Acad. Sci. U.S.A. **90**, 1493 (1993).
3. K. R. Prowse and C. W. Greider, *Ibid.* **92**, 4818 (1995).
4. A. Macieira-Coelho and B. Azzarone, *Anticancer Res.* **8**, 669 (1988).
5. To clone the mouse telomerase RNA, we probed a genomic library from D3 embryonic stem cells in λ EMBL3 (Stratagene) with a 450-bp PCR product from the human telomerase RNA gene (primers used to generate the probe were as follows: u3b, 5′-GCCTGGAGGGGTGGTGGCCATTTTTTG-3′; and ol3, 5′-GATCGGCGTTCCCCCCACCAAC-3′). Hybridization was done at 55°C in a moderate stringency buffer containing 1% bovine serum albumin, 500 mM $NaPO_4$, 15% formamide, 1 mM EDTA, and 7% SDS. The filters were washed at 55°C in 0.4× saline sodium citrate and 0.1% SDS. Positive clones were plaque-purified and rescreened under the same conditions. After screening approximately five genome equivalents (1×10^6 λ phage), three identical clones were identified by restriction mapping. Sequence (1.2 kb) was obtained from one of the clones with standard dideoxy sequencing methods.
6. J. Feng *et al.*, unpublished results.
7. The 5′ end of the mouse RNA was mapped by primer extension and ribonuclease (RNase) protection with the use of standard methods, and the 3′ end was mapped by reverse transcriptase–PCR (RT-PCR). Two primers that hybridize at nt 501 to 518 and nt 523 and 543 did not generate products by RT-PCR; however, the primer that hybridized between nt 413 and 435 did generate RT-PCR products. On the basis of the alignment of the conserved region in the human and mouse RNAs, we estimate that the 3′ end is near position 453 (marked with 3′ in Fig. 1) and that the mouse RNA is ~430 nt long. The GenBank accession number for the mTR sequence is U33831.
8. G. Shumyatsky and R. Reddy, *Nucleic Acids Res.* **20**, 2159 (1992).
9. The sequence around the template region for the mouse RNA is 5′-CCUAACCCUGAU-3′, for the rat RNA it is 5′-UCUAACCCUAUU-3′, and for the Chinese hamster RNA it is 5′-UCUAACCCUGAA-3′.
10. C. W. Greider and E. H. Blackburn, *Nature* **337**, 331 (1989).
11. D. Shippen-Lentz and E. H. Blackburn, *Science* **247**, 546 (1990).
12. J. Lingner, L. L. Hendrick, T. R. Cech, *Genes Dev.* **8**, 1984 (1994).
13. The sequences of the oligonucleotides used for inhibition and elongation experiments were as follows: MI-2, ATGAAAATCAGGGTTAGG; MP-1, CCACAGCTAATGAAAATC; MP-4, CCACAGCTAATGAAAATCAGGGTTAGG; MI-3, TCACGTTCAAGGGTTAGG; and MI-5, ATGAAAATCGCTACCTAA. In addition, two oligonucleotides that hybridize to the RNA 3′ of the template region had no effect on inhibition or elongation. The sequences for MP-3 and MP-4 were as follows: MP-3, CCCACAGCTAATGAAAAT; MP-4, CCCCACAGCTAATGAAAA.
14. To further characterize the function of the cloned mouse sequence, we used several approaches. Genomic Southern (DNA) blots probed at high stringency with the potential transcribed region of the mouse gene identified a single band, which suggests that the gene for the mouse telomerase RNA is a single copy like the human gene (*6*). To confirm that the gene we cloned is expressed, we used RT-PCR from total mouse RNA with or without the initial reverse transcription step. One band with the expected size and sequence was amplified from total RNA after reverse transcription; no band was generated in the absence of reverse transcriptase. The conditions for RT-PCR were as follows. First-strand complementary DNA (cDNA) synthesis was primed by random hexamers, $p(dN_6)$, and the products were amplified by PCR with the use of two specific primers: mTR5b, 5′-CGTCGACTAGGGTCGAGGGCGGCTAGGCCT-3′; and mTR3, 5′-GGAGGCGGCCGCAGGTGCACTTCCCACAGCTCAG-3′. This reaction was followed by a second round of PCR with primers mTR5b and an internal nested primer called "nest B" (5′-GGAGGCGGCCGCAGACGTTTGTTTTTTGAGGC-3′). Total