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Menin uncouples Elk-1, JunD and c-Jun phosphorylation from MAP kinase activation

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Menin, a nuclear protein encoded by the tumor suppressor gene MEN1, interacts with the AP-1 transcription factor JunD and inhibits its transcriptional activity. In addition, overexpression of Menin counteracts Ras-induced tumorigenesis. We show that Menin inhibits ERK-dependent phosphorylation and activation of both JunD and the Ets-domain transcription factor Elk-1. We also show that Menin represses the inducible activity of the c-*fos* promoter. Furthermore, Menin expression inhibits Jun N-terminal kinase (JNK)-mediated phosphorylation of both JunD and c-Jun. Kinase assays show that Menin overexpression does not interfere with activation of either ERK2 or JNK1, suggesting that Menin acts at a level downstream of MAPK activation. An N-terminal deletion mutant of Menin that cannot inhibit JunD phosphorylation by JNK, can still repress JunD phosphorylation by ERK2, suggesting that Menin interferes with ERK and JNK pathways through two distinct inhibitory mechanisms. Taken together, our data suggest that Menin uncouples ERK and JNK activation from phosphorylation of their nuclear targets Elk-1, JunD and c-Jun, hence inhibiting accumulation of active Fos/Jun heterodimers. This study provides new molecular insights into the tumor suppressor function of Menin and suggests a mechanism by which Menin may interfere with Ras-dependent cell transformation and oncogenesis.
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Introduction

The autosomal dominant syndrome multiple endocrine neoplasia 1 (MEN1) is characterized by pancreatic, parathyroid and anterior pituitary adenomas (Marx *et al*., 1999). The causal gene locus MEN1 was identified by positional cloning (Chandrasekharappa *et al*., 1997). The identification of germline mutations affecting the MEN1 gene in familiar MEN1 (Heppner *et al*., 1997), as well as somatic mutations in sporadic tumors (Marx *et al*., 1998) suggest that MEN1 is a tumor suppressor gene. Recently, it has been shown that mice which are heterozygous for a MEN-1 null allele display a phenotype that is strikingly similar to that of the human disorder MEN1 (Crabtree *et al*., 2001), further supporting the role of MEN1 as an onco-suppressor gene. Menin, the product of the MEN1 gene, is a 610-amino acid protein that mainly localizes in the nucleus (Guru *et al*., 1998). Menin over-expression inhibits proliferation and clonogenicity of Ras-transformed fibroblasts in soft agar, as well as Ras-induced tumor growth in nude mice (Kim *et al*., 1999). Furthermore, Menin inactivation by antisense RNA antagonizes cell growth inhibition mediated by TGF-β (Kaji *et al*., 2001). Conversely, Menin interacts with the AP-1 transcription factor JunD and with NF-κB proteins and represses both NF-κB-mediated and JunD-dependent transcription (Agarwal *et al*., 1999; Gobl *et al*., 1999; Heppner *et al*., 2001).

AP-1 is a dimeric protein complex whose components are the products of the Jun and Fos gene families (Angel and Karin, 1991; Karin *et al*., 1997). In vertebrates, the Jun family of transcription factors is composed of c-Jun, JunB and JunD (reviewed in Mechta-Grigoriou *et al*., 2001). JunD is known to antagonize the growth-promoting functions of c-Jun, to slow down the proliferation of immortalized fibroblasts and to counteract Ras-dependent transformation (Pfarr *et al*., 1994). Although JunD seems to have growth inhibitory property in some conditions, it is growth promoting under other conditions. In fact, the study of JunD-deficient mice has shown that JunD is essential for the survival of primary embryonic fibroblasts and can inhibit stress-induced apoptosis by modulating a p53-dependent pathway (Wentzman *et al*., 2000).

While numerous studies on the phosphorylation of c-Jun have been reported, JunD phosphorylation and its possible regulatory function has not been characterized in great detail so far. However, it has been shown that JunD is phosphorylated *in vivo* in response to PDGF and anisomycin, and *in vitro* by active JNK (Gupta *et al*., 1996; Kallunki *et al*., 1996; Wang *et al*., 1996). It

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has also been shown that the increase of JunD phosphorylation by okadaic acid correlates with induction of JunD/FosB dependent transcription (Rosenberger *et al.*, 1999).

AP-1 transcriptional activity is stimulated by extracellular signals such as growth factors, cytokines, tumor-promoters and UV-irradiation (Karin *et al.*, 1997; Shaulian and Karin, 2001). Presumably, the main growth factor response is mediated by the ERK MAP kinase, which can phosphorylate transcription factors of the TCF family such as Elk-1, and thus cause induction of the c-*fos* gene. The subsequently expressed c-*fos* protein can then associate with Jun proteins to form stable AP-1 heterodimers. It is well-established that Ras-induced transformation requires c-Jun (Johnson *et al.*, 1996), and that c-Jun phosphorylation on sites that are mainly phosphorylated by JNK plays a crucial role in Ras-oncogenic transformation (Behrens *et al.*, 2000; Smeal *et al.*, 1991). Conceivably, oncogenic Ras may cause activation of both ERK and JNK, leading to elevated expression of Fos proteins and N-terminal phosphorylation of c-Jun (reviewed in Davis, 2000; Shaulian and Karin, 2001).

In the present study we have investigated whether Menin expression affects phosphorylation and activation of nuclear MAPK substrates such as JunD, Elk-1 and c-Jun. We provide evidence that Menin inhibits ERK-dependent phosphorylation and activation of both JunD and Elk-1 transcription factors, as well as c-Jun and JunD phosphorylation induced by JNK signaling. Furthermore, we show that Menin expression represses the serum-, or Ras^{Val12}-induced activity of a c-*fos* promoter construct. The finding that Menin can suppress the signal output of MAP kinase cascades reveals a potential mechanism by which MEN1 evokes tumor suppressor activity.

Results

JunD is specifically phosphorylated by ERK

The N-terminal domain of JunD contains three candidate sites for MAP kinase phosphorylation: the serine residues 90 and 100 and threonine 117, which share a conserved sequence context with the well characterized regulatory phosphorylation sites located in the N-terminal region of c-Jun (serines 63 and 73, and threonine 91). We have examined whether activation of the MEK-ERK pathway modulates the phosphorylation state of JunD-N-terminal domain. To this end, we tested the effect of serum stimulation on JunD phosphorylation, either in the absence or presence of a specific MEK inhibitor such as UO126 (Favata *et al.*, 1998). HEK-293 cells were transiently transfected with a CMV-vector expressing a mouse JunD protein tagged with a heme-agglutinine (HA) epitope (Musti *et al.*, 1996), Phosphorylation of JunD-N-terminal domain was monitored by immunoblot assays, using an anti-phospho Jun antibody (α-73) that recognizes JunD when it is phosphorylated at serine 100. Figure 1a shows that cellular treatment with UO126 abolished the enhancement of JunD phosphorylation by serum stimulation, indicating that MEK activation is required for the effect of serum. Since basal level of JunD phosphorylation is detected in exponentially growing HEK-293 cells, we tested the effect of MEK inhibition also in cycling HEK-293 cells. As shown in Figure 2b, JunD phosphorylation was impaired by cellular-treatments with the MEK inhibitor PD98059 (Alessi *et al.*, 1995) (Figure 1b). PD98059 treatment also impaired the phosphorylation of ERK1/2 that is normally detected in H-293 cycling cells (data not shown). To provide additional evidence that JunD is phosphorylated in a MEK-ERK-dependent manner, we examined the effect of a constitutively active form of ERK2, the Myc-tagged ERK2-MEK1-LA fusion protein. This fusion-protein kinase can induce neurite outgrowth in PC12 cells in the absence of upstream signals and can mediate cellular transformation (Robinson *et al.*, 1998). ERK2-MEK1-LA expression caused a robust phosphorylation of the N-terminal domain of JunD, as shown by immunoblotting with anti phospho-Jun antibody and by protein mobility shift (Figure 1a). Taken together these results suggest that MEK-dependent activation of ERK1/2 catalyses JunD phosphorylation in its N-terminal domain. To validate this conclusion, we analysed phosphorylation of endogenous JunD in response to serum stimulation. Since JunD is poorly expressed in H-293 cells, this analysis was performed in NIH3T3 cells. Figure 1c (upper panel) shows weak phosphorylation of JunD serine 100 already detectable after 5 min of serum stimulation, reaching a maximal level after 30 min, at which time a protein mobility shift was also visible. This effect of serum-stimulation was impaired in presence of PD98059, indicating that MEK activation is necessary for serum-induced phosphorylation of JunD. As shown in Figure 1c (lower panel) serum stimulation and inhibitors had no effect on JunD steady-state levels. PD98059 treatments also impaired phosphorylation of ERK1/2, but had no effect on the phosphorylation state of anisomycin-induced JNK1/2 (data not shown). Immunoblot-analysis with an anti-phospho c-Jun/specific antibody (α-63), that recognizes phosphorylated c-Jun at serine 63, but not phosphorylated JunD, showed that c-Jun phosphorylation is not detectable in the experimental conditions presented in Figure 1c (data not shown). These results demonstrate that activation of the MEK-ERK pathway elicits JunD phosphorylation at endogenous levels of expression.

