ. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267:389–393.

Gupta S, Davis RJ. 1994. MAP kinase binds to the NH_2-terminal activation domain of c-Myc. FEBS Lett 353:281–285.

Hann SR, Abrams HD, Rohrschneider LR, Eisenman RN. 1983. Proteins encoded by v-Myc and c-Myc oncogenes: Identification and localization in acute leukemia transformants and bursal lymphoma cell lines. Cell 34:789–798.

Henricksson M, Bakardjiev A, Klein G, Luscher B. 1993. Phosphorylation sites mapping in the N-terminal domain of c-Myc modulate its transforming potential. Oncogene 8:3199–3209.

Hibi M, Lin A, Smeal T, Minden A, Karin M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation doamin. Genes Dev 7:2135–2148.

Hoang A, Lutterbach B, Lewis BC, Yano T, Chou T-Y, Barrett J, Raffeld M, Hann SR, Dang CV. 1995. A link between increased transforming activity of lymphoma-derived MYC mutant alleles, their defective regulation by p107, and altered phosphorylation of the c-Myc transactivation domain. Mol Cell Biol 15:4031–4042.

Hunter T, Karin M. 1992. The regulation of transcription by phosphorylation. Cell 70:375–387.

Kameshita I, Fujisawa H. 1989. A sensitive method for detection of calmodulin-dependent protein kinase II in sodium dodecyl sulfate-polyacrylamide gel. Anal Biochem 183:139–143.

Kazlauskas A, Cooper JA. 1988. Protein kinaase C mediates platelet-derived growth factor-induced tyrosine phosphorylation of p42. J Cell Biol 106:1395–1402.

Kunkel TA. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82:488–492.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahamd MF, Avruch J, Woodgett JR. 1994. The stress-activated protein kinase subfamily of kinases. Nature 369:156–160.

Luscher B, Kuenzel EA, Krebs EG, Eisenman RN. 1989. Myc proteins are phosphorylated by casein kinase II. EMBO J 8:1111–1119.

Lutterbach B, Hann SR. 1997. Overexpression of c-Myc and cell immortalization alters c-Myc phosphorylation. Oncogene 14:967–975.

Lutterbach B, Hann SR. 1994. Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. Mol Cell Biol 14:5510–5522.

Meek DW, Street AJ. 1992. Nuclear protein phosphorylation and growth control. Biochem J 287:1–15.

Palmieri S, Kahn P, Graf T. 1983. Quail embryo fibroblasts transformed by four v-myc isolates show enhanced proliferation but are non-tumorigenic. EMBO J 2:2385–2389.

Papas TS, Lautenberger JA. 1985. Sequence curiosity in v-myc oncogene. Nature 318:237.

Pulverer BJ, Fischer K, Vousden K, Littlewood T, Evan G, Woodgett JR. 1994. Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. Oncogene 9:59–70.

Rossomando AJ, Payne DM, Weber MJ, Pelech SL, Sturgill TW. 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc Natl Acad Sci USA 86:6940–6943.

Seth A, Gonzalez S, Gupta S, Raden DL, Davis RJ. 1992. Signal transduction within the nucleus by mitogen activated protein kinase. J Biol Chem 267:24796–24804.

Sherr CJ. 1994. G1 phase progression: Cycling on cue. Cell 79:551–555.

Smeal T, Binetruy B, Mercola D, Grover-Bardwick A, Heidecker G, Rapp UR, Karin M. 1992. Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. Mol Cell Biol 12: 3507–3513.

Smith D, Johnson K. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67:31–40.

Street AJ, Blackwood E, Luscher B, Eisenman RN. 1990. Mutational analysis of the carboxy-terminal casein kinase II phosphorylation site in human c-myc. Curr Top Microbiol Immunol 166:251–258.

Symonds G, Hartshorn A, Kennewell A, O'Mara M-A, Bruskin A, Bishop JM. 1989. Transformation of murine myelomonocytic cells by myc: Point mutations in v-myc contribute synergistically to transforming potential. Oncogene 4:285–294.