RNA (3 μg) was mixed with random hexamers (8 ng) in a final volume of 10 μl, denatured at 95°C for 10 min, and chilled on ice and then incubated for 60 min at 50°C. We stopped the reactions by heating the tubes at 95°C for 10 min. The first-strand cDNA product (1 μl) was used for each PCR reaction. PCR reactions contained 1× PCR buffer, 5 mM dNTPs, 100 ng of each primer, 0.1 μg of T4 gene 32 protein, and 2 units of Taq polymerase from Perkin-Elmer. The conditions of PCR amplification were as follows: 94°C for 1 min; 60°C for 45 s; and 72°C for 1.5 min. Typically, 35 to 40 cycles were carried out for a first amplification. For nested amplification, 1 μl of the first PCR product was used in a second PCR. For cloning, the PCR products were phenol-extracted, precipitated, and digested with restriction enzymes Not I and Sal I and cloned in KS+ Bluescript that was previously digested with Not I and Sal I. We also assayed for copurification of the RNA with telomerase activity. We purified mouse telomerase activity over four columns and did Northern blots of RNA extracted from the fractions. An RNA of the expected size copurified with telomerase activity over each column.
15. G.-L. Yu, J. D. Bradley, L. D. Attardi, E. H. Blackburn, *Nature* **344**, 126 (1990).
16. M. S. Singer and D. E. Gottschling, *Science* **266**, 404 (1994); M. J. McEachern and E. H. Blackburn, *Nature* **346**, 403 (1995).
17. Transient transfection of plasmids containing 3.2 kbp of genomic flanking sequence and a 100-bp internal deletion of the transcribed region into NIH 3T3 cells generated an RNA of the expected size on Northern blots, which indicates that the cloned genomic DNA is sufficient to direct transcription of mTR.
18. The mutant mouse telomerase RNA gene with the template sequence 5′-CCCAACCCCAA-3′ was generated by in vitro mutagenesis (Amersham) and cloned into Bluescript in the context of a 5.5-kb genomic mTR clone. The mTR gene coding region was sequenced to confirm the template mutation.
19. For both wild-type and mutant extracts, a conventional telomerase reaction was done as described (*2*) with the use of the TS primer (*20*), dGTP (2 mM), dTTP (2 mM), and for some reactions dATP (2 mM) or ddATP (0.5 mM) as indicated (Fig. 3). Telomerase products were then amplified with the TS primer and either a $(A_2TC_3)_3$ primer or a $(A_2C_4)_3$ primer. Hot star PCR was used with the following conditions: 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min for 25 cycles.
20. N. W. Kim *et al.*, *Science* **266**, 2011 (1994).
21. C. Chadeneau, P. Siegel, C. B. Harley, W. Muller, S. Bacchetti, *Oncogene* **11**, 841 (1995).
22. Although mTR was not detected in the adult mouse kidney in this study, a low level of telomerase activity in this tissue has been reported (*3*). Such a difference may be attributed to the low activity levels detected or to the low sensitivity of Northern blot analysis of mTR.
23. The mouse species *Mus spretus* was used here because the standard laboratory species *Mus musculus* has very long telomeres that make detection of telomere length changes difficult [D. Kipling and H. J. Cooke, *Nature* **347**, 400 (1990)].
24. We thank S. K. Smith and R. Adams for excellent technical assistance, J. Rodriguez and A. Samuelson for generating PCR fragments from rat and hamster telomerase RNAs, J. Feng and W. Andrews for providing the original TRC3 clone; A. Zaug and T. Cech for advice about the 3′ end; D. Hanahan for critical reading of the manuscript, K. Collins and M. Serrano for helpful discussions; and R. DePinho for providing RNA . This work was supported by NIH grant AG09383 (C.W.G.), the Allied Signal Award for Aging Research, Geron Corporation, and the Ministerio de Educación y Ciencia from Spain (M.A.B.).

