pacity of these proteins resulted in a decreased amount of translation of the *psbA* mRNA, we analyzed the synthesis of D1 protein in vivo by pulse labeling proteins with [^{14}C]acetate. Synthesis of the D1 protein, as well as of other light-regulated proteins (D2, CP47, and CP43), was less in *cc703* cells than in wt cells (Fig. 4). In contrast, the synthesis of the α and β subunits of adenosine triphosphatase (ATPase), which do not show light-regulated translation (*3*), was not affected in *cc703* cells (Fig. 4A). The synthesis of proteins in light-grown *cc703* cells (Fig. 4A) is similar to the synthesis of proteins in dark-grown wt cells (*3*) and is in agreement with the hypothesis that reducing power, generated by PS I, is responsible for the activation of translation of the light-regulated chloroplast mRNAs. Analysis of amounts of *psbA*, *psbD*, and *atpB* mRNAs by RNA-blot analysis (Fig. 4B) showed no difference between wt and *cc703* cells. Thus, differences between wt and *cc703* cells in the synthesis of D1 and other proteins were due to differences in the rate of translation and not to altered amounts of mRNAs.

Thioredoxin serves as a transducer of redox potential generated by the "light-reactions" of photosynthesis (*15*), providing the chloroplast with a mechanism to coordinate the activity of various components of photosynthesis to light. Our results show that the formation of *psbA* RNP is also modulated by redox in vitro and in vivo and that translation of the core proteins of PS II is regulated by redox in vivo. These data suggest that thioredoxin may act as a link between the "light-reactions" of photosynthesis and the amount of *psbA* mRNA binding and hence *psbA* mRNA translation in vivo. According to this model, translation of light-regulated proteins is activated in response to changes in redox potential generated by photosynthesis. Following illumination, the reducing power generated by photosynthesis is used to reduce thioredoxin through a series of oxidation-reduction reactions involving ferredoxin (Fd), ferredoxin-thioredoxin reductase, and thioredoxin. Reduced thioredoxin drives reduction of the *psbA* mRNA–binding proteins, increasing their capacity to bind the 5′-UTR of *psbA* mRNA. Binding of this protein complex to the *psbA* mRNA allows for recruitment of *psbA* mRNAs onto polysomes and subsequent translation of the D1 protein. This direct link between light and the translation of the D1 protein, which is part of the PS II reaction center, provides the cell with a capacity to respond to fluctuating light levels, replacing photooxidized reaction center proteins (*21*, *22*) at a rate that is appropriate to the rate of photosynthesis.

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Differential Activation of ERK and JNK Mitogen-Activated Protein Kinases by Raf-1 and MEKK

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Growth factors activate mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinases (ERKs) and Jun kinases (JNKs). Although the signaling cascade from growth factor receptors to ERKs is relatively well understood, the pathway leading to JNK activation is more obscure. Activation of JNK by epidermal growth factor (EGF) or nerve growth factor (NGF) was dependent on H-Ras activation, whereas JNK activation by tumor necrosis factor α (TNF-α) was Ras-independent. Ras activates two protein kinases, Raf-1 and MEK (MAPK, or ERK, kinase) kinase (MEKK). Raf-1 contributes directly to ERK activation but not to JNK activation, whereas MEKK participated in JNK activation but caused ERK activation only after overexpression. These results demonstrate the existence of two distinct Ras-dependent MAPK cascades—one initiated by Raf-1 leading to ERK activation, and the other initiated by MEKK leading to JNK activation.

The transactivation function of c-Jun, a component of AP-1, is stimulated by phosphorylation at serines 73 and 63 (*1*, *2*) in response to H-Ras activation (*1*). Phosphorylation at Ser^{63} and Ser^{73} is also stimulated by other oncoproteins, including v-Sis and v-Raf (*3*), or by exposure of cells to ultraviolet radiation (*4*), TNF-α (*5*), growth factors (*6*), or factors that activate T cells (*7*). Both Ser^{63} and Ser^{73} of c-Jun are efficiently phosphorylated by a single type of protein kinase termed JNK, which exists in several isoforms (*8*–*10*). The JNKs are members of the MAPK family (*9*, *10*) and, like ERK1 (pp44) and ERK2 (pp42) (*11*), are activated through phosphorylation at conserved Thr and Tyr residues (*9*). Both the ERKs (*12*) and JNKs (*8*, *9*) are activated by EGF and oncogenic H-Ras (*6*–*8*). The two forms of JNK expressed by human cells, JNK1 and JNK2, are also efficiently activated in cells exposed to ultraviolet radiation or TNF-α, which are weak ERK activators (*6*). In contrast, 12-O-tetradecanoyl phorbol-13-acetate (TPA), a potent ERK activator (*12*), has little effect on JNK activity in fibroblasts and epithelial cells (*6*). These differences suggest that the JNKs are activated by a different signaling cascade than that which

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mediates ERK activation. However, the activation of JNKs by H-Ras (*8*, *9*) suggests that at least one pathway leading to their activation is Ras-dependent. The relation of this pathway to the Ras-dependent pathway that mediates ERK activation (*12*–*14*) is unknown.

