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11. Purified p48 (150 μg) from *Xenopus* eggs was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The transferred p48 was reduced and *S*-carboxymethylated in situ (*24*) and digested with *Achromobacter* protease I. Peptides released from the membrane were fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a Wakosil-II 5C18 AR column (Wako Pure Chemical Industries, Osaka, Japan) and subjected to amino acid sequence analysis with a Shimadzu gas-phase sequencer, model PSQ-10 (Shimadzu, Kyoto, Japan). Molecular mass determination was carried out on a Sciex API-III mass spectrometer (Perkin-Elmer Sciex, Thorn Hill, Ontario, Canada) with an ionspray ion source and a quadrupole mass analyzer.
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16. The anti-p48 serum was raised in rabbits against purified *Xenopus* p48 (S-Sepharose fraction; see Fig. 1). The antibody was affinity-purified and used for immunoaffinity purification of p48 as described (*25*).
17. Affinity-purified anti-p48 was concentrated and dialyzed against phosphate-buffered saline (PBS). This antibody solution (15 μl) or PBS (15 μl) was added to 50 μl of M-phase extracts from *Xenopus* eggs. After incubation of the mixtures for 1.5 hours at 4°C, protein A beads (10 μl) were added and the incubation at 4°C continued for 1 hour with gentle shaking. The samples were then centrifuged at 10,000*g* for 15 s at 4°C and the supernatants were assayed for microtubule-severing activity.
18. The open reading frame (ORF) of human EF-1α (*26*) was amplified by PCR with the 5′ primer 5′-CGCGGGATCCAATGGGAAAGGAAAAGAC-3′ and the 3′ primer 5′-GGAGGGATCCTATTCATTTAGCCTTCTG-3′, which produce Bam HI sites at both ends of the EF-1α ORF. The resultant Bam HI fragment was cloned into pET-16b (Novagen, Madison, WI) and used to transfect the BL21 (DE3) pLysS strain of *Escherichia coli*. The expression of histidine-tagged EF-1α was induced for 10 hours at 30°C in the presence of 0.7 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted and lysed in a solution containing 5 mM imidazole, 500 mM NaCl, 20 mM tris-Cl (pH 7.9), 0.1% NP40, 1 mM PMSF, 1% aprotinin, 10 mM $MgCl_2$, and deoxyribonuclease I (20 μg/ml). After sonication and centrifugation at 39,000*g* for 20 min, the supernatant was applied to ProBond resin (Invitrogen), and the recombinant EF-1α was eluted with a gradient of 60 to 500 mM imidazole and dialyzed against 80PME.
19. Rat 3Y1 cells on glass cover slips were treated with 80PME containing 0.5% Triton X-100, 10 mM EGTA, and 5 μM taxol for 2 min at 37°C. After washing the cells with 80PME containing 10 mM EGTA and 0.4 M NaCl, we incubated them with 80PME or human recombinant EF-1α (150 μg/ml) for 10 min at 20°C. The cells were then washed with 80PME and fixed with 1% glutaraldehyde in PBS for 15 min. After blocking with PBS containing 3% bovine serum albumin and goat immunoglobulin (1 mg/ml), the cells were incubated with an antibody to α-tubulin (anti-α-tubulin) (DM1A, 1:150 dilution) and anti-p48 (1:5 dilution) and then washed with PBS. Immunoreactive proteins were detected by rhodamine isothiocyanate (RITC)–coupled goat antibody to mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)–coupled goat antibody to rabbit IgG, respectively.
20. Microinjection was done as in (*27*). FITC-labeled goat IgG was included in the EF-1α solution or in control buffer to distinguish injected from uninjected cells. The cells were fixed with 3.7% formaldehyde in PBS for 10 min. After blocking, they were stained with anti–α-tubulin (DM1A, 1:100 dilution) and RITC goat antibody to mouse IgG (anti-mouse IgG).
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Inhibition of Ras-Induced DNA Synthesis by Expression of the Phosphatase MKP-1

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Mitogen-activated protein kinases (MAP kinases) are common components of signaling pathways induced by diverse growth stimuli. Although the guanidine nucleotide-binding Ras proteins are known to be upstream activators of MAP kinases, the extent to which MAP kinases directly contribute to the mitogenic effect of Ras is as yet undefined. In this study, inhibition of MAP kinases by the MAP kinase phosphatase MKP-1 blocked the induction of DNA synthesis in quiescent rat embryonic fibroblast REF-52 cells by an activated mutant of Ras, V^{12}Ras. These results suggest an essential role for activation of MAP kinases in the transition from the quiescent to the DNA replication phase of the eukaryotic cell cycle.