Menin expression inhibits JunD phosphorylation induced by the MEK-ERK signaling

To investigate whether there is a link between Menin expression and modulation of MEK-ERK signaling, we investigated the effect of Menin on JunD phosphorylation. Figure 1b shows that overexpression of Menin caused a strong reduction of JunD phosphorylation, reminiscent of PD98059 treatment without affecting its expression level (middle panel).

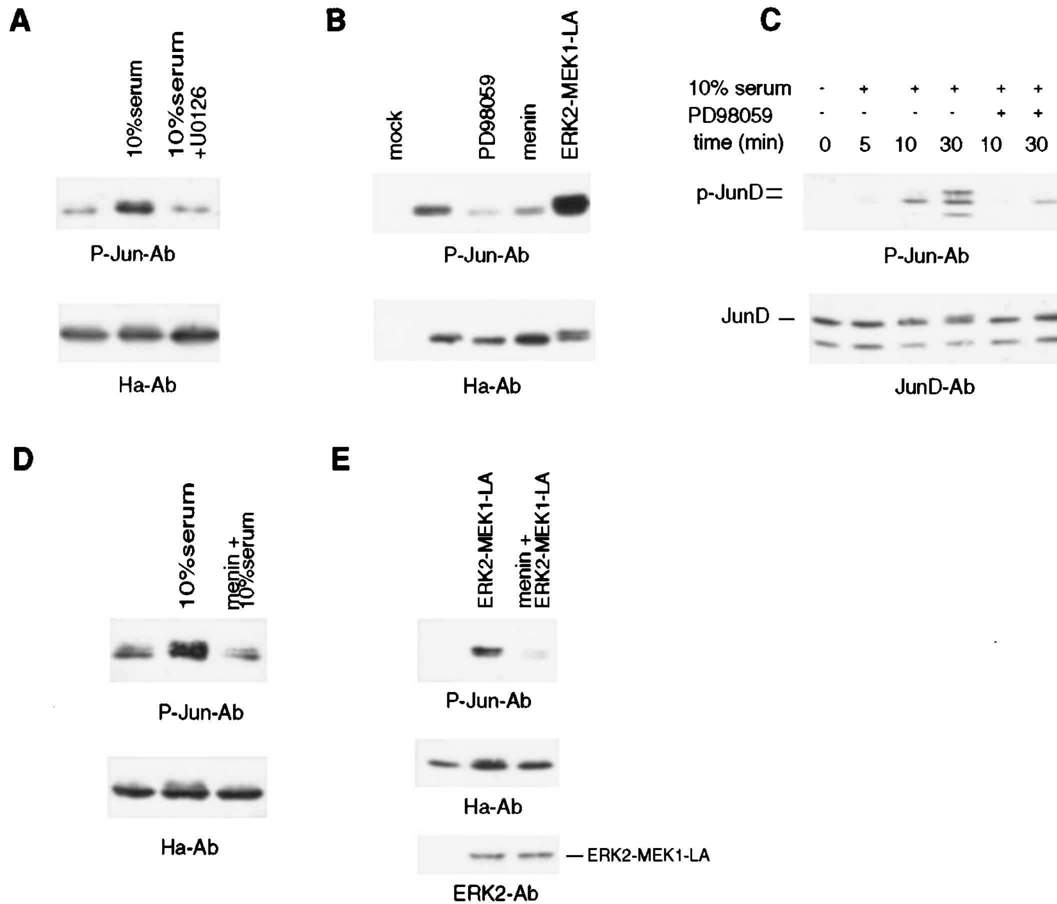

Figure 1 Menin expression inhibits JunD phosphorylation. (**a**) HEK-293 cells were transfected with JunD-HA (1.5 μg) plasmid, either alone or together with plasmids expressing Menin (1.5 μg) as indicated. Twenty hours after transfection, cells were serum-starved for 24 h and then stimulated (where indicated) with 10% fetal calf serum or 10% fetal calf serum and UO126 (10 μM), immunoblots were performed either with anti-phospho-Jun (P-Jun) (upper panel **a**), or with anti-HA antibodies (lower panel). (**b**) HEK-293 cells were transiently transfected with JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing Menin (1.5 μg), or ERK2-MEK1-LA (1.5 μg), as indicated. Where indicated, cells were treated with 100 μM PD98059 for 6 h. (**c**) NIH3T3 cells were serum-starved for 24 h and then treated with 10% calf-serum for different times, as indicated. Where indicated, cells were treated with 50 μM PD98059. (**d**) HEK-293 cells were transfected with JunD-HA (1.5 μg) plasmid, either alone or together with plasmids expressing Menin (1.5 μg) as indicated. Twenty hours after transfection, cells were serum-starved for 24 h and then stimulated (where indicated) with 10% fetal calf serum. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (lower panel). (**e**) HEK-293 cells were transfected with a JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing Menin (1.5 μg) or ERK2-MEK1-LA (0.7 μg), as indicated. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (lower panel), or with ERK1-2 antibodies to monitor the expression level of ERK2-MEK1-LA (middle panel)

This finding suggests that Menin inhibits JunD phosphorylation induced by chronic activation of the MEK-ERK pathway. To examine whether Menin can also block JunD phosphorylation that is induced by acute activation of MEK-ERK signaling, we analysed JunD phosphorylation in response to serum stimulation. HA-tagged JunD protein was transiently expressed in HEK either alone or in the presence of Menin. Transfected cells were serum-starved for 24 h and then stimulated for 30 min with 10% serum. JunD phosphorylation was subsequently monitored as described above. As shown in Figure 1d, Menin expression repressed serum-induced phosphorylation of JunD.

We also examined the effect of Menin on JunD phosphorylation induced by the ERK2-MEK1-LA fusion kinase. As shown in Figure 1e, JunD phosphorylation observed upon expression of ERK2-MEK1-LA was blocked by co-expression of Menin, with no change in either JunD-HA (middle panel) or ERK2-MEK1-LA (lower panel) expression levels. Taken together, these findings suggest that Menin overexpression inhibits ERK-dependent phosphorylation of JunD by acting at a level downstream of ERK activation.