We have now shown that activation of JNK by the growth factors EGF and NGF is dependent on H-Ras. Although the activation of ERKs by Ras is mediated via Raf-1 (*13*) and the MAPK, or ERK, kinases (MEKs) (*14*), neither Raf-1 nor MEK1 or MEK2 mediate JNK activation. Instead, we showed that JNK activation was mediated by another Ras-responsive protein kinase, MEK kinase (MEKK) (*15*, *16*). Activation of MEKK resulted in preferential activation of JNK rather than ERK. Hence, Ras activation triggers two divergent signaling cascades that activate distinct MAPKs with different substrate specificities and transcriptional functions (*17*).

Phosphorylation of c-Jun (*1*, *3*) and JNK activity (*8*, *9*) are both stimulated by oncogenic H-Ras. To determine whether c-H-Ras is an important intermediate in JNK activation, we examined the effect of the dominant interfering *Ha-ras(Asn17)* mutant (*18*) on JNK activation by EGF, NGF, or TNF-α. JNK activity was measured by the ability of the protein to bind and phosphorylate a glutathione-S-transferase (GST)–c-Jun(1–223) fusion protein immobilized on glutathione-agarose beads (*8*). In rat pheochromocytoma PC12 cells harboring a glucocorticoid-inducible *Ha-ras(Asn17)* construct (*18*), treatment with dexamethasone blocked JNK activation by EGF and NGF but not by TNF-α (Fig. 1A). These differences correlate with the different abilities of EGF, NGF, and TNF-α to activate H-Ras in PC12 cells (6). Kinase assays of proteins separated by SDS–polyacrylamide gel electrophoresis (PAGE) (*7*–*10*) or immunoprecipitated with an antiserum to JNK confirmed that *Ha-ras(Asn17)* expression blocked activation of both JNK1 and JNK2 (*19*). *Ha-ras(Asn17)* induction also blocked the activation of ERK2 by EGF and NGF (Fig. 1A) (*12*). Consistent with its in- ability to activate Ras (6), TNF-α had only a small effect on ERK activity. Inhibition of both JNK and ERK activation occurred with little delay and with similar kinetics after induction of *Ha-ras(Asn17)* (Fig. 1B), suggesting that inhibition is attributable to direct interference with Ras function. Transient cotransfection of an expression vector encoding epitope-tagged JNK1 (9) with an *Ha-ras(Asn17)* expression vector (*18*) into HeLa cells inhibited JNK activation by EGF but not by TNF-α (Fig. 1C). Hence, JNK activation by growth factors such as EGF and NGF is dependent on Ras function (*8*, 9). However, JNK is also activated by Ras-independent stimuli, such as TNF-α.

TPA is an efficient activator of the ERKs (*6*, *12*), acting most likely through activation of Raf-1 by protein kinase C (*20*). The small effect of TPA on JNK activity in cells in which it activates ERK2 (6) suggests that Raf-1 does not participate in JNK activation. However, expression of activated Raf-1 stimulated c-Jun phosphorylation at Ser^{63} and Ser^{73} (*3*). Because the effect of TPA on JNK activity was determined during the first hour after its addition (6), whereas the effect of activated Raf-1 on c-Jun phosphorylation was examined 36 to 48 hours after coexpression of c-Jun and Raf-1 (*3*), we examined the effect of Raf-1 activation on JNK in more detail. Coexpression of epitope-tagged JNK1 and activated Raf-1 resulted in weak activation of JNK (Fig. 2A). Greater JNK1 activation was obtained by cotransfection with an activated H-Ras expression vector, but even this response was weaker than the response to EGF. In contrast, ERK2 was efficiently activated by both the H-Ras and Raf-1 vectors, and the response was comparable to that induced by EGF (Fig. 2A). We used an NIH 3T3 cell line expressing a fusion protein between activated Raf-1 and the ligand-binding domain of the estrogen receptor (3T3:hRafER) (*21*) to examine the kinetics of JNK and ERK activation in response to hRafER activation by the antiestrogen ICI 164-384 (*21*). Although addition of ICI 164-384 resulted in rapid ERK2 activation, JNK activation did not occur until 16 to 24 hours later (Fig. 2B).