Mitogen-activated protein kinases or extracellular signal–regulated kinases ($p42^{mapk}$ or ERK2 and $p44^{mapk}$ or ERK1, hereafter referred to as MAP kinase) are protein serine-threonine kinases that are activated by various mitogenic stimuli (*1*). The binding of a growth factor to its receptor tyrosine kinase induces formation of multiprotein complexes in the plasma membrane that trigger conversion of Ras from an inactive guanosine diphosphate (GDP)–bound form to an active guanosine triphosphate (GTP)–bound state (*2*). Activated Ras then initiates a cascade of sequential phosphorylation events in which the serine-threonine kinase Raf phosphorylates and activates MAP kinase kinase (also known as MEK) (*3*), a dual specificity kinase, that in turn phosphorylates both threonine and tyrosine regulatory sites in MAP kinase (*4*), thus leading to activation of its kinase function. Once activated, MAP kinase can phosphorylate other serine-threonine kinases such as $p90^{rsk}$ and transcription factors including $p62^{TCF}$ or Elk-1 (*5*). Expression of oncogenic V^{12}Ras, in which Gly^{12} is mutated to Val and the resulting protein is locked in the GTP-bound form, leads to constitutive activation of MAP kinase (*6*). However, whether activation of MAP kinase is an obligatory step for the mitogenic effect of Ras has yet to be established.

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MAP kinase phosphatase 1 (MKP-1) is encoded by the mitogen-inducible gene *3CH134* (*7*). MKP-1 inactivates $p42^{mapk}$ and $p44^{mapk}$ by dephosphorylating both phosphothreonine and phosphotyrosine regulatory sites in the enzymes (*8*). MKP-1 appears to be highly specific for the dephosphorylation of MAP kinase, because transient expression of the phosphatase in COS cells leads to selective dephosphorylation of MAP kinase from the spectrum of phosphotyrosine-containing proteins. Furthermore, we have demonstrated that a catalytically inactive mutant phosphatase in which Cys^{258} is changed to Ser forms a specific complex with its substrate, the phosphorylated form of MAP kinase. The selectivity of MKP-1 toward MAP kinase has also been reported for CL100, the human homolog of MKP-1 (*9*, *10*), and the related phosphatases PAC1 in lymphocytes (*11*) and MSG5 in yeast *Saccharomyces cerevisiae* (*12*). In addition, MKP-1 or CL100 does not dephosphorylate $p34^{cdc2}$, another serine-threonine kinase regulated by phosphorylation of threonine and tyrosine residues (*9*, *13*).

Recently, members of the Jun kinase (JNK) (*14*) or stress-activated protein kinase (SAPK) (*15*) family have been identified. These enzymes are distantly related to MAP kinase, and they are also regulated by threonine and tyrosine phosphorylation. We therefore examined whether MKP-1 had the capacity to dephosphorylate these enzymes. In parallel in vitro assays, approx-

imately 30 times more recombinant MKP-1 was needed to inactivate JNKs than to inactivate p42^{mapk} (*16*). In addition, we compared the sensitivity of p42^{mapk} or JNK1 toward MKP-1 when they were co-expressed in rat embryonic fibroblast REF-52 cells. Following transfection, the ectopically expressed kinases were isolated by immunoprecipitation and assayed for kinase activity. In the absence of MKP-1, p42^{mapk} and JNK1 could each be activated when cells were treated with 12-O-tetradecanoyl phorbol-13-acetate (TPA), or ultraviolet (UV) radiation, respectively (Fig. 1A). Co-expression of MKP-1 readily inhibited the TPA-induced p42^{mapk} activity, whereas the UV-induced JNK1 activity was largely unaffected by MKP-1 (Fig. 1A). These data further indicate the high degree of selectivity of MKP-1 for MAP kinase.