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An Essential Role for Rho, Rac, and Cdc42 GTPases in Cell Cycle Progression Through G_1

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Members of the Rho family of small guanosine triphosphatases (GTPases) regulate the organization of the actin cytoskeleton; Rho controls the assembly of actin stress fibers and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia. When microinjected into quiescent fibroblasts, Rho, Rac, and Cdc42 stimulated cell cycle progression through G_1 and subsequent DNA synthesis. Furthermore, microinjection of dominant negative forms of Rac and Cdc42 or of the Rho inhibitor C3 transferase blocked serum-induced DNA synthesis. Unlike Ras, none of the Rho GTPases activated the mitogen-activated protein kinase (MAPK) cascade that contains the protein kinases c-Raf1, MEK (MAPK or ERK kinase), and ERK (extracellular signal–regulated kinase). Instead, Rac and Cdc42, but not Rho, stimulated a distinct MAP kinase, the c-Jun kinase JNK/SAPK (Jun NH_2-terminal kinase or stress-activated protein kinase). Rho, Rac, and Cdc42 control signal transduction pathways that are essential for cell growth.

Constitutively active V12Cdc42 (Cdc42 with valine substituted for glycine at position 12), V14Rho, V12Rac, and V12Ras recombinant proteins were each microinjected with rat immunoglobulin G (IgG) into the cytoplasm of quiescent Swiss 3T3 fibroblasts, and the incorporation of bromodeoxyuridine (BrdU) into nascent DNA was measured after 40 to 48 hours (*1*). Microinjection of rat IgG alone (Fig. 1, A and B) had no effect on DNA synthesis; ~2% of the cells showed BrdU incorporation (Fig. 1C). Microinjection of V12Cdc42 efficiently stimulated DNA synthesis (Fig. 1, A and B); ~90% of injected cells were positive for BrdU incorporation (Fig. 1C). V12Ras, V14Rho, and V12Rac also stimulated BrdU incorporation in the majority of the injected cells (Fig. 1C).

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Two small GTPases are essential during the G_1 phase of the cell cycle. Microinjection of the neutralizing antibody to Ras Y13-259 into quiescent cells blocks subsequent mitogenic stimulation (*2*), and inhibition of Rho, by adenosine diphosphate (ADP) ribosylation with C3 transferase, arrests cells in G_1 (*3*). To determine whether Rac or Cdc42 is required for cell cycle progression, we microinjected dominant negative forms of Rac and Cdc42 into quiescent Swiss fibroblasts before addition of serum. Eukaryotic expression vectors encoding epitope-tagged V12N17Rac and N17Cdc42 were injected into the nuclei of quiescent fibroblasts, and cells were incubated for 16 hours to allow for expression of the mutant GTPases (*4*). The cells were then stimulated with fetal calf serum (20%) for 24 hours in the presence of BrdU. Expression of N17Cdc42 (Fig. 2A) blocked BrdU incorporation in response to serum (Fig. 2B). Approximately 80 to 90% of cells expressing N17Cdc42 or V12N17Rac were inhibited in BrdU incorporation, whereas over 95% of uninjected cells showed BrdU incorporation. In addition, microinjection of C3 transferase or the antibody to Ras Y13-259 also blocked serum-induced DNA synthesis. Our results indicate that Rho, Rac, and Cdc42, along with Ras, each play an essential role in G_1 progression in response to mitogenic stimulation.