The slow kinetics of JNK activation by Raf-1 most likely reflected the operation of an autocrine loop. Addition of conditioned medium from ICI 164-384–treated 3T3:hRafER cells to HeLa cells resulted in rapid and efficient JNK activation (Fig. 2C). Medium conditioned by uninduced 3T3:hRafER cells or by ICI 164-384–treated parental NIH 3T3 cells did not induce JNK activation in HeLa cells. Thus, although Raf-1 activation may result in delayed JNK activation through an autocrine loop, Raf-1 is not a component of the JNK activation cascade. Raf-1 also does not participate in the response of JNK to TNF-α, because it was not activated by this cytokine (*22*). These conclusions are also consistent with the inability of recombinant MEK1 and MEK2 (*23*), the ERK kinases activated by Raf-1 (*13*, *14*), to phosphorylate and activate either JNK1 isolated from nonstimu-

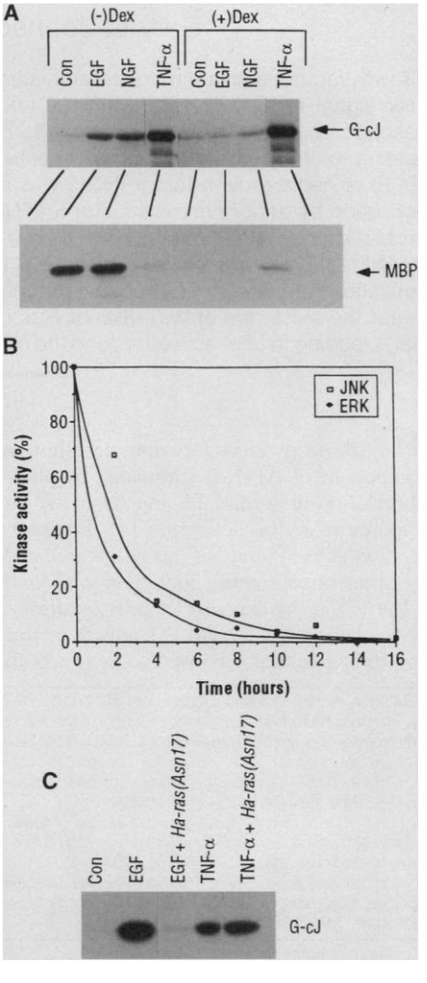

Fig. 1. Dependence of JNK activation by growth factors on Ras. (**A**) PC12 cells expressing dominant negative *Ha-ras(Asn17)* under the control of the murine mammary tumor virus (MMTV) promoter (*18*) were incubated with 1 μM dexamethasone or vehicle alone for 16 hours and then treated with EGF (15 ng/ml), NGF (50 ng/ml), TNF-α (10 ng/ml), or buffer alone (Con) for 15 min. Cells were collected and assayed for JNK activity by solid-phase kinase assay (*6*, *8*) with GST–c-Jun(1–223) as a substrate (G-cJ), or for ERK2 activity in an immune complex kinase assay with myelin basic protein (MBP) as a substrate (*6*). The phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. (**B**) PC12 cells harboring the glucocorticoid-inducible *Ha-ras(Asn17)* construct (*18*) were incubated with 1 μM dexamethasone for the indicated times, after which they were treated with EGF (15 ng/ml) for 15 min. The cells were collected, and lysates were assayed for JNK or ERK2 activity as described in (A). GST–c-Jun(1–223) or MBP phosphorylation was quantitated with a Bio-Rad Phosphoimager. The amount of kinase activity present in extracts of cells incubated in the absence of dexamethasone was taken as 100%. (**C**) HeLa cells were transfected with an HA-JNK1 expression vector (*30*), together with either an empty expression vector or a *Ha-ras(Asn17)* expression vector (*18*). After 48 hours, the cells were incubated in the absence (Con) or presence of either EGF (15 ng/ml) or TNF-α (10 ng/ml) for 15 min. The cells were then harvested, and JNK1 activity was measured in an immune complex kinase assay (*9*, *31*) with GST–c-Jun(1–79) as a substrate (G-cJ).

lated human cells (Fig. 3A) or recombinant JNK1 expressed in *Escherichia coli* (*24*). Both MEK1 and MEK2 efficiently activated ERK2 (Fig. 3B) (*14*).

