constituent species, the macroscopic features of the liquid-crystalline phases obtained from interwound DNA were found to be directly and exclusively determined by the density and handedness of the supercoiling. Because the supercoiling density responds to environmental and genetic parameters (*23*), we propose that in addition to its role in genetic transactions (*24*), DNA supercoiling promotes ordered DNA segregation and serves as a regulatory link between cellular determinants and DNA supramolecular packaging. The demonstrated propensity of supercoiled DNA species to form liquid crystalline phases and to regulate their properties—in vitro as well as in bacteria, viruses (*25*), and mitochondria (*26*)—is consistent with the notion that supercoiling-dependent liquid crystallinity provides an effective and general packaging mode for interwound, nucleosome-free DNA molecules in vivo.

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4. We calculated this with the Sherrer equation [N. M. Henry, H. Lipson, W. A. Wooster, *The Interpretation of X-ray Diffraction Photographs* (Macmillan, London, 1961), pp. 212–218.
5. In vitro CD studies conducted on nicked-circular DNA molecules (obtained by exposing the supercoiled plasmids to a moderate deoxyribonuclease I activity) indicated that these species do not aggregate into chiral clusters (*27*). This result supports the assumption that radiation-induced DNA nicking is responsible for the time-dependent decay of the scattering peak.
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16. The degree and sense of supercoiling in interwound DNA species are defined by the relation Lk = Tw + Wr (*16*), where Lk is the linking number, Tw is the twist of the DNA strands, and Wr, or writhe, provides the extent and handedness of the supercoiling [J. H. White, *Am. J. Math.* **91**, 693 (1969)].
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21. Our proposed correlation between a decreased superhelical density and increased magnitudes of the cholesteric optical properties is supported by the enhanced CD signals exhibited under packaging conditions by partially relaxed topoisomers, obtained by use of topoisomerase II; the magnitude of these signals is four to five times larger than that of those displayed by native, nonrelaxed plasmids (*27*).
22. The previously reported dependence of twist handedness in cholesteric liquid crystals on the helical sense of polymers that form this phase (*13*) corroborates the assumption that a right-to-left reversal of the supercoiling sense does indeed occur at moderately elevated pH values. Notably, for a supercoiling density of 0.05 (characteristic of isolated plasmids), only 150 base pairs, which constitute 5% of the plasmid total length, must be titrated to effect complete relaxation; additional titration of a few base pairs would lead to a low positive superhelical density. Such a limited titration can be done at a moderately elevated pH [J. C. Wang, *J. Mol. Biol.* **89**, 783 (1974)]. The very large positive CD signals observed under packaging conditions at these pH values are clearly inconsistent with the occurrence of extensive DNA denaturation and point toward a right-handed cholesteric twist.
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28. The osmotic pressure exerted by 25% (w/w) polyethylene glycol is comparable to the osmotic pressure within *Escherichia coli* cells [P. Mitchell and J. Moyle, in *Bacterial Anatomy*, 6th symposium of the Society for General Microbiology, E. T. C. Spooner and B. A. D. Stocker, Eds. (Cambridge Univ. Press, Cambridge, 1956), vol. 6, pp. 150–179]. Thus, polyethylene glycol should not effect ordered DNA packaging within bacteria.
29. We derived binding constants of ethidium bromide to supercoiled DNA molecules under the specific experimental conditions used for the reported CD studies by determining the concentration of unbound ethidium bromide. Under such conditions, the drug to base pair molar ratio of 0.4 (at which the plasmid-derived liquid-crystalline phase is no longer chiral) corresponds to a binding ratio of 0.05, which was found to induce plasmid relaxation (*18*), thus supporting the suggested correlation between the supercoiling parameters and the properties of the plasmid cholesteric organization.

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Activation of Raf as a Result of Recruitment to the Plasma Membrane

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The small guanine nucleotide binding protein Ras participates in a growth promoting signal transduction pathway. The mechanism by which interaction of Ras with the protein kinase Raf leads to activation of Raf was studied. Raf was targeted to the plasma membrane by addition of the COOH-terminal localization signals of K-ras. This modified form of Raf (RafCAAX) was activated to the same extent as Raf coexpressed with oncogenic mutant Ras. Plasma membrane localization rather than farnesylation or the presence of the additional COOH-terminal sequence accounted for the activation of RafCAAX. The activation of RafCAAX was completely independent of Ras; it was neither potentiated by oncogenic mutant Ras nor abrogated by dominant negative Ras. Raf, once recruited to the plasma membrane, was not anchored there by Ras; most activated Raf in cells was associated with plasma membrane cytoskeletal elements, not the lipid bilayer. Thus, Ras functions in the activation of Raf by recruiting Raf to the plasma membrane where a separate, Ras-independent, activation of Raf occurs.