Ras activates the mitogen-activated protein kinase (MAPK) cascade that contains the protein kinases c-Raf1, MEK (MAPK or ERK kinase), and ERK1/2 (extracellular signal–regulated kinases 1 and 2) which leads to cellular growth in quiescent fibroblasts and differentiation in PC12 cells (*5*). We examined whether the stimulation of DNA synthesis induced by Rho, Rac, and Cdc42 could be accounted for by activation of this MAPK pathway. A transient transfection protocol was used in which COS-1 monkey kidney cells were cotransfected with DNA encoding NH_2-terminal Myc epitope–tagged ERK2 along with Myc epitope–tagged V12Cdc42, V14Rho, V12Rac, D12Ras, or RafCAAX [a c-Raf1 kinase targeted to the plasma membrane by a COOH-terminal lipid modification signal from Ki-Ras (6)] (*7*). Expression of all transfected constructs was confirmed by protein immunoblotting with monoclonal antibody (mAb) 9E10 to the Myc epitope (anti-Myc). Activated D12Ras and RafCAAX stimulated the kinase activity of immunoprecipitated ERK2 (Fig. 3A) at least 25-fold to phosphorylate myelin basic protein (MBP) (Fig. 3B). However, despite comparable amounts of ERK2 being immunoprecipitated from transfected cells (Fig. 3A), V12Rac, V14Rho, and V12Cdc42 did not increase MBP phosphorylation (Fig. 3B).

We also tested whether Rho, Rac, and Cdc42 could stimulate other MAPK-like cascades. Stimulation of the MAPK cascade that contains the protein kinases MEKK (MEK kinase), JNKK/SEK (JNK kinase or SAPK/ERK kinase), and JNK/SAPK (Jun NH_2-terminal kinase or stress-activated protein kinase) results in phosphorylation of the c-Jun transcription factor (*8*) at two sites in the NH_2-terminus of the protein (*9*). These phosphorylation events increase the transactivation activity of the protein and result in increased transcription from AP-1 promoter sequences (*10*). A transient transfection protocol was again used in which COS-1 monkey kidney cells were cotransfected with DNA encoding an NH_2-terminal epitope–tagged (FLAG epitope; Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) version of JNK1 and Myc epitope–tagged V12Cdc42, V14Rho, V12Rac, D12Ras, the COOH-terminal kinase domain of MEKK, or RafCAAX (*11*). Expression of all transfected constructs was confirmed by protein immunoblotting with anti-Myc and mAb M2 to the FLAG epitope. Treatment of cells with ultraviolet light (UV) or transfection with the COOH-terminal domain of MEKK increased the ability of immunoprecipitated JNK1 (Fig. 4A) to phosphorylate recombinant GST (glutathione-S-transferase)–c-Jun (Fig. 4B). In addition, V12Cdc42, V12Rac, and D12Ras also stimulated JNK1 activity.