It has been reported that JNKs can be partially activated by Ras in certain systems (*14*, *17*). Therefore, we also compared the ability of p42^{mapk} and JNK1 to be activated by V^{12}Ras in REF-52 cells. Plasmids encoding p42^{mapk} or JNK1 were transfected into REF-52 cells together with V^{12}Ras expression plasmid. The relevant kinases were immunoprecipitated and assayed for kinase activity. V^{12}Ras was a potent activator of p42^{mapk}, resulting in a greater than 40-fold activation of its kinase activity (Fig. 1B). In contrast, JNK1 was poorly activated by V^{12}Ras (approximately twofold activation), but could be efficiently activated by UV (Fig. 1B). These data suggest that in REF-52 cells, activation of MAP kinase, but not JNKs, constitutes a preferential signaling pathway for Ras. Therefore, JNKs do not represent potential targets for MKP-1 in this experimental system.

In order to determine whether MKP-1 can inhibit Ras signaling events mediated by MAP kinase, we used a transient expression assay based on a reporter plasmid, 5XSRE-CAT, which contains five copies of the serum response element (SRE) (*18*) placed adjacent to a basic promoter element and linked to CAT (chloramphenicol acetyltransferase). The SRE from the c-*fos* promoter binds the transcription factor p62^{TCF}, which on phosphorylation by MAP kinase can activate transcription through the SRE promoter (*19*). The pm18 mutation, which contains a single base substitution in the SRE, abolishes p62^{TCF} binding, and thus the mutant SRE is not induced in cells stimulated with TPA (*18*). We observed induction of CAT activity in HeLa cells when V^{12}Ras was expressed with the 5XSRE-CAT reporter (Fig. 1C). However, the pm18 mutant derivative was not responsive to V^{12}Ras, which suggests that all of the signals from Ras to the SRE require p62^{TCF}. Hence the activation of SRE by V^{12}Ras can be used as a readout of the activation of MAP kinase. Co-expression of V^{12}Ras with the MAP kinase-specific phosphatase MKP-1 abolished Ras-induced activation of the SRE, whereas expression of a broad specificity tyrosine phosphatase, PTP1B, had no apparent effect (Fig. 1C). Therefore, these results demonstrate that MKP-1 can effectively block signaling responses downstream of MAP kinase.

We also used the 5XSRE-CAT reporter to monitor activation of MAP kinase in single cells. Quiescent REF-52 cells that had been grown to confluence and deprived of serum were microinjected with the reporter plasmid. Activation of the promoter was then detected by immunocytochemical staining with an antibody to CAT. When V^{12}Ras expression plasmid and 5XSRE-CAT were injected with either a vector control (*20*) or a plasmid expressing the tyrosine phosphatase PTP1B (Fig. 2B), expression of CAT was readily detected. However, when V^{12}Ras expression plasmid and 5XSRE-CAT were injected with a plasmid

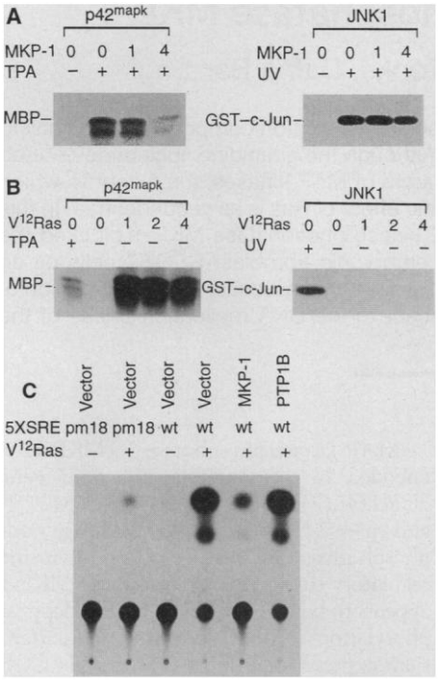

Fig. 1. (**A**) Comparison of sensitivity of p42^{mapk} and JNK1 toward MKP-1 in REF-52 cells. Plasmid expressing epitope-tagged p42^{mapk} or JNK1 was transfected into REF-52 cells together with 0, 1, or 4 μg of plasmid expressing MKP-1. Where indicated, cells were exposed to 200 nM TPA or 80 J/m^2 UV before harvesting. The ectopically expressed p42^{mapk} (left panel) or JNK1 (right panel) was isolated by immunoprecipitation and assayed for kinase activity with the substrate myelin basic protein (MBP) or GST–c-Jun(5-89), respectively (*27*). (**B**) Comparison of activation of p42^{mapk} and JNK1 by V^{12}Ras in REF-52 cells. The plasmid expressing epitope-tagged p42^{mapk} or JNK1 was transfected into REF-52 cells together with 0, 1, 2, or 4 μg of plasmid expressing V^{12}Ras. Cells were treated with TPA or UV where indicated. The relevant kinases were isolated by immunoprecipitation and assayed with either MBP (left panel) or GST–c-Jun(5-89) (right panel), respectively. (**C**) Inhibition of Ras-induced activation of 5XSRE-CAT reporter by MKP-1 in a transient transfection assay in HeLa cells. CAT activity was measured from HeLa cells transfected with wild-type 5XSRE-CAT plasmid or its pm18 mutant derivative together with either plasmid vector, MKP-1 or PTP1B expression plasmids, in the presence or absence of V^{12}Ras expression plasmid (*28*).