We next investigated whether JunD phosphorylation induced by ERK2-MEK1-LA might correlate with modulation of JunD-dependent transcription. JunD-transactivation assays were performed by measuring the activity of Gal4 fusion proteins containing either wild type mouse JunD protein that lacks the bZIP region (Gal4/JunD), or a corresponding fragment that

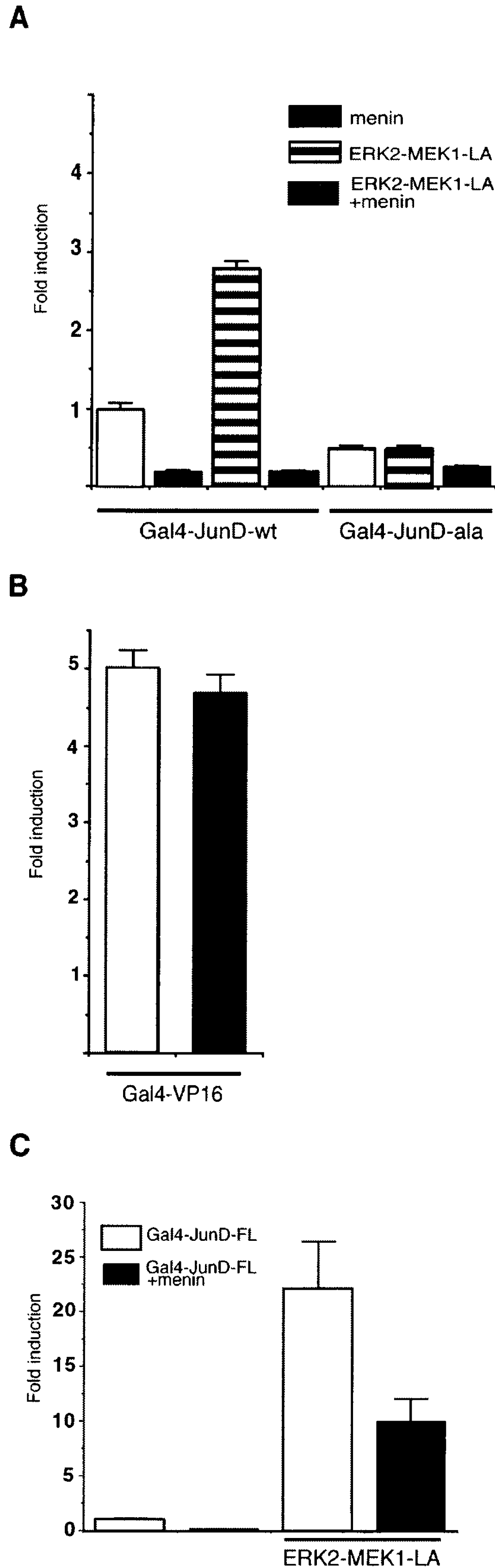

Figure 2 ERK-dependent activation of JunD requires phosphorylation-sites in the N-terminal domain and it is repressed by Menin. (**a**) CHO cells were transiently transfected with plasmids encoding Gal4 fusion proteins containing a zipper-truncated form of either wt-JunD protein, or of a JunD-ala mutant (Gal4/JunD-ala), together with the reporter plasmid Gal4-luciferase, and a Renilla-Luciferase expression vector pRL-TK (Promega). Gal4/JunD-wt plasmid (100 ng) was expressed either alone or with ERK2-MEK1-LA plasmid (0.5 μg or both with ERK2-MEK1-LA (0.5 μg) and Menin plasmids (1 μg) as indicated. Gal4/JunD-ala plasmid was expressed either alone or with ERK-MEK1-LA plasmid. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase signal. Error bars indicate s.d. from four independent experiments. (**b**) CHO cells were transiently transfected with Gal4/VP16 plasmid (100 μg) together with the reporter plasmid Gal4-luciferase and a Renilla-Luciferase expression vector pRL-TK. Where indicated Menin plasmid (1 μg) was cotransfected. Luciferase specific activity was determined as described above (**c**) HEK-293 cells were transiently transfected with plasmids encoding Gal4-JunD-full length fusion protein (Gal4/JunD-FL) (1.5 μg), the reporter plasmid Gal4-CAT (5 μg), and a pSV2-LacZ plasmid (1 μg). Gal4/JunD-FL plasmid was expressed alone or in combination with Menin plasmid (1.5 μg) and ERK2-MEK1-LA plasmid (0.7 μg) as indicated. Chloramphenicol acetyltransferase (CAT) activity was measured and normalized against the cotransfected β-galactosidase activity.. Error bars indicate s.d. from four independent experiments

bears alanine-substitutions of serine residues 90 and 100 and of threonine 117 (Gal4/JunD-ala) (see Materials and methods). As shown in Figure 2a, coexpression of ERK2-MEK1-LA increased the basal activity of the Gal4/JunD-wt, but had no effect on Gal4/JunD-ala fusion protein. These results indicate that active ERK1/2 is able to enhance JunD-dependent transcription by modulating the phosphorylation state of JunD-N-terminal domain. Likewise, Gal4/JunD-wt fusion protein showed a higher basal activity than Gal4/JunD-ala (Figure 2a), presumably resulting basal level of phosphorylation that is present in cycling cells (see Figure 1b).

Since our data indicate that Menin represses ERK-dependent phosphorylation of JunD, we tested the effect of Menin on JunD-dependent transcription induced by ERK-MEK1-LA. Figure 2a shows that Menin completely abrogated the enhancement of Gal4/JunD activity by ERK-MEK1-LA. Menin also reduced the basal activity of Gal4/JunD-wt and Gal4/JunD-ala basal activities to levels that are similar for both proteins (Figure 2a). These results suggest that Menin represses base level as well as phosphorylation induced transactivation potential of JunD. To test the specificity of Menin-mediated inhibition of transactivation, we tested the effect of Menin on the Gal4-VP16 fusion protein. As shown in Figure 2b, Menin did not cause significant variations of Gal4-VP16 activity in the reporter assay.

The inhibitory effect of Menin on JunD-dependent transcription has been previously characterized in HEK-293 cells by assaying a fusion protein consisting of the Gal4 DNA-binding domain fused to full-length human JunD (Gal4/JunD-FL) (Agarwal *et al*., 1999). To compare the published studies with our findings, we also tested the effect of ERK2-MEK1-LA on Gal4/JunD-FL activity, in the absence or presence of Menin

overexpression. As shown in Figure 2c the activity of Gal4/JunD-FL was strongly enhanced by coexpression of ERK2-MEK1-LA. However, Menin overexpression was inhibited 60% of this enhancing effect, suggesting that both the truncated and the full-length form of JunD are similarly regulated by Menin.

Menin inhibits ERK-dependent transcriptional activation of Elk-1

To investigate whether the Menin-mediated inhibition of the ERK response is restricted to JunD phosphorylation, we examined the effect of Menin on the activity of a well-characterized ERK substrate, the ternary complex factor Elk-1. ERK-dependent activation of Elk-1 was evaluated by transfecting HEK-293 cells with a Gal4-Elk-1/Gal4-CAT reporter system. The C-terminal ERK-responsive domain of Elk-1 was fused to the DNA binding domain of Gal4 (Marais *et al.*, 1993). The Gal4-Elk-1/Gal4-CAT reporter system was expressed either alone, along with a Myc-tagged ERK2 protein (Cowley *et al.*, 1994), or with the ERK2-MEK1-LA fusion protein. As shown in Figure 3a, Menin co-transfection represses both the basal and the ERK-stimulated activity of Gal4-Elk-1. To a lesser degree, Menin co-expression also inhibited CAT activity when it was stimulated by expression of ERK2-MEK1-LA (Figure 3a). Analysis of ERK-Myc and ERK2-MEK1-LA protein expression revealed that Menin co-expression did not alter their steady-state levels (data not shown).