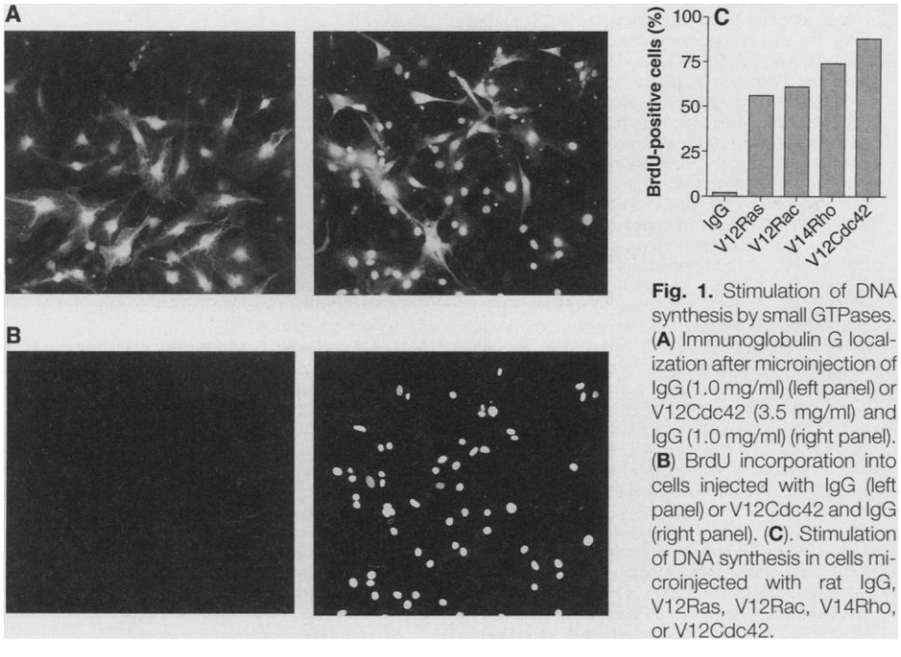

Fig. 1. Stimulation of DNA synthesis by small GTPases. (**A**) Immunoglobulin G localization after microinjection of IgG (1.0 mg/ml) (left panel) or V12Cdc42 (3.5 mg/ml) and IgG (1.0 mg/ml) (right panel). (**B**) BrdU incorporation into cells injected with IgG (left panel) or V12Cdc42 and IgG (right panel). (**C**). Stimulation of DNA synthesis in cells microinjected with rat IgG, V12Ras, V12Rac, V14Rho, or V12Cdc42.

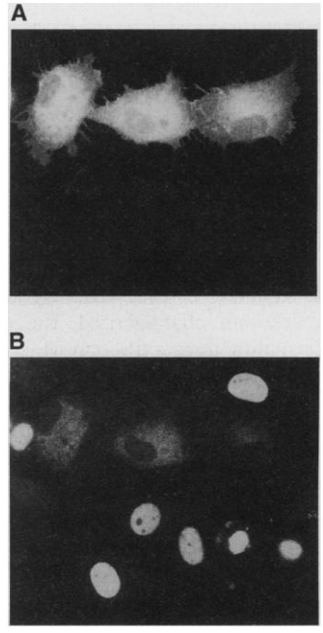

Fig 2. Cdc42 is essential for serum-stimulated DNA synthesis. (**A**) Localization of Myc-N17Cdc42 expressed from pEXV-Myc-N17Cdc42 after microinjection. (**B**) BrdU incorporation into cells injected with pEXV-Myc-N17Cdc42.

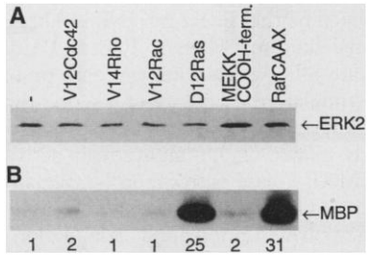

Fig. 3. Activation of ERK2 by Ras and RafCAAX but not by the Rho family of GTPases. (**A**) Immunoblot with antibody to the Myc epitope showing levels of ERK2 immunoprecipitated from cell lysates of COS-1 cells cotransfected with pEXV-Myc-ERK2 and potential activators. (**B**) MBP phosphorylation by immunoprecipitated ERK2. Fold stimulation above the activity of ERK2 transfected alone (-) is indicated.

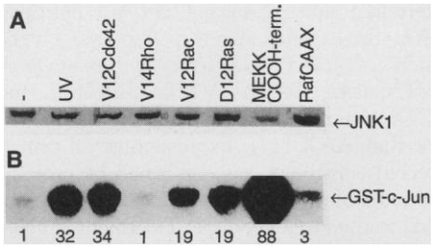

Fig. 4. Activation of JNK1 by Cdc42 and Rac but not by Rho. (**A**) Immunoblot with antibody to the FLAG epitope showing levels of JNK1 immunoprecipitated from cell lysates of COS-1 cells cotransfected with pCMV-FLAG-JNK1 and potential activators. (**B**) Phosphorylation of GST–c-Jun by immunoprecipitated JNK1. Fold stimulation above the activity of JNK1 transfected alone (-) is indicated.