Recently, MEKK was identified as another MEK-activating kinase (*15*). Except for features common to all protein kinases, MEKK is unrelated to Raf-1. However, it is quite similar to Ste11, a yeast protein kinase from the kinase cascade that activates the Fus3 and Kss1 MAPKs (*25*). MEKK activity is stimulated in cells treated with EGF or NGF in a Ras-dependent manner and is weakly responsive to TPA (*16*). To determine whether MEKK participates in the JNK activation cascade, we constructed an expression vector encoding truncated MEKK (MEKKΔ) under the control of a glucocorticoid-inducible promoter (*26*). Cotransfection of this vector into HeLa cells with expression vectors encoding epitope-tagged JNK1 or ERK2 resulted in dexamethasone-inducible JNK1 activation after 4 hours, a time at which little MEKKΔ had accumulated (Fig. 4A). However, ERK2 activation was delayed and did not occur until 12 hours after dexamethasone addition, by which time a substantial amount of MEKKΔ had accumulated. We observed JNK1 activation in cells transfected with as little as 2.5 ng of an expression vector encoding full-length MEKK rather than MEKKΔ (Fig. 4B). ERK2 activation, however, was not observed until 500 ng of the MEKK expression vector were transfected. With the expression vector encoding MEKKΔ, virtually complete activation of JNK1 was obtained with 2.5 ng of DNA, whereas full ERK2 activation required 25 ng of DNA (Fig. 4B). Therefore, the truncation of MEKK resulted in a partial loss of specificity and also increased its stability or the efficiency of its translation (*26*).

To examine whether MEKK activity could be augmented in response to Ras activation, we transfected cells with the JNK1 or ERK2 vectors and expression vectors encoding MEKK, Raf-1, or activated H-Ras (Fig. 4C). Activated H-Ras alone increased JNK1 activity 2.7-fold and increased the activation of JNK1 by MEKK by 2.9-fold. Although activated Raf-1 alone weakly activated JNK1, it had no effect on JNK1 activation by MEKK. In contrast, ERK2 activity was markedly stimulated by either activated H-Ras or Raf-1 and no further activation was obtained in the presence of MEKK (Fig. 4C); in fact, MEKK attenuated the activation of ERK2 by Raf-1.

We also examined the effect of a catalytically inactive MEKK mutant, MEKKΔ(K432M) (*26*), on JNK and ERK activation by EGF in the same transient transfection assay. Whereas coexpression of MEKKΔ(K432M) resulted in at least 80% inhibition of JNK1 activation by EGF, ERK2 activation was inhibited by less than 20% (Fig. 4D).

These results and those described elsewhere (*16*) demonstrate that Ras proteins activate two divergent signaling cascades, one initiated by Raf-1 and the other by MEKK. The first pathway results in activation of the ERKs (*13*, *14*, *21*), and the second pathway activates the JNKs. Expression of dominant negative H-Ras resulted in rapid and complete inhibition of JNK activation by EGF and NGF. Activated H-Ras activates Raf-1 (*27*) and is essential for activation of MEKK (*16*). Raf-1 does not participate in the kinase cascade leading to JNK activation, although it is an efficient activator of the ERK pathway. In contrast, MEKK was a potent activator of JNK in the absence of ERK activation, and kinase-defective MEKK inhibited JNK, but not ERK, activation by EGF. However, when overexpressed, MEKK also activated the ERK pathway, suggesting the possibility of a cross-talk between the two pathways. The activity of full-length MEKK was potentiated by coexpression of activated H-Ras, resulting in marked JNK activation.

Activation of Raf-1 can result in delayed JNK activation. Although the effect of excessive MEKK expression on ERK activity is likely attributable to activation of MEK1 or MEK2 (*15*, *16*), the effect of Raf-1 on JNK is mediated through an autocrine loop. Because MEK1 and MEK2 do not activate JNK1, the in vivo targets of MEKK are likely to be different dual-specificity protein kinases responsible for JNK activation (JNKKs).