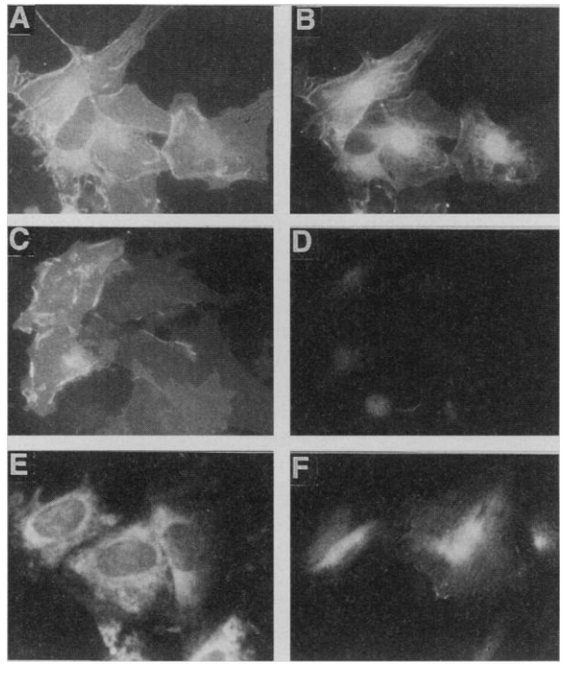

Fig. 2. Block of V^{12}Ras-induced expression of 5XSRE-CAT by microinjection of MKP-1 in REF-52 cells. Quiescent REF-52 cells were microinjected with 5XSRE-CAT reporter plasmid, plasmid expressing V^{12}Ras, and plasmid expressing PTP1B (**A** and **B**) or MKP-1 (**C** and **D**). Cells were immunostained simultaneously with antibody to Ras [(A) and (C), fluorescein staining] and antibody to CAT [(B) and (D), rhodamine staining]. In parallel, cells were examined for the expression of the phosphatase with anti-PTP1B staining (**E**) or anti–MKP-1 staining (**F**). The fields were visualized and photographed with a Zeiss Axiophot fluorescence microscope (*29*).

expressing MKP-1, SRE induction was blocked, as judged by the lack of CAT staining (Fig. 2D). The amount of V^{12}Ras expressed was comparable under both conditions, as determined by simultaneous counterstaining with an antibody to Ras (Fig. 2, A and C). Expression of each phosphatase was also verified by immunostaining (Fig. 2, E and F). Microinjection of recombinant MKP-1 protein also antagonized the induction of 5XSRE-CAT reporter mediated by recombinant V^{12}Ras protein, TPA, or epidermal growth factor (*20*). These data indicate that we can observe the ability of MKP-1 to antagonize MAP kinase function in single cells.

To address the question of whether the activity of MAP kinase is essential for the transition from the quiescent (G_0) to the DNA replication (S) phase of the cell cycle, we used microinjection and immunocytochemistry to examine the effects of MKP-1 on V^{12}Ras-induced DNA synthesis in quiescent REF-52 cells. DNA synthesis was monitored by immunostaining of bromodeoxyuridine (BrdU) incorporated into newly synthesized DNA. In quiescent REF-52 cells injected with vector alone, less than 5% of the cells stained positive for BrdU (*20*). However, of the cells microinjected with V^{12}Ras expression plasmid, ~40% were BrdU-positive, which indicates that they had entered S phase (Fig. 3C). This stimulation of DNA synthesis was inhibited by ~90% when MKP-1 was expressed with V^{12}Ras (Fig. 3, A and C). In contrast, the expression of PTP1B had no apparent effect on V^{12}Ras-induced DNA synthesis (Fig. 3, A and C). Again, the expression of Ras was verified under both conditions by simultaneous staining with antibody to Ras (Fig. 3A). To ensure that the inhibitory effect of MKP-1 on V^{12}Ras-induced DNA synthesis does not reflect a difference in the onset of expression of the two proteins from their respective plasmids, we co-injected purified recombinant V^{12}Ras protein together with PTP1B or MKP-1 expression plasmids (Fig. 3, B and C). Again, Ras-induced DNA synthesis was blocked by expression of MKP-1 but not of PTP1B.