JNK1 immunoprecipitated from cells coexpressing V14Rho or RafCAAX did not have increased GST–c-Jun phosphorylation activity. Thus, activation of JNK1 does not result from nonspecific effects of overexpressing GTPases or of ERK activation. Furthermore, the COOH-terminal domain of MEKK did not activate ERK2 (Fig. 3).

Although the downstream elements of the Ras signaling cascade required for cell growth are well characterized, the downstream signaling targets through which Rho, Rac, and Cdc42 GTPases influence either cell growth or the organization of the actin cytoskeleton are unclear (*12*, *13*). Our results support the idea that Rac and Cdc42 are regulators of the JNK MAP kinase–like cascade. Ras can activate Rac in Swiss 3T3 cells (*13*), and Rac has an essential role in Ras-mediated transformation (*14*). Given that Ras and RafCAAX stimulated ERK2 but only Ras stimulated JNK1 [Figs. 3 and 4 (*15*)], there is an apparent bifurcation in the signaling pathways resulting in the activation of these MAP kinases by Ras. We propose, therefore, that the activation of JNK1 by Ras is mediated by Rac and that this is required for Ras-induced transformation. Rho, which can also induce DNA synthesis, activated neither ERK2 nor JNK1. Although the mechanisms whereby Rac and Cdc42 activate JNK1 are not known, both proteins can stimulate the activity of a serine-threonine kinase, p65PAK (*16*), a close relative of which, STE20 (*17*), is an upstream activator of a MAP kinase pathway in *Saccharomyces cerevisiae* (*18*). Whether Rac/Cdc42-induced actin polymerization and stimulation of growth are two distinct downstream effects of the GTPases or whether they are both dependent on a common target such as p65PAK will require further investigation.