Since phosphorylation of Elk-1 at the C-terminal serine 383 is required for Elk-1 transcriptional activity (Gille *et al.*, 1995; Janknecht *et al.*, 1993; Price *et al.*, 1995), we examined the effect of Menin on ERK-dependent phosphorylation of Elk-1-serine 383. Flag-tagged Elk-1 protein was expressed in HEK-293 cells, either alone, or in combination with Menin, or ERK2-Myc. Elk-1 phosphorylation was monitored by an anti-phospho ELK-1 (ser383) antibody. As shown in Figure 3b, Menin expression inhibited ERK2-dependent phosphorylation of Elk-1-Flag without altering the expression level of either Elk-1-Flag (middle panel) or ERK2-Myc (lower panel). These results indicate that Menin represses ERK-dependent activation of Elk-1 by inhibiting Elk-1-phosphorylation at serine 383.

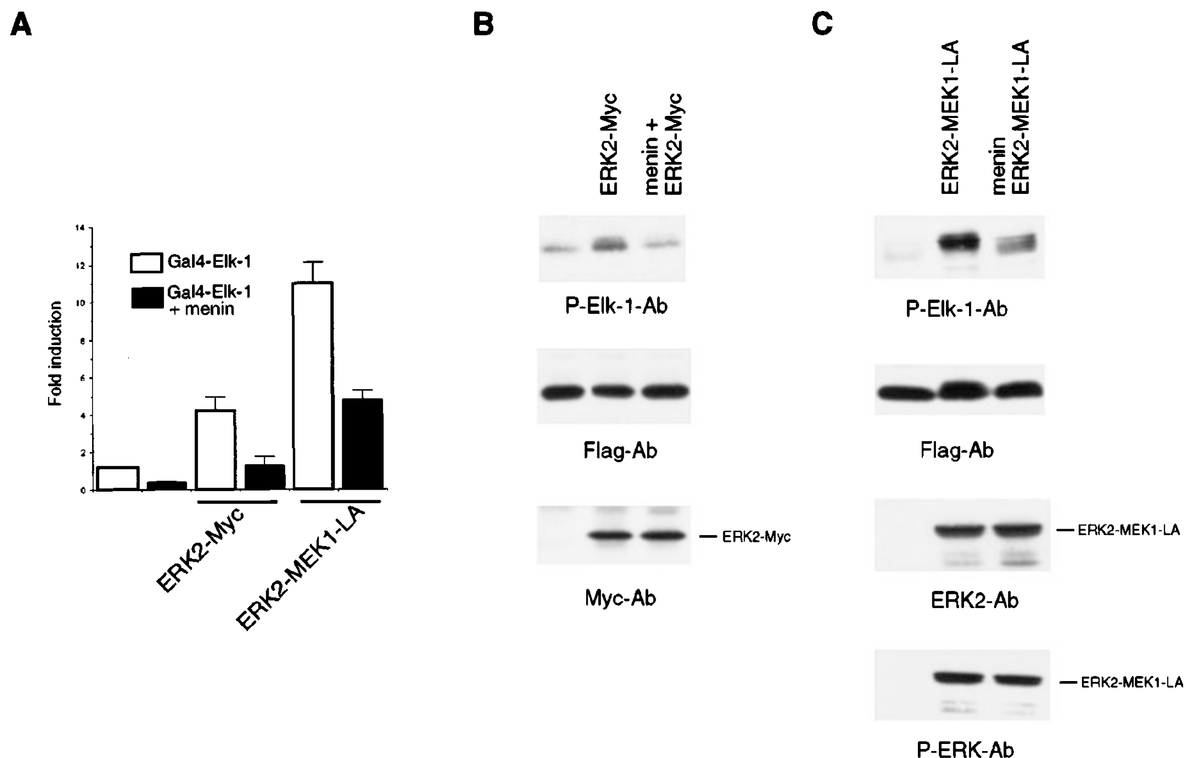

Figure 3 Menin inhibits Elk-1-dependent transcription and phosphorylation induced by ERK signaling. (**a**) HEK-293 cells were transiently transfected with plasmids encoding the Gal4-Elk-1 fusion protein (1 μg), the reporter plasmid Gal4-CAT (5 μg), and a pSV2-LacZ plasmid (1 μg). Where indicated, cells were cotransfected with Menin plasmid (1.5 μg), ERK2-Myc plasmid (2 μg), or ERK2-MEK1-LA plasmid (0.7 μg). CAT activity was measured and normalized against the co-transfected β-galactosidase activity. Error bars indicate s.d. from four independent experiments. (**b**) HEK-293 cells were transiently transfected with Elk-1-Flag plasmid (1 μg), either alone or in combination with ERK2-Myc plasmid (2 μg), or with Menin plasmid (1.5 μg), as indicated. Immunoblots were performed with anti p-Elk-1 (upper panel), or with anti Flag (middle panel), or anti-Myc antibodies (lower panel). (**c**) HEK-293 cells were transiently transfected with Elk-1-Flag plasmid (1 μg), either alone or in combination with Myc-tagged ERK2-MEK1-LA plasmid (0.7 μg), or Menin plasmid, as indicated. Elk-1 phosphorylation and expression were detected respectively by using anti-p-Elk-1 (upper panel) or anti-Flag antibody (middle panel) as indicated. ERK2-MEK1-LA expression and phosphorylation were respectively detected by using ERK2-1 and anti-phospho-ERK antibody as indicated (lower panels)

It has been reported that ERK2-MEK1-LA catalyses phosphorylation of Elk-1 on ser383 (Robinson *et al*., 1998). Figure 3c shows that the ERK2-MEK1-LA mediated phosphorylation of Elk-1, as monitored by immunoblotting with anti-phospho-Elk-1 antibody (upper panel), was inhibited by Menin co-expression. No change in either Elk-1-Flag or ERK1-MEK1-LA expression (middle panels), or in the phosphorylation state of ERK2-MEK1-LA (lower panel) was detectable. These findings indicate that Menin overexpression can uncouple ERK activation from the phosphorylation of its physiological substrate Elk-1.

Menin represses induction of a c-fos *reporter gene*

The time-course of Elk-1 phosphorylation after triggering the ERK pathway correlates tightly with the activity of Elk target genes (reviewed in Janknecht *et al*., 1994). The c-*fos* promoter contains a functional Elk-1-responsive element (Hill and Treisman, 1995a). Therefore, we examined whether Menin might influence the transcription of a c-*fos* reporter gene (Watanabe *et al*., 1996) that is inducible either by serum stimulation, or by co-expression of a constitutively active mutant of Ras (RasV12). As shown in Figure 4, Menin expression completely blocked both serum-induced and Ras12-stimulated expression of this reporter. Furthermore, we tested the inhibitory effect of Menin on c-*fos* promoter activity induced by c-AMP signaling, that is mediated by the transcription factor CREB in a TCF-independent manner (Sassone-Corsi *et al*., 1988). As shown in Figure 4b, the inhibitory effect of Menin observed on serum-dependent activation of c-*fos* promoter activity was significantly reduced when the c-*fos* promoter was induced by forskolin, a strong activator c-AMP signaling. Since c-AMP promotes ERK activity in several cell types (Houslay and Kolch, 2000; Qiu *et al*., 2000), including those used in our studies, the limited repression of c-AMP dependent c-*fos* promoter activation by Menin probably results from inhibition of ERK-dependent activation of Elk1. These results indicate that Menin specifically inhibits ERK-dependent induction of a physiological promoter that requires endogenous Elk-1 for expression.