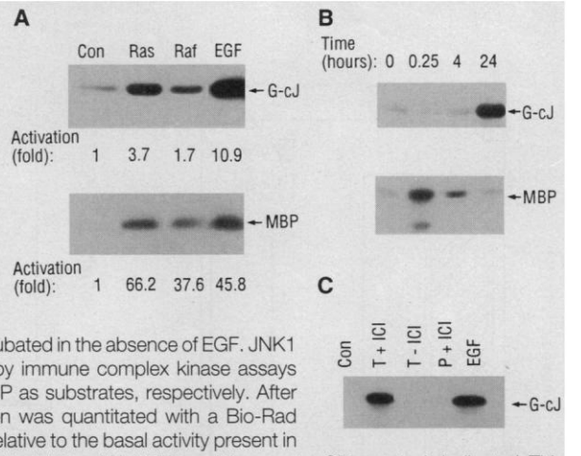

Fig. 2. Effect of Raf-1 on the JNK activation cascade. (**A**) HeLa cells were transfected with either an HA-JNK1 or an HA-ERK2 (*30*) expression vector and the pSRα (*32*) expression vector with no insert or with activated H-Ras(Leu^{61}) (*33*) or activated Raf-1(BXB) (*34*) inserts. After 48 hours, cells transfected with JNK1 or ERK2 constructs and the empty expression vector were incubated without (Con) or with EGF (15 ng/ml) for 15 min; cells transfected with JNK1 or ERK2 constructs and either H-Ras or Raf-1 vectors were incubated in the absence of EGF. JNK1 and ERK2 activities were measured by immune complex kinase assays with 2 μg of GST–c-Jun(1–79) or MBP as substrates, respectively. After SDS-PAGE, substrate phosphorylation was quantitated with a Bio-Rad Phosphoimager. The activation (fold) relative to the basal activity present in unstimulated cells cotransfected with HA-JNK1 or HA-ERK2 and the empty pSRα vector is indicated. This experiment was repeated at least three times with similar results. (**B**) 3T3:hRafER cells (*21*) were incubated with 1 μM ICI 164-384 for the indicated times. Cell extracts were prepared and assayed for JNK activity by the solid-phase kinase assay with GST–c-Jun(1–79) as substrate (*6*, *8*), or for ERK2 activity by immune complex kinase assay with MBP as substrate (*6*). After SDS-PAGE, phosphorylated proteins were visualized by autoradiography. (**C**) 3T3:hRafER cells (*21*) were maintained in serum-free medium in the absence (T−ICI) or presence (T+ICI) of 1 μM ICI 164-384 for 24 hours. Parental NIH 3T3 cells were incubated with ICI 164-384 for 24 hours in serum-free medium (P+ICI). Conditioned medium was removed and added to serum-deprived HeLa cells. As a positive control, HeLa cells were treated with EGF (15 ng/ml); as a negative control, HeLa cells were incubated in serum-free medium (Con). After 15 min, the HeLa cells were harvested and JNK activity was measured by the solid-phase kinase assay (*6*, *8*).

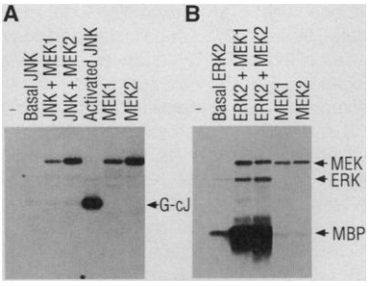

Fig. 3. Activation of ERK2 but not JNK1 by MEK1 and MEK2. (**A**) Failure of MEK1 and MEK2 to activate JNK1. HA-JNK1 was immunopurified from either untreated (basal JNK) or EGF-treated (15 ng/ml, 15 min) (activated JNK) HeLa cells that were transiently transfected with an HA-JNK1 expression vector (1 μg/10^6 cells). Immunopurified HA-JNK1 from untreated cells (basal JNK) was incubated with or without purified GST-MEK1 or GST-MEK2 (1 μg each), prepared as described (*35*), for 20 min at 30°C in kinase buffer [20 mM tris-HCl (pH 7.6), 20 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM dithiothreitol, 1 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate] containing 50 μM adenosine triphosphate (ATP) and [γ-^{32}P]ATP. GST–c-Jun(1–79) (2 μg) was then added for another 30 min to assay JNK1 activity. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. (**B**) Phosphorylation and activation of ERK2 by MEK1 and MEK2. Purified recombinant histidine-tagged ERK2 (0.2 μg), expressed in and purified from *E. coli* (*23*), was preincubated with or without purified GST-MEK1 or GST-MEK2 (1 μg each) for 20 min at 30°C in kinase buffer containing 50 μM ATP and [γ-^{32}P]ATP. MBP (2 μg) was added to the reaction mixture for another 15 min to assay ERK2 activity. Phosphorylated proteins were resolved and visualized as in (A).