REFERENCES AND NOTES

1. Swiss 3T3 cells (5×10^5) were plated onto 13-mm-diameter glass coverslips and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) for 7 to 10 days. After microinjection, coverslips were incubated in serum-free DMEM with BrdU (10 μg/ml) for 40 to 48 hours, and then cells were fixed and stained for IgG and BrdU incorporation. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then incubated for 90 min at 30°C with BrdU mAb (5 μg/ml), deoxyribonuclease I (DNase I, 0.5 mg/ml), and 0.1% bovine serum albumin (BSA). Injected cells were visualized with a 1:300 dilution of fluorescein isothiocyanate (FITC)–conjugated anti-rat IgG and BrdU incorporation was visualized with a 1:100 dilution of TRITC (tetramethyl-rhodamine isothiocyanate)–conjugated anti-mouse IgG. The protein concentrations microinjected and the number of BrdU-positive cells per total number of cells injected in Fig. 1C were as follows: 1.0 mg/ml IgG (8/321); 1.0 mg/ml Ras (135/241); 0.6 mg/ml Rac (96/158); 1.4 mg/ml Rho (140/190); and 3.5 mg/ml Cdc42 (148/168).
2. L. S. Mulcahy, M.R. Smith, D. W. Stacey, *Nature* **313**, 241 (1985).
3. T. Nishiki *et al.*, *Biochem Biophys. Res. Commun.* **167**, 265 (1990); M. Yamamoto *et al.*, *Oncogene* **8**, 1449 (1993).
4. Plasmid pEXV-Myc-N17Cdc42 (200 ng/ml) was microinjected into the nuclei of Swiss 3T3 cells, grown as in (*1*). After 16 hours of incubation, cells were stimulated with 20% FCS in the presence of BrdU (10 μg/ml). After an additional 24 hours cells were fixed and stained for N17Cdc42 expression and BrdU incorporation. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, incubated for 60 min with mAb 9E10 [1:50 dilution (*19*)], 60 min with a 1:100 dilution of TRITC-conjugated anti-mouse IgG, and then 90 min at 30°C with FITC-conjugated BrdU mAb (5 μg/ml), DNase I (0.5 mg/ml), and 0.1% BSA.
5. C. J. Marshall, *Curr. Opin. Genet. Dev.* **4**, 82 (1994); *Cell* **80**, 179 (1995).
6. S. J. Leevers, H. F. Paterson, C. J. Marshall, *Nature* **369**, 411 (1994); D. Stokoe, S. G. Macdonald, K. Cadwallader, M. Symons, J. F. Hancock, *Science* **264**, 1463 (1994).
7. COS-1 cells were cultured and transfected as described (*20*). Plasmid concentrations per 10-cm petri dish were as follows: 10 μg of pEXV-Myc-ERK2 and 5 μg each of pEXV-Myc-V12Cdc42, pEXV-Myc-V14Rho, pEXV-Myc-V12Rac, pEXV-Myc-D12Ras, pMT-Myc-MEKK COOH-terminal domain (*21*), and pMT-Myc-RafCAAX. After incubation for 16 hours in serum-free DMEM, cell lysates were harvested and the phosphorylation of MBP by immunopurified ERK2 was determined. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in 25 mM Hepes (pH 7.6) buffer containing 1% Triton X-100 (v/v), 1% sodium deoxycholate, 0.1% SDS, 0.3 M NaCl, 50 mM NaF, 0.1 mM Na_3VO_4, 5 mM EDTA, 20 mM EGTA, leupeptin (20 μg/ml), aprotinin (20 μg/ml), 3 mM phenylmethylsulfonyl fluoride (PMSF), and 40 mM $Na_4P_2O_7$. Fifty micrograms of protein from each lysate were electrophoresed on 15% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Immunoblot analysis of the epitope-tagged proteins was done with mAb 9E10 (*19*). ERK2 was immunoprecipitated from lysates by incubation with 10 μg of mAb 9E10 for 1 hour at 4°C in 20 mM Hepes (pH 7.6) binding buffer (50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100) containing 40 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, leupeptin (20 μg/ml), aprotinin (20 μg/ml), and 3 mM PMSF. Protein G–Sepharose 4B was added and lysates were incubated for an additional 1 hour. Beads were washed three times with Hepes binding buffer and once with kinase wash buffer [30 mM tris (pH 8.0), 20 mM $MgCl_2$, 2 mM $MnCl_2$]. ERK2 kinase activity was assayed in 30 mM tris (pH 8.0), 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM adenosine triphosphate (ATP), 7.5 μg of MBP, and 1 μCi of [γ-^{32}P]ATP at 30°C. Kinase reaction products were electrophoresed on 15% SDS-PAGE and transferred to nitrocellulose. [^{32}P] incorporation was determined by autoradiography, and the relative levels of MBP phosphorylation were quantitated with a Phospho-Imager. Levels of ERK2 immunoprecipitated were determined with mAb 9E10 and alkaline phosphatase–conjugated anti-mouse IgG.