Ras functions in a signal transduction pathway from the cell membrane to the nucleus. Binding of growth factors to their receptors results in receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic tail of the receptor. Among the multiple proteins that bind to the phosphorylated tail of the epidermal growth factor receptor is the Grb2-Sos complex. The translocation of Sos to the plas-

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ma membrane allows it access to Ras. Sos activates Ras by catalyzing release of guanosine diphosphate and binding of guanosine triphosphate (GTP) (*1–3*). The subsequent interaction of RasGTP with the serine-threonine kinase c-Raf-1 leads to the activation of Raf. Raf phosphorylates and activates MEK (also known as MAP kinase kinase or MAPKK), the dual specificity kinase that phosphorylates and activates mitogen activated protein kinase (MAPK) (*4–6*).

MEK and MAPK are activated by phosphorylation of critical serine, threonine, and tyrosine residues (*7*), but the mechanism of activation of Raf remains unknown, and the precise role of Ras in the Raf activation process is unclear. In vitro, Ras binds with high affinity through its effector domain to the NH_2-terminal CR-1 domain of Raf in a GTP-dependent manner (*8–12*), and complexes between Ras and Raf can be immunoprecipitated from Ras transformed fibroblasts and activated T cells (*13*). However, formation of a complex between Ras and Raf in vitro does not lead to the activation of Raf, and the incubation of such Ras-Raf complexes with lysates prepared from cells transformed with Src or Ras does not lead to appreciable Raf activation (*14*).

Ras and Raf co-localize in the plasma membrane of Ras-transformed retinal cells (*15*). Taken together with data showing that Ras proteins require localization to the plasma membrane in order to be biologically active (*16*), these results suggest that the simplest role for Ras in Raf activation could be the recruitment of Raf to the plasma membrane. If this is the function of Ras, then Raf targeted to the plasma membrane should be constitutively active and this activity should be independent of Ras.

Coexpression of Raf with oncogenic mutant Ras resulted in the translocation of Raf from the cytosol to the plasma membrane (Fig. 1). This translocation was apparently not dependent on the activation of Raf because catalytically inactive Raf also localized to the plasma membrane in the presence of oncogenic mutant Ras (Fig. 1). However, oncogenic mutant Ras C186S (in which cysteine 186 is replaced with serine), which does not undergo posttranslational processing and mislocalizes to the cytosol (*16, 17*), had no effect on the subcellular localization of Raf (Fig. 1). To engineer a Raf protein that was constitutively associated with the plasma membrane, the COOH-terminal 17 amino acids of K-Ras were cloned onto the COOH-terminal of c-Raf-1. This sequence contains the CAAX motif (in which A means aliphatic acid and X refers to any amino acid) and a polylysine domain, which together comprise the plasma membrane targeting signals for K-Ras

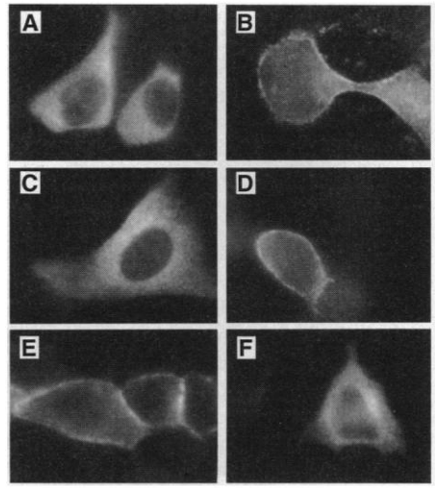

Fig. 1. Subcellular localization of Raf and Raf-CAAX. Raf-1 was tagged at the NH_2-terminus with a Myc epitope (MEQKLISEEDL) (*23*) or a Glu-Glu epitope (MEYMPME) (*24*). Coding sequences for the COOH-terminal 17 amino acids of wild-type K-Ras or K-Ras K(175–180)Q (K6Q) were cloned onto the COOH-terminus of epitope-tagged Raf. Wild-type K-Ras localizes to the plasma membrane, whereas K6Q, although farnesylated, is cytosolic, because the polylysine domain