Although normal Ras function is necessary for JNK activation by EGF, expression of oncogenic H-Ras does not activate JNK as efficiently as EGF, suggesting that activation of the EGF receptor generates a second signal that potentiates JNK activation. Similarly, activation of JNK by H-Ras activators in T cells is potentiated by Ca^{2+}, a second signal generated by the T cell receptor (*7*). However, Ca^{2+} ionophore has no effect on JNK activation by H-Ras in fibroblasts or HeLa cells (*19*). Furthermore, we have not observed any potentiation of the response of JNK to H-Ras by treatment with TPA (*19*). Thus, the nature of the second signal generated by the EGF receptor is currently unknown. JNK is also activated by cytokines, such as TNF-α, whose effect is Ras independent. The protein kinases that mediate JNK activation by TNF-α have not been identified. However, because the response of JNK to EGF and TNF-α together is more than additive (*19*), it is likely that the TNF-α receptor uses signaling molecules different from those activated by the EGF receptor.

Both the ERKs and the JNKs phosphorylate transcription factors (*17*). However, their substrates differ; ERK1 and ERK2 phosphorylate and potentiate the activity of TCF/Elk-1 and thereby induce c-*fos* (*28*), whereas the JNKs phosphorylate and potentiate the activity of c-Jun (*7–9*) and ATF-2 (*29*). The activation of two divergent MAPK cascades that contribute to transcriptional regulation explains at least part of the pleiotropic effects of growth factors on cell proliferation, differentiation, and survival.