8. A. Lin *et al.*, *Science* **268**, 286 (1995); A. Minden *et al.*, *Mol. Cell. Biol.* **14**, 6683 (1994); I. Sanchez *et al.*, *Nature* **372**, 794 (1994); M. Yan *et al.*, *ibid.*, p. 798.
9. M. Hibi, A. Lin, T. Smeal, A. Minden, M. Karin, *Genes Dev.* **7**, 2135 (1993).
10. T. Smeal *et al.*, *Mol. Cell. Biol.* **12**, 3507 (1992); T. Smeal, B. Binetruy, D. A. Mercola, M. Birrer, M. Karin, *Nature* **354**, 494 (1991); B. Binetruy, T. Smeal, M. Karin, *ibid.* **351**, 122 (1991).
11. Transfections of COS-1 cells and harvesting of cell lysates were done as described (*7*). Plasmid concentrations per 10-cm petri dish were as follows: 10 μg of pCMV-FLAG-JNK1 and 5 μg each of pEXV-Myc-V12Cdc42, pEXV-Myc-V14Rho, pEXV-Myc-V12Rac, pEXV-Myc-D12Ras, pMT-Myc-MEKK COOH-terminal domain (*21*), and pMT-Myc-RafCAAX. JNK1 was immunoprecipitated from lysates as described in Fig. 3 with 4.5 μg of mAb M2 (anti-FLAG) and assayed for GST-c-Jun phosphorylation activity. Protein G–Sepharose 4B beads were washed three times with Hepes binding buffer and then resuspended in 30 μl of kinase reaction mixture containing 20 mM Hepes (pH 7.6), 20 mM $MgCl_2$, 4 mM NaF, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 20 μM ATP, 10 μg GST–c-Jun, and 5 μCi [γ-^{32}P]ATP. After incubation at 30°C for 30 min, kinase reaction products were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose. [^{32}P] incorporation was determined by autoradiography and the relative amounts of GST–c-Jun phosphorylation were quantitated with a Phospho-Imager. Immunoprecipitation of JNK1 was determined with mAb M2 (anti-FLAG) and alkaline phosphatase–conjugated anti-mouse IgG. To address the possibility that JNK1 activation was mediated by an autocrine loop, we used conditioned medium from dishes of V12Cdc42, V12Rac, or D12Ras COS-1 transfectants to stimulate JNK1-transfected cells for 5, 20, or 60 min before harvesting. Under these conditions no evidence of autocrine stimulation of JNK1 was observed.
12. A. J. Ridley and A. Hall, *Cell* **70**, 389 (1992); C. D. Nobes and A. Hall, *ibid.* **81**, 53 (1995).
13. A. J. Ridley, H. F. Paterson, C. L. Johnston, D. Diekmann, A. Hall, *ibid.* **70**, 401 (1992).
14. R.-G. Qiu, J. Chen, D. Kirn, R. McCormick, M. Symons, *Nature* **374**, 457 (1995).
15. A. Minden *et al.*, *Science* **266**, 1719 (1994).
16. E. Manser, T. Leung, H. Salihuddin, Z. S. Zhao, L. Lim, *Nature* **367**, 40 (1994).
17. E. Leberer, D. Dignard, D. Harcus, D. Y. Thomas, M. Whiteway, *EMBO J.* **11**, 4815 (1992); S. W. Ramer and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 452 (1993).
18. R. L. Roberts and G. R. Fink, *Genes Dev.* **8**, 2974 (1994).
19. G. I. Evan, G. K. Lewis, G. Ramsay, J. M. Bishop, *Mol. Cell. Biol.* **5**, 3610 (1985).
20. L. R. Howe *et al.*, *Cell* **71**, 335 (1992).
21. The MEKK COOH-terminal domain was generated by polymerase chain reaction (PCR) with complementary DNA made from A431 human epidermoid carcinoma cell–derived RNA. Oligonucleotides used were 5′-CAGCTGCAGGTGGAAAATGG-3′, corresponding to positions 1585 to 1604 of the published sequence of MEKK (*22*), and 5′-ATTAACTACCAGGTGGTACG-3′, corresponding to positions 2491 to 2510. The PCR fragment generated was subcloned into pBluescript, verified by DNA sequencing, and then subcloned into pMT-SM (*23*) with an NH_2-terminal Myc-epitope tag.
22. C. Lange-Carter, C. M. Pleiman, A. M. Gardner, K. J. Blumer, G. L. Johnson, *Science* **260**, 315 (1993).
23. D. Schaap, J. van der Wal, L. R. Howe, C. J. Marshall, W. J. van Blitterswijk, *J. Biol. Chem.* **268**, 20232 (1993).

24. We thank C. J. Marshall for pEXV-Myc-ERK2, pEXV-Myc-D12Ras, and pMT-Myc-RafCAAX; M. Karin and J. Ham for pCMV-FLAG-JNK1 and pGEX–c-Jun; C. Nobes for advice on microinjection; and A. Self, T. Bridges, and D. Drechsel for recombinant proteins. Supported by a program grant from the Cancer Research Campaign (SP2249) and the Human Frontiers Science Program.

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