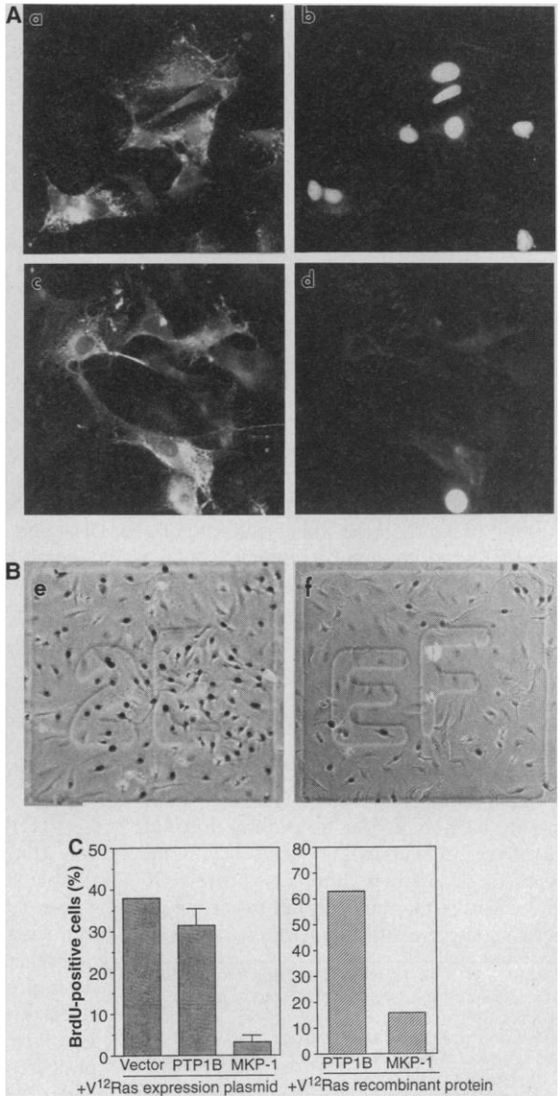

Fig. 3. Inhibition of V^{12}Ras-induced DNA synthesis by MKP-1. (**A**) Quiescent REF-52 cells were microinjected with plasmids expressing V^{12}Ras and PTP1B (a and b) or plasmids expressing V^{12}Ras and MKP-1 (c and d). Cells were immunostained simultaneously with antibody to Ras (a and c, fluorescein staining) and antibody to BrdU (b and d, rhodamine staining). The fields were visualized and photographed with a Zeiss Axiophot fluorescence microscope. (**B**) Quiescent REF-52 cells were microinjected with bacterially expressed V^{12}Ras protein together with plasmid expressing PTP1B (e) or MKP-1 (f). BrdU incorporation was detected by immunohistochemical staining. The fields were visualized and photographed with phase-contrast microscopy. (**C**) Quantitation of cells stained positively for BrdU. BrdU-positive cells are expressed as a percentage of the total number of cells injected. In one set of experiments (left panel), V^{12}Ras expression plasmid was injected together with expression plasmid for PTP1B or MKP-1. The results shown represent an average of three independent experiments in which 250 to 350 cells were injected for each condition. The error bars indicate standard deviation. The percentage of BrdU-positive cells in one determination in which ~250 cells were injected with V^{12}Ras expression plasmid and a vector control, illustrates that expression of PTP1B did not significantly affect V^{12}Ras-induced DNA synthesis. In a second set of experiments (right panel), recombinant V^{12}Ras protein was injected together with PTP1B or MKP-1 expression plasmid using ~400 cells for each condition.