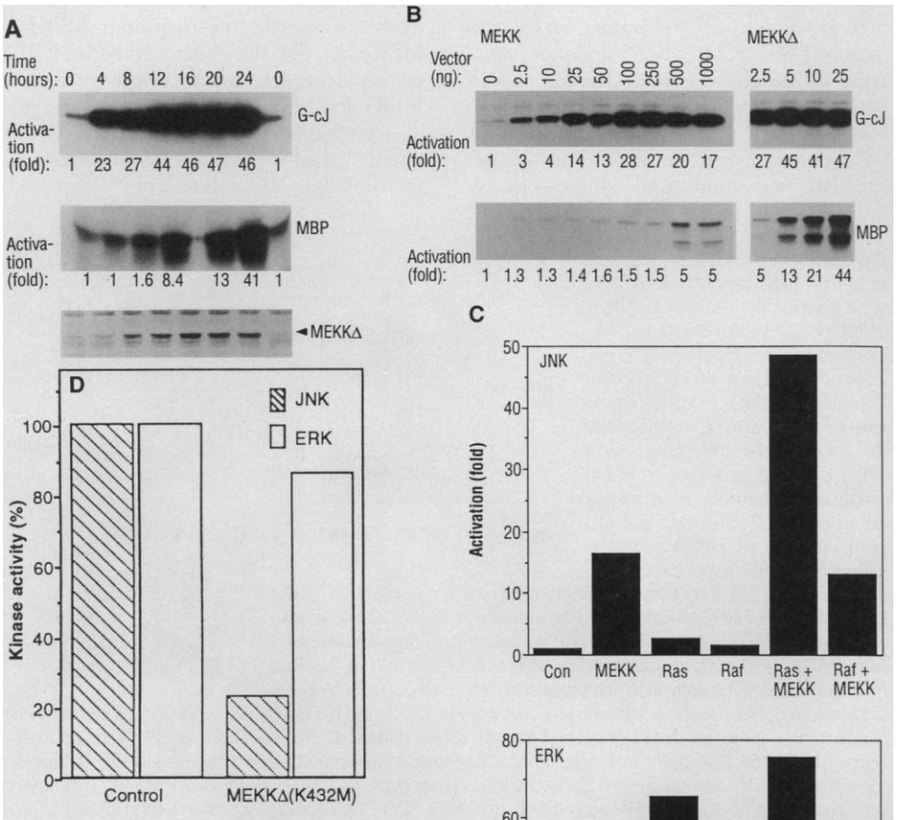

Fig. 4. Preferential activation of the JNK activation cascade by MEKK. (**A**) HeLa cells were transiently cotransfected with expression vectors encoding the truncated MEKKΔ, under the control of the inducible MMTV promoter (*26*), and the rat glucocorticoid receptor (*36*), together with an expression vector encoding either HA-JNK1 or HA-ERK2. The following day, MEKKΔ expression was induced with 1 μM dexamethasone. At the indicated times, cells were collected and assayed for either JNK1 or ERK2 activity as described in the legend to Fig. 2A. Fold activation relative to unstimulated cells is indicated. Extracts from these cells were also analyzed by immunoblotting (*37*) to visualize MEKKΔ expression. (**B**) HeLa cells were transfected with 1 μg of expression vector encoding either HA-JNK1 or HA-ERK2, together with various concentrations of CMV5 vectors encoding either full-length MEKK or MEKKΔ. The amount of DNA was kept constant by supplementation with pCMV5. After 48 hours, cell extracts were prepared, and JNK1 or ERK2 activity was measured. Fold activation relative to control cells transfected with HA-JNK1 or HA-ERK2 expression vectors alone is indicated. (**C**) HeLa cells were transfected with either HA-JNK1 or HA-ERK2 expression vectors (0.6 μg), together with pSRα(Con) or with 0.6 μg of expression vectors encoding full-length MEKK, activated H-Ras(Leu^{61}), or activated Raf-1(BXB) either alone or in combination. DNA concentration was kept constant with pSRα. After 48 hours, the cells were collected and JNK1 and ERK2 activities were measured. This experiment was repeated three times with essentially identical results. (**D**) HeLa cells were transfected with 0.5 μg of either HA-JNK1 or HA-ERK2 expression vectors, and 250 ng of either a MEKKΔ(K432M) expression vector or pSRα (control). After 48 hours, cells were stimulated with EGF (15 ng/ml) for 15 min, extracts were prepared, and JNK1 and ERK2 activities were measured. Kinase activity is expressed as a percentage of that observed in control cells. This experiment was repeated twice with similar results.

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22. A. Minden, A. Lin, C. Lange-Carter, unpublished results. Raf-1 activation was examined in HeLa cells by an immune complex kinase assay, in which transiently expressed epitope-tagged Raf-1 was immunoprecipitated and catalytically inactive MEK1 was used as a substrate [M. Wartmann and R. J. Davis, *J. Biol. Chem.* **269**, 6695 (1994)]. Although EGF activated Raf-1 approximately 10-fold, Raf-1 was not activated

by TNF-α. However, in the same cells, TNF-α activated JNK 10-fold. Similar results were obtained when endogenous Raf-1 and B-Raf were immunoprecipitated from PC12 cells and catalytically inactive MEK1 was used as a substrate; although EGF activated Raf-1 and B-Raf, TNF-α did not.
23. Purified recombinant GST-MEK1 and GST-MEK2 were provided by K. L. Guan (*35*). Recombinant histidine-tagged ERK2 protein was expressed as described [T. G. Boulton *et al.*, *Cell* **65**, 664 (1991)] and purified by a Nickel-NTA-agarose column (Qiagen).
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26. A. Minden and C. Lange-Carter, unpublished results. MEKKΔ is a truncated form of MEKK that lacks amino acids 1 to 351 and in which translation is initiated at an internal Nco I site. Complementary DNA encoding the COOH-terminal portion (1.7 kb) of MEKK was subcloned into pBluescript KS⁻ (Stratagene) between the Eco RI and Xho I sites. A Bam HI–Kpn I fragment encoding the entire COOH-terminal portion of MEKK was then subcloned between the Bam HI and Kpn I sites of pJ5Ω [J. P. Morgenstern and H. Land, *Nucleic Acids Res.* **18**, 1068 (1990)], in which expression is under the control of the MMTV long terminal repeat. MEKKΔ(K432M) was constructed like MEKKΔ, but it also contains a $Lys^{432} \rightarrow Met$ substitution. To compare expression of MEKK and MEKKΔ, we transfected COS-1 cells with equal amounts of MEKK and MEKKΔ CMV5 expression vectors (*15*) by the calcium phosphate coprecipitation technique [S. Osawa *et al.*, *Cell* **63**, 697 (1990)]. Cell extracts were prepared and analyzed after 48 hours by immunoblotting (*37*), and the amounts of MEKK and MEKKΔ were quantitated by densitometry. The extent of MEKKΔ expression was five- to sixfold that of MEKK.
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30. T. Deng, personal communication. HA-tagged JNK1 and ERK2 contain the influenza virus hemagglutinin epitope fused to the NH_2-termini of JNK1 and ERK2, respectively.
31. Transfections were done with lipofectamine (Gibco/BRL). Cells on 35-mm plates were transfected with 1 μg of each plasmid and collected 48 hours later (*9*). Cell extract (100 μg of protein) was analyzed by immune complex kinase assays, after precipitation with antibodies to HA (Boehringer Mannheim), in the presence of kinase buffer containing 20 μM ATP, [γ-^{32}P]ATP, and either 2 μg of GST–c-Jun(1–79) or MBP as substrate (*9*).
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37. For immunoblot analysis, whole-cell extract (25 μg of protein) was resolved by SDS-PAGE and transferred to Immobilon P membranes (Millipore). After blocking of nonspecific sites, the filters were incubated with antibodies to a COOH-terminal peptide from MEKK (C-22; Santa Cruz Biotechnology), and antibody-antigen complexes were visualized by the chemiluminescence detection system (Amersham), as described (*7–9*).
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Suppression of Hyphal Formation in *Candida albicans* by Mutation of a *STE12* Homolog