Menin inhibits c-Jun and JunD phosphorylation that is induced by JNK signaling

Next we investigated whether Menin also interferes with the JNK group of MAP kinases. Activation of the JNK pathway regulates AP-1 transcription activity mainly through phosphorylation of c-Jun and ATF2 (Karin, 1995). However, JunB and JunD have also been shown to be phosphorylated in response to JNK activation (Gomez and Cohen, 1991; Kallunki *et al*., 1996). We asked whether Menin expression affects c-Jun and JunD phosphorylation in response to anisomycin activation of JNK. JunD-HA or c-Jun-HA were transiently expressed in HEK-293 cells, either alone or together with Menin. Transfected cells were then treated with anisomycin for 30 min. N-terminal phosphorylation of c-Jun and JunD proteins was monitored by Western blot experiments, using the anti-phospho Jun antibody. As shown in Figure 5a, JunD phosphorylation is greatly induced by anisomycin treatment. In contrast, Menin co-expression inhibits JNK-induced phosphorylation of JunD-HA (upper panel) without affecting its expression level (lower panel). As shown in Figure 5b, Menin expression had a similar inhibitory effect on anisomycin-induced c-Jun phosphorylation (upper panel), without effecting c-Jun-HA expression levels (lower panel). These results suggest that Menin inhibits JNK-dependent activation of AP-1 by impairing phosphorylation of either c-Jun or JunD within their transactivation domains.

Menin expression does not affect MAPK activation

The results presented above indicate that Menin suppresses signal-dependent phosphorylation of MAPK targets. To examine whether Menin acts at the level of MAPK catalytic function we analysed the effect of Menin expression on signal-dependent activation of the MAP kinases ERK2 and JNK1. Myc-tagged ERK2 or JNK1-HA were transiently expressed in HEK-293 cells, either alone or in combination with Menin. Transfected cells were then stimulated either with 10% serum for 10 min (Figure 6a) or with anisomycin for 20 min (Figure 6b). Figure 6a shows that Menin coexpression had no effect on either the basal or serum-stimulated activity of ERK2-Myc, assayed by using GST-Elk-1 as substrate. ERK2-Myc specific activity (Figure 6a, upper panel) was determined by normalizing Elk-1 phosphorylation values for the levels of ERK2-Myc protein present in the anti-Myc immunoprecipitates (Figure 6a, middle panel). Similarly, JNK1-HA activity was assayed in JNK1-HA expressing cells by using GST-c-Jun as substrate (Figure 6b). As shown in Figure 6b (upper panel), Menin expression had no effect on anisomycin-dependent activation of JNK1-HA.

These results suggest that Menin inhibits phosphorylation of ERK and JNK nuclear targets by acting downstream of MAPK activation, presumably at the level of their substrates.

The N-terminal region of Menin is required for inhibition of JNK-dependent, but not ERK-dependent phosphorylation of JunD

To test whether Menin needs to physically bind to JunD in order to inhibit its phosphorylation by MAPK, we generated a deletion mutant of Menin (Menin-ΔNH_2, see Materials and methods) that lacks 228 amino acids at the N-terminus and therefore is incompetent for JunD binding (Figure 7a) (Agarwal *et al*., 1999). We expressed Menin-ΔNH_2 in HEK-293 cells and examined its ability to inhibit JunD phosphorylation by MAPK. JunD phosphorylation at

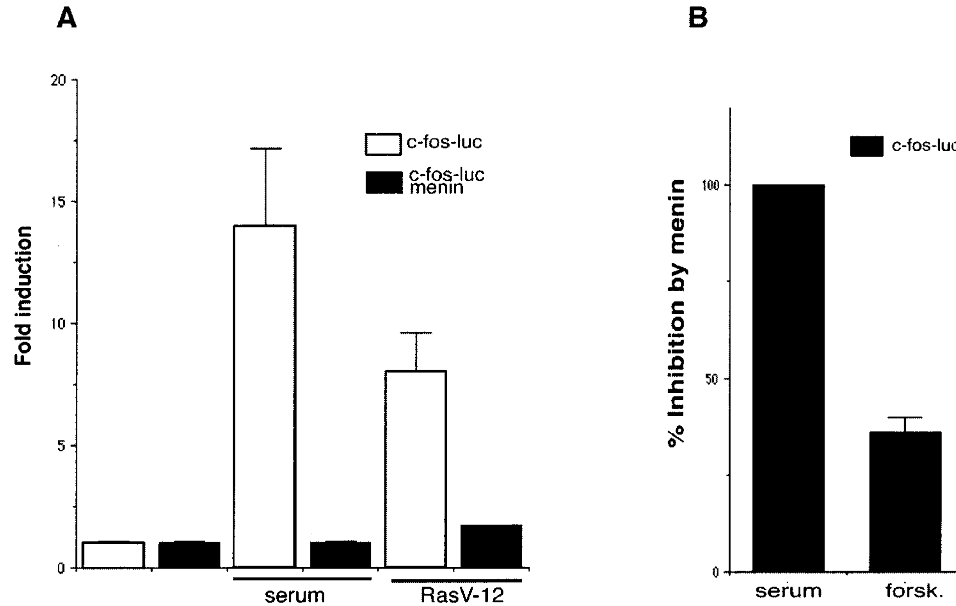

Figure 4 Menin affects the activity of the c-*fos* promoter. (**a**) CHO cells were transiently transfected with a luciferase reporter controlled by the human c-*fos* promoter (0.5 μg) containing the SRE responsive element ($-361/+157$), and a Renilla Luciferase expression vector pRL-CMV (50 ng) (Promega). As indicated, cells were co-transfected either with Menin (0.5 μg) or with RasV12 (0.5 μg). Transfected cells were kept in serum-deprived medium or treated with 10% FCS, where indicated. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase. Error bars indicate s.d. from five independent experiments. (**b**) CHO cells were transfected as described in (**b**). Transfected cells were kept in serum-deprived medium and then treated, as indicated, either with 10% FCS or 10 μM Forskolin. Histograms represent percentage of Menin-mediated inhibition of c-*fos* promoter activity in response to serum or forskolin. Firefly luciferase was determined as described above

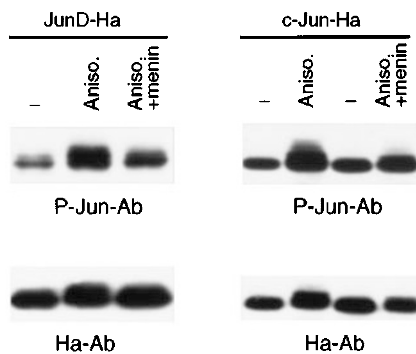

Figure 5 Menin inhibits c-Jun and JunD phosphorylation that is induced by JNK activation. (**a**) HEK-293 cells were transiently transfected with JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing Menin (1.5 μg), 20 h after transfection, cells were stimulated (where indicated) with anisomycin (50 ng/ml) for 30 min. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (lower panel). (**b**) HEK-293 cells were transiently transfected with c-Jun-HA plasmid (2 μg), either alone or together with plasmids expressing Menin (1.5 μg), 20 h after transfection, cells were stimulated (where indicated) with anisomycin (50 ng/ml) for 30 min. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (lower panel)

serine 100 was assayed as described above. As shown in Figure 7b, Menin-ΔNH_2 represses JunD phosphorylation induced by co-expression of ERK2-MEK1-LA as effectively as Menin-wt. Furthermore, Menin-ΔNH_2 was also able to inhibit JunD phosphorylation induced by a constitutively active form of MEK1, MEKEE (Cowley *et al*., 1994), that stimulates endogenous ERKs (Figure 7c). This result demonstrates that Menin does not require JunD binding to repress ERK-dependent phosphorylation of JunD. Interestingly, Menin-ΔNH_2 failed to suppress JunD phosphorylation that is induced by anisomycin-activated JNK (Figure 7c). This observation suggests that JunD binding may be crucial for the ability of Menin to inhibit nuclear JNK signaling. Moreover, Menin apparently interferes with ERK and JNK signaling through two distinct inhibitory mechanisms.

Discussion

It is well established that the AP-1 transcription factor plays a crucial role in growth regulation and transformation by oncogenic Ras (reviewed in Davis, 2000; Rosenberg *et al*., 1999; Mechta-Grigoriou *et al*., 2001). Both ERK and JNK MAP kinase cascades are probably involved in the activation of AP-1 by oncogenic Ras: the first leading to elevated expression of Fos genes through phosphorylation of TCFs, the latter inducing N-terminal phosphorylation of c-Jun (reviewed in Shaulian and Karin, 2001). In the present study we show that Menin functions as suppressor of MAPK-induced phosphorylation of nuclear factors such as Elk-1, JunD and c-Jun, without directly altering the activity of either ERK2 or JNK1. Our

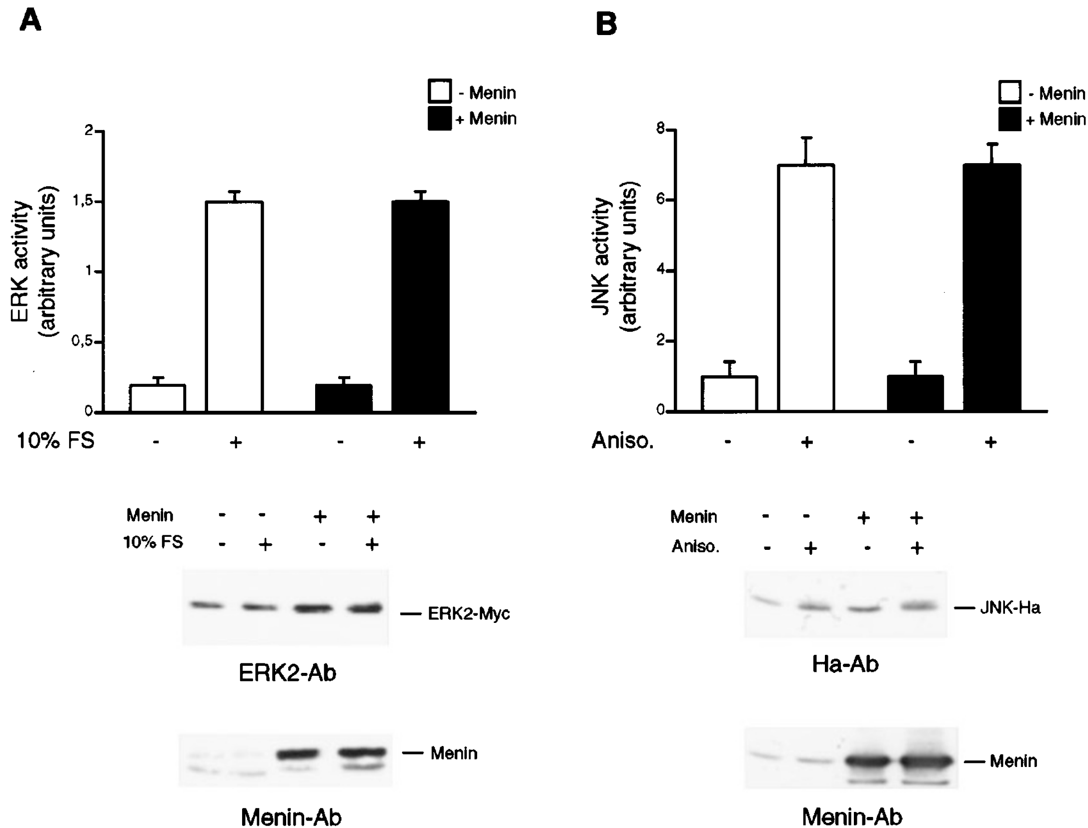

Figure 6 Effect of Menin on ERK2 and JNK1 activation. (**a**) HEK-293 cells were transiently transfected with ERK2-Myc plasmid (2 μg) alone or together with Menin plasmid (1.5 μg). Transfected cells were serum-starved for 24 h and then stimulated with 10% calf serum for 10 min. Cell lysates were immunoprecipitated with Myc antibody and ERK2-myc activity was determined by measuring *in vitro* phosphorylation of GST-Elk-1 using anti-phospho-Elk-1 antibody (upper panel). Levels of ERK2-Myc present in the immunoprecipitates were determined by Western-blot analysis with anti-ERK2 antibody (middle panel). Expression levels of Menin were determined by Western blot analysis of total extracts with anti Menin antibody (lower panel). (**b**) HEK-293 cells were transiently transfected with a JNK1-HA plasmid (2 μg) alone or together with Menin plasmid (1.5 μg). Transfected cells were then stimulated (where indicated) with anisomycin (50 ng/ml) for 20 min. Cell lysates were immunoprecipitated with anti HA antibody and JNK-HA activity was determined by measuring *in vitro* phosphorylation of GST-Jun using anti-phospho-c-Jun antibody (upper panel). Levels of JNK-HA present in the immunoprecipitates were determined by Western-blot analysis with anti-JNK antibody (middle panel). Expression levels of Menin were determined by Western blot analysis of total extracts with anti Menin antibody (lower panel)

results indicate that Menin uncouples ERK and JNK activation from phosphorylation of their nuclear targets through distinct inhibitory mechanisms.

JunD activation by ERK signaling

The transcription factor AP-1 mediates the nuclear response to several extracellular stimuli by acting as a target for MAP kinases (Karin *et al*., 1997; Shaulian and Karin, 2001). In contrast to c-Jun, whose activation by MAPK has been extensively studied, JunD activation by extracellular stimuli has not been yet elucidated. In the present study we show that signal-induced activation of the MEK-ERK pathway leads to phosphorylation of ectopically expressed as well as endogenous JunD in its N-terminal domain (Figure 1). The positive role of MEK activation in JunD phosphorylation was documented by the use of MEK specific inhibitors, PD98059 or UO126, and by expression of a constitutively active form of MEK1 (Figure 7c). Furthermore, the evidence that JunD phosphorylation is enhanced by expression of a constitutively active form of ERK2 kinase confirms that ERK1/2 activation leads to phosphorylation of the JunD transactivation domain.

The analysis of Gal4/JunD fusion proteins, which lacks the leucin zipper domain reveals that phosphorylation of the JunD-N-terminal domain, induced by ERK signals, promotes JunD-dependent transcription, without a requirement for dimerization with other members of the AP-1 family. These findings support a model in which activation of the MEK-ERK pathway leads to ERK-mediated phosphorylation and activation of JunD. Since JunD functions as a negative regulator of Ras-mediated transformation (Pfarr *et al*., 1994), it appears counterintuitive that JunD should act as a nuclear target of MEK-ERK signaling. However, the role of JunD in the control of cell proliferation is complex, since it can have either positive or negative effects depending on the cellular and genetic context (Weitzman *et al*., 2000; reviewed in Shaulian and Karin, 2001).

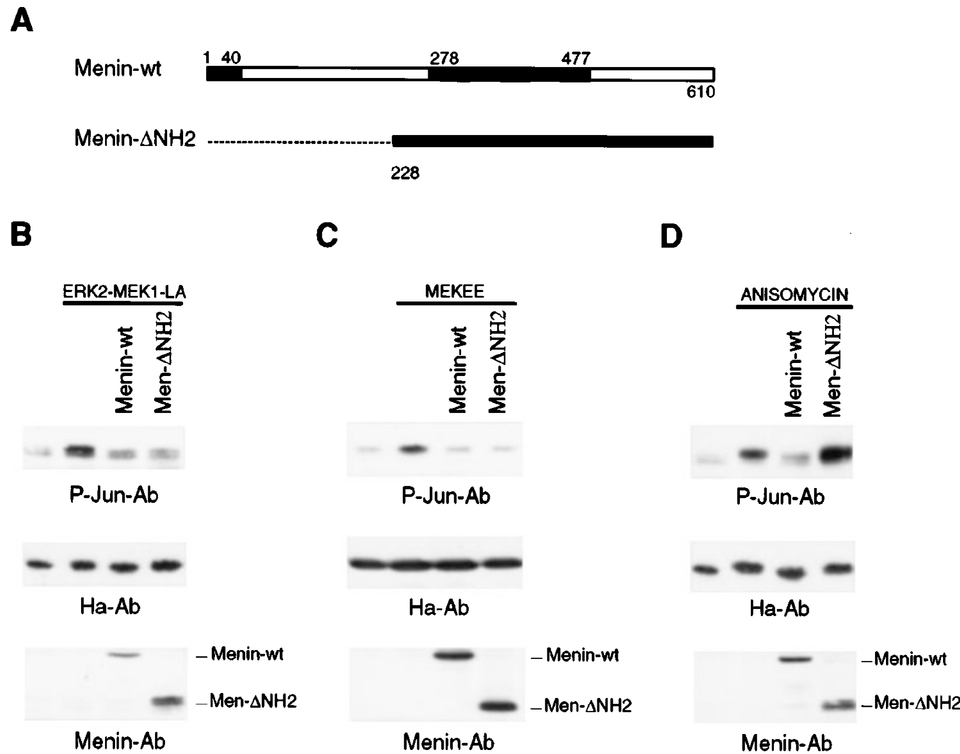

Figure 7 The N-terminal region of Menin is required for inhibition of JNK-dependent, but not ERK-dependent phosphorylation of JunD. (**a**) Structure of full-length Menin and deletion mutant Menin-ΔNH_2. Black boxes in Menin-wt represent the regions that are required for JunD binding. Dotted lines in Menin-ΔNH_2 represent the region that has been deleted. Numbers represent amino acid residues. (**b**) HEK-293 cells were transfected with a JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing CMV-Menin wt (1.5 μg), or CMV-Menin-ΔNH_2 (1.5 μg) or ERK2-MEK1-LA (0.7 μg), as indicated. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (middle panel), or with anti Menin antibody (lower panel). (**c**) HEK-293 cells were transfected with a JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing CMV-Menin wt (1.5 μg), or CMV-Menin-ΔNH_2 (1.5 μg) or MEKEE (2 μg), as indicated. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (middle panel), or with anti Menin antibody (lower panel). (**d**) HEK-293 cells were transfected with a JunD-HA plasmid (1.5 μg), either alone or together with plasmids expressing CMV-Menin wt (1.5 μg), or CMV-Menin-ΔNH_2 (1.5 μg) as indicated. Twenty hours after transfection cells were treated, as indicated, with anisomycin (50 ng/ml) for 30 min. Immunoblots were performed with either anti-P-Jun (upper panel), or with anti HA antibodies (middle panel), or with anti Menin antibody (lower panel)

Menin abrogates JunD phosphorylation and activation by the MEK-ERK pathway

Several lines of evidence indicate that Menin inhibits JunD phosphorylation induced by active MEK-ERK pathway: (a) Menin inhibits JunD phosphorylation that is detected in cycling cells or induced by serum stimulation as efficiently as MEK inhibitors; (b) Menin inhibits JunD phosphorylation that is induced by expression of constitutively active forms of either ERK2 kinase or MEK1 kinase. The N-terminus and the middle region of Menin are both required for binding to JunD (Agarwal *et al*., 1999). The finding that a N-terminal deletion of Menin (Menin-ΔNH_2) is still capable of repressing JunD phosphorylation by ERK signals (Figure 7b,c), demonstrates that physical interaction between JunD and Menin is not required for this inhibitory effect.

It is known that Menin physically interacts and represses JunD-dependent transcription (Agarwal *et al*., 1999; Gobl *et al*., 1999). From the study of Agarwal *et al*. it emerges that JunD/Menin interaction is important, although not sufficient, for mediating repression of JunD-dependent transcription. On the other hand, the study by Gobl *et al*. reveals that a histone deacetylase activity might be implicated in the repression of JunD-activated transcription. Together these studies suggest a model where Menin represses JunD-dependent transcription by binding and delaying histone deacetylases to JunD specific promoters. The present study demonstrates that the phosphorylation state of the JunD-N-terminal domain modulates JunD-dependent transcription. Hence, the evidence that Menin represses both JunD phosphorylation and JunD-dependent transcription induced by ERK signals suggests that Menin acts as modulator of JunD activity by repressing its phosphorylation. On the other side, Menin inhibits Gal4/JunD-ala activity, indicating that it also impairs JunD-dependent transcription that is independent of the phosphorylation state of JunD. On the other hand, repression of JunD phosphorylation evidently mediates the inhibitory effect of Menin on JunD-dependent transcription when it is induced by MAPK signals. The relevance of these two mechanisms

for the Menin-mediated inhibition of Ras-dependent transformation remains to be defined. However some naturally occurring oncogenic mutant alleles of Menin disrupt Menin/JunD interaction (Agarwal *et al*., 1999) and/or lacks the ability to repress signal-induced phosphorylation of JunD (A Gallo and AM Musti unpublished observations), suggesting that both mechanisms may be crucial for Menin oncosuppressor activity.

*Menin affects Elk-1 activation and the induction of the c-*fos *promoter*

The ternary complex factor (TCF) component Elk-1 is a physiological target of ERK-MEK signaling (Gille *et al*., 1995; Janknecht *et al*., 1993; Price *et al*., 1995; Yamg *et al*., 1998). Elk-1 is phosphorylated by ERK2 within its C-terminal domain. We show that Menin expression inhibits ERK-dependent transcriptional activation of Elk-1 and its C-terminal phosphorylation. As for JunD, Menin inhibits both Elk-1 phosphorylation and transcriptional activity that are induced by constitutively active ERK2. This observation suggests that Menin acts downstream of ERK activation. This interpretation is further supported by the finding that Menin overexpression does not alter the activation of ERK2 by serum stimulation (Figure 6a).

Activation of the TCF family of transcription factors by MEK-ERK signaling mediates transcriptional activation of targets gene of the Ras-MAP pathway, such as c-*fos* (Janknecht *et al*., 1995). Our findings that Menin efficiently blocks either serum stimulated or Ras-dependent expression of the c-*fos* promoter, indicate that Menin-dependent inhibition of Elk-1 activation is physiologically relevant and suggest that Menin prevents Fos accumulation in response to ERK signals. The c-*fos* promoter also contains cis-acting elements responsive to different transcription factors activated by distinct signal transduction pathways (Hill and Treisman, 1995b). An example is the c-AMP responsive element (CRE) that mediates c-*fos* expression in response to the c-AMP responsive element binding protein (CREB) (Sassone-Corsi *et al*., 1988). Induction of c-*fos* expression by active CREB is independent of TCF. The inefficient repression of c-AMP-stimulated c-*fos* promoter activity by Menin indicates that it interferes specifically with MAPK signals.

Menin prevents JunD and c-Jun phosphorylation by JNK

Several lines of evidences suggest that regulation of JNK activity is crucial for both AP1 activity and tumor development (reviewed in Davis, 2000). Phosphorylation of c-Jun on sites that are mainly phosphorylated by JNK (Ser-63 and Ser73) is required for Ras-dependent transformation (Smeal *et al*., 1991). Our results show that Menin impairs JNK-dependent phosphorylation of both c-Jun and JunD on JNK-specific sites that are present in their respective transactivation domains. Since c-Jun activity correlates with JNK-dependent phosphorylation, our findings suggest that Menin overexpression may lead to inhibition of signal-dependent activation of c-Jun.

Interestingly, the N-terminal deletion mutant Menin-ΔNH2, which lacks JunD binding activity (Agarwal *et al*., 1999), fails to inhibit JunD phosphorylation by JNK, but efficiently inhibits ERK-dependent phosphorylation of JunD. These findings indicate that distinct mechanisms account for the inhibition signal-induced phosphorylation of JunD. However, both mechanisms act at a level that are downstream of ERK and JNK activation, since Menin overexpression does not alter signal-dependent activation of either ERK2 or JNK1.

Based on results presented here, we cannot distinguish whether Menin inhibits phosphorylation or promotes dephosphorylation of JunD, Elk-1 and c-Jun. However, *in vitro* kinase assays with purified ERK2 and GST-Elk-1 proteins showed that excess of purified GST-Menin protein did not inhibit ERK2 activity (data not shown), suggesting that Menin does not compete with ERK2 specific substrates. Most likely, the inhibitory mechanism is complicated, involving several, yet unidentified, cellular components.

In conclusion, our study reveals that Menin uncouples ERK and JNK activation from phosphorylation of their nuclear targets, such as Elk-1, JunD and c-Jun. This will inhibit accumulation of Fos/Jun heterodimers by two mechanisms: inhibition of transcription (i.e. Elk-1/TCF) and inhibition of c-Jun phosphorylation, which modulates its stability (Musti *et al*., 1997). Presumably, this will lead to repression of AP-1 activity in response to chronic stimulation of MAPK pathways. These observations suggest a mechanism by which Menin might interfere with transcriptional aspects of cell cycle control.

Materials and methods

Cell culture and transfection

Human embryonic kidney cell line HEK-293 and Chinese hamster ovary cell line CHO were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Life Technology). Mouse fibroblast cell line NIH3T3 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) (Life Technology).

HEK-293 cells were seeded at 8×10^5 cells/100 mm plate and transfected with the indicated plasmids DNA on the following day by calcium-phosphate co-precipitation procedure (Graham and van der Eb, 1973). Transfected cells were harvested 36–48 h after transfection.

CHO cells were seeded into 24-well plates 20–24 h before transfection which was performed using Fugene-6 reagent as recommended by the manufacturer (Roche-Diagnostics). After 5–6 h the medium was replaced with DMEM serum-free (prior to serum stimulation) or supplemented with 2% charcoal-stripped FCS. Cells were incubated for additional 18–20 h before harvesting and assaying for reporter activity.

Plasmids

All DNA manipulations were performed essentially as described (Sambrook *et al*., 1989). The expression vectors

for JunD-HA, c-Jun-HA, JNK1-HA, Gal4-JunD-FL, Myc–ERK2,Myc–ERK-MEK1-LA, MEKEE, HA-RasV12, and the c-*fos*-luc reporter plasmid have been described (Agarwal *et al*., 1999; Cowley *et al*., 1994; Derijard *et al*., 1994; Joneson *et al*., 1996; Musti *et al*., 1996; Robinson *et al*., 1998; Watanabe *et al*., 1996). Full-length Flag-tagged Elk-1 was provided by RJ Davis. The expression vector Gal4-Elk-1, Gal4-VP16 and the reporter plasmids Gal4-luc and Gal4-CAT were supplied by Stratagene.

The expression plasmid for the Gal4-JunD-wt fusion protein was generated by subcloning a DNA fragment corresponding to a Zip-truncated form of mouse JunD (amino acid residues 1–266) into a mammal expression vector in frame with an Ubiquitin-promoter driven Gal4 DNA binding domain. The expression plasmid Gal4-JunD-ala fusion protein was generated as follows: codons for serine 90, serine 100 and Threonine 117 of the expression plasmid JunD-Ha were replaced with codons for alanine, by a PCR-based oligonucleotide-directed mutagenesis system (Landt *et al*., 1990). The resulting mutated plasmid was then used to generate a DNA fragment corresponding to a Zip-truncated form of mutated JunD (amino acid residues 1–266) into a mammal expression vector in frame with an Ubiquitin-promoter driven Gal4 DNA binding domain.

Reporter analysis

Luciferase assays Gal4-JunD and Gal4-JunD-ala constructs containing a Zip-truncate form of mouse JunD were cotransfected with Menin plasmid or ERK2-MEK1-LA plasmid, with the reporter plasmid Gal4-luciferase, and a Renilla Luciferase expression vector pRL-CMV (Promega) in CHO cells. Transient transfections were performed using Fugene-6 reagent as recommended by the manufacturer (Roche-Diagnostics). Cells were incubated for 18–20 h and then luciferase activity was measured with Dual Luciferase Kit (Promega) according to the recommendations of the manufacturer. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase signal. Same procedures were followed for transfections of CHO cells with c-*fos*-luciferase reporter and RasV12 plasmids.

Chloranphenicol acetyltransferase (CAT) assays HEK-293 cells were transfected by the calcium-phosphate method (Graham and van der Eb, 1973). Thirty-six to forty-eight hours after transfection cells were harvested and cell extract was prepared as described (Sambrook *et al*., 1989). CAT and β-galactosidase assays were determined as described (Gorman *et al*., 1982; Sambrook *et al*., 1989).

Immunoblotting

Cell extracts from HEK-293 cells were prepared in a buffer containing 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1% N-P40 and Protease inhibitors (Boehringer Mannheim). Samples were then boiled in SDS-sample buffer for 5 min, and the proteins were separated on 10% SDS–PAGE gels and detected by immunoblot as previously described (Musti *et al*., 1996). HA-tagged proteins (JunD-HA, c-Jun-HA and JNK-HA) were detected by using a mouse monoclonal antibody anti-HA (Santa Cruz). Phosphorylated JunD-HA and c-Jun-HA were detected by using an anti phospho-specific c-Jun/JunD antibody (NEB) that recognizes JunD when it is phosphorylated at serine 100, and c-*jun* when it is phosphorylated at serine 73. Menin expression was detected by immunoblot using a polyclonal anti-goat antibody anti-Menin (C-19) that is raised against a peptide mapping at the carboxy terminus of human Menin (Santa Cruz). Myc-tagged ERK2 and ERK2-MEK1-LA were detected by immunoblot using a mouse monoclonal antibody anti-Myc 9E10 (Sigma) or a polyclonal anti-rabbit antibody anti-ERK2(C-14) (Santa Cruz). Phosphorylated ERK2-MEK1-LA was detected by using a mouse monoclonal anti p-ERK(E-A) (Santa Cruz). Endogenously expressed ERK1-2 kinase and JNK1-2 were detected respectively by using rabbit polyclonal anti-ERK2(C-14) and rabbit polyclonal anti-JNK1(FL) (Santa Cruz). FLAG-tagged Elk-1 was detected by immunoblot analysis using a mouse monoclonal anti-M2 FLAG antibody (Sigma) or anti-phospho Elk-1 (S383) antibody (NEB). Antibodies were used according to instructions of manufacturers. Immune complexes were detected by using horseradish peroxidase-conjugated secondary antibody followed by ECL (Amersham).

Kinase assay

HEK-293 cells were transfected with ERK2-Myc or JNK1-HA in the presence or absence of Menin. After transfection, cells were serum starved for 24 h followed by stimulation with either 10% FCS for 10 min, or with anisomycin (50 ng/ml) (Sigma). Transfected cells were lysed in the following buffer: 10 mM Tris-Cl pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and Protease inhibitors mix (Boehringer Mannheim) was added as recommended by the manufacturers. Cell lysates were incubated with agarose conjugate monoclonal anti-Myc (9E10) (Santa Cruz) or agarose conjugate monoclonal HA (F-7) (Santa Cruz) antibody for 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and incubated in kinase buffer containing either 1.5 μg of either GST-Elk-1 (NEB) or GST-c-Jun (NEB). Phosphorylation of GST-Elk-1 and GST-c-Jun was examined by Western blot analysis, as described above, using anti-P-Elk-1 (NEB) and anti-P-c-Jun (NEB). GST-Elk-1 and GST-c-Jun phosphorylation values were determined by densitometric analysis of exposed films.

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