Our results demonstrate that MKP-1 can antagonize MAP kinase activation and block V^{12}Ras-induced mitogenesis in quiescent cells. Expression of inactive mutants of $p42^{mapk}$ (ERK2) or $p44^{mapk}$ (ERK1), in which the lysine residue that is essential for binding adenosine triphosphate has been mutated to arginine, inhibited the activation of an AP-1 reporter by mitogenic stimuli, including Ras (*21*). However, the effects of such mutants on cell-cycle progression have not been addressed. Expression of inactive point mutants of $p44^{mapk}$, mutated at the regulatory threonine and tyrosine phosphorylation sites, inhibited cell growth as assessed by the number of viable colonies recovered in a transient transfection assay (*22*), but the point of the cell cycle at which these mutants exert their effect has not been established. We have demonstrated a high degree of specificity of MKP-1 for MAP kinase and have exploited this specificity to establish that the activation of MAP kinase is required for V^{12}Ras-induced transition from quiescence to the S phase of the cell cycle. Our results not only establish an essential role for MAP kinase in the mitogenic effects of Ras but also suggest that MKP-1 may now be used to ascertain whether the activation of MAP kinase is essential for normal mitogenic signaling and for transformation by cytoplasmic oncoproteins.

Note added in proof: While this manuscript was under review, Cowley and colleagues and Mansour and colleagues (*30*) reported that constitutively active MAP kinase kinase can transform NIH 3T3 cells. Their conclusion is consistent with that discussed in this report.

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16. Recombinant histidine-tagged $p42^{mapk}$, purified after expression in *Escherichia coli*, was activated in *Xenopus* egg extracts as described previously (*8*). Jun kinases (a partially purified preparation, containing both $p46^{JNK}$ and $p54^{SAPK}$ isoforms) were provided by C. Franklin and A. Kraft at the University of Alabama, Birmingham. The $p42^{mapk}$ (~6 nM) or Jun kinases (~2 nM) were incubated with purified recombinant MKP-1 protein in phosphatase assay buffer [20 mM Hepes (pH 7.5), and 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA)] for 5 min at 30°C then assayed for kinase activity for 10 min in kinase assay buffer [20 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, 1 mM vanadate, 100 μM adenosine triphosphate (ATP) and [γ-^{32}P]ATP at ~2000 cpm/pmol] with either myelin basic protein (0.2 mg/ml) or GST–c-Jun(5-89) (0.2 mg/ml) (*23*) as substrate, respectively. Reaction products were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography, then quantitated with the Fuji imaging system. Fifty percent dephosphorylation of $p42^{mapk}$ was achieved with 0.4 ng of MKP-1, whereas 35 ng of the phosphatase was required to produce 50% dephosphorylation of Jun kinases.
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27. REF-52 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected by the standard calcium phosphate ($CaPO_4$) method with 8 μg of pEXV3-$p42^{mapk}$-Myc (*24*) or pCMV5-Flag-JNK1 (*14*) and 1, 2, or 4 μg of pDCR-V^{12}Ras, supplemented with vector pDCR to total 16 μg of DNA. Alternatively, cells were transfected using 8 μg of pCG-HA-$p42^{mapk}$ or pSG5-Flag-JNK1 and 1 or 4 μg of pCEP4-MKP-1-Myc, supplemented with vector pCEP4 to give a total of 16 μg of DNA. After an 12-hour incubation with DNA-$CaPO_4$ precipitates, cells were incubated in DMEM containing FBS (0.5%) for 24 hours. Where indicated, cells were treated with TPA or UV (wavelength, 254 nm) before harvesting. Cells were lysed in lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM DTT, 1 mM vanadate, 1 μM okadaic acid, 1 mM benzamidine, 1 mM phenylethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)], and lysates were clarified by centrifugation. Myc epitope–tagged $p42^{mapk}$ was immunoprecipitated with monoclonal antibody 9E10 (American Type Culture Collection), HA eptiope–tagged $p42^{mapk}$ was immunoprecipitated with monoclonal antibody 12CA5 (Babco), and Flag epitope–tagged JNK1 was immunoprecipitated with monoclonal antibody M2 (IBI-Kodak). Immune complexes were collected by binding to protein A–Sepharose, washed extensively in lysis buffer, then assayed for 10 min at 30°C in kinase assay buffer with either myelin basic protein or GST–c-Jun(5-89) as described above (*16*).
28. HeLa cells were cultured in DMEM supplemented with FBS (5%) and transfected by means of the lipofection method with 1 μg of 5XSRE-CAT reporter plasmid or its pm18 mutant derivative; 3 μg of vector pCEP4, pCEP4–MKP-1–Myc, or pCEP4-PTP1B; and 1 μg of pDCR or pDCR-V^{12}Ras, together with 2 μg of SV40–β-Gal. After an 18-hour incubation with Lipofectin (BRL), cells were incubated in DMEM containing FBS (0.5%) for 24 hours. Lysates were then prepared, normalized for β-galactosidase activity, and assayed for CAT activity. The sequence of SRE is 5′-GGATGTCCATATTAGGACATCT-3′, and its pm18 derivative contains a single G to T base change at position 2 (*18*).
29. REF-52 cells were plated onto glass cover slips and cultured in DMEM supplemented with FBS (10%). The cells were grown to confluence, then placed in starvation medium (DMEM with 0.5% FBS) for 24 hours before microinjection. A plasmid mixture containing 5XSRE-CAT (62 μg/ml), pDCR-V^{12}Ras (5 μg/ml), and pCEP4-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml) in microinjection buffer [50 mM Hepes (pH 7.2), 100 mM KCl, and 5 mM $NaHPO_4$] was microinjected into cell nuclei. Sixteen hours after injection, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 hour, then permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. The cover slips were incubated with a mixture of rat antibody to Ras (Y13-259) (*25*) and rabbit antibody to CAT (5 prime, 3 prime) in PBS containing BSA (2 mg/ml) and then with a mixture of fluorescein-conjugated goat antibody to rat immunoglobin G (IgG) and rhodamine-conjugated goat antibody to rabbit IgG (Cappel). PTP1B was detected with monoclonal antibody FG6 (Oncogene Sciences), and Myc epitope–tagged MKP-1 was detected with monoclonal antibody 9E10 and the appropriate fluorescein-conjugated secondary antibodies. To monitor DNA synthesis, quiescent REF-52 cells were microinjected with a plasmid mixture containing pDCR-V^{12}Ras (5 μg/ml) together with pCEP-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml). Alternatively, bacterially expressed V^{12}Ras protein (1.7 mg/ml) (*26*) was injected together with plasmid pCEP4-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml). Although microinjections are done into cell nuclei, some spillover to the cytoplasm occurs, thus enabling the recombinant proteins to be delivered to the cytosol. After injection, BrdU (10 μM) was added to the medium, and cells were fixed 28 hours after injection in acid alcohol (ethanol:water:acetic acid, 90:5:5) at −20°C for 1 hour. The cover slips were first incubated with rat antibody to Ras (Y13-259), then with mouse antibody to BrdU (Cell Proliferation Kit, Amersham), and finally with a mixture of fluorescein-conjugated goat antibody to rat IgG and rhodamine-conjugated goat antibody to mouse IgG. Alternatively, after antibody to BrdU was applied, horseradish peroxidase–conjugated antibody to mouse IgG was used and detected by histocytochemical color development with Amersham's Cell Proliferation Kit.
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Restoration of X-ray Resistance and V(D)J Recombination in Mutant Cells by Ku cDNA

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Three genetic complementation groups of rodent cells are defective for both repair of x-ray–induced double-strand breaks and V(D)J recombination. Cells from one group lack a DNA end–binding activity that is biochemically and antigenically similar to the Ku autoantigen. Transfection of complementary DNA (cDNA) that encoded the 86-kilodalton subunit of Ku rescued these mutant cells for DNA end-binding activity, x-ray resistance, and V(D)J recombination activity. These results establish a role for Ku in DNA repair and recombination. Furthermore, as a component of a DNA-dependent protein kinase, Ku may initiate a signaling pathway induced by DNA damage.

X-rays and oxidative metabolism induce DNA double-strand breaks, which must be repaired if cells are to survive. In yeast, double-strand break repair (DSBR) occurs mainly by homologous recombination (*1*). However, in mammalian cells, DSBR appears to occur by a different pathway: X-ray–sensitive mutant cells fall into nine genetic complementation groups; three of these (groups 4, 5, and 9) are severely defective for DSBR (*2*) but show at most a mild deficit in homologous recombination (*3*, *4*).

V(D)J recombination is another pathway that involves the resolution of DNA double-strand breaks. The pathway rearranges DNA by cleavage and rejoining of segments from the immunoglobulin or T cell receptor genes in a way that is independent of extensive DNA homology (*5*). Little is known about the proteins or genes involved, but early steps require the lymphocyte-specific recombination-activating genes *RAG-1* and *RAG-2*, which are sufficient for activating V(D)J recombination in nonlymphoid cells.

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