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A *Candida albicans* gene (*CPH1*) was cloned that encodes a protein homologous to *Saccharomyces cerevisiae* Ste12p, a transcription factor that is the target of the pheromone response mitogen-activated protein kinase cascade. *CPH1* complements both the mating defect of *ste12* haploids and the filamentous growth defect of *ste12/ste12* diploids. *Candida albicans* strains without a functional *CPH1* gene (*cph1/cph1*) show suppressed hyphal formation on solid medium. However, *cph1/cph1* strains can still form hyphae in liquid culture and in response to serum. Thus, filamentous growth may be activated in *C. albicans* by the same signaling kinase cascade that activates Ste12p in *S. cerevisiae*; however, alternative pathways may exist in *C. albicans*.

Candida albicans is the most frequently isolated fungal pathogen in humans. The ability to switch between the yeast and filamentous form has been postulated to contribute to the virulence of this organism (*1*). Difficulties in the genetic manipulation of *C. albicans* have hindered the identification of factors that contribute to the dimorphic switch. *Candida albicans* has no known sexual cycle and is at least diploid. Thus, it is difficult to isolate and identify mutations that affect pathogenicity (*1*).

The observation that *S. cerevisiae* is also dimorphic has permitted the analysis of the switch from budding cells to filaments in a genetically more tractable fungus. Under conditions of nitrogen starvation on solid medium, *S. cerevisiae* diploids switch their growth motif from round cells to pseudohyphae, which are chains of elongated cells that remain attached to each other (*2*). Elements of the yeast mating signal-transduction pathway [the mitogen-activated protein (MAP) kinase cascade] are required for pseudohyphal growth (*3*). The kinases Ste20p, Ste11p(MEKK), and Ste7p(MEK) are required in haploids for the phosphorylation of the transcription factor Ste12p, which stimulates the expression of mating-specific genes (*4–6*). Pseudohyphal formation is greatly suppressed in diploids homozygous for mutations in *STE20*, *STE11*, *STE7*, or *STE12* (*3*).

The existence of a filamentous phase in *S. cerevisiae* provides a suitable background in which to clone *Candida* genes that enhance pseudohyphal growth in *S. cerevisiae*. A diploid *S. cerevisiae* strain, CGx69 (**a**/α *ura3-52/ura3-52*) (*7*), was transformed by electroporation with a *C. albicans* genomic library constructed in a high–copy number *S. cerevisiae* vector, and Ura^+ transformants were selected on a medium that suppresses pseudohyphal formation (*8*). Nine transformants that formed pseudohyphae on this medium were isolated by their agar invasion phenotype and elongated cell morphology (*9*). Restriction analysis of the plasmids isolated from these nine clones indicated that they represented two *C. albicans* genes, *CPH1* and *CPH2* (*Candida* pseudohyphal regulator). *CPH1* markedly enhanced the pseudohyphal growth of *Saccharomyces* on nitrogen starvation medium (Fig. 1, A and B).

The DNA sequence from the open reading frame of the *CPH1* clones showed that the predicted amino acids 20 to 187 of Cph1p are 74% identical to residues 32 to 200 of *S. cerevisiae* Ste12p and 83% identical to residues 27 to 194 of *Kluyveromyces lactis* Ste12p (Fig. 2) (*10–12*). This region contains the DNA-binding domain of *Saccharomyces* Ste12p (*13*). Another stretch of eight residues (Cph1p residues 291 to 298) that are identical between Cph1p and Ste12p of *S. cerevisiae* or *K. lactis* is located in the middle of the region required for pheromone-inducible transcriptional activation (*4*, *14*). *CPH1* complements both the defect in filament formation and the mating defect of *S. cerevisiae ste12* mutants. The *ste12/ste12* diploid strains that carry the *CPH1* gene on a plasmid show restored filament formation (Fig. 1, C and D). The suppression of the *Saccharomyces* mating defect by *Candida* *CPH1* is striking because no sexual cycle is known in *Candida*. The existence of a functional *Candida* homolog of the *S. cerevisiae* mating pathway component Ste12p suggests that *C. albicans* may have an undetected sexual cycle. In a manner similar to *K. lactis* *STE12*, *Candida* *CPH1* complements the *ste12* mating defect better in *MAT***a** strains than in *MAT*α strains (Fig. 3), which presumably reflects the additional requirement for α1 protein to interact with Ste12p in *MAT*α strains for successful mating (*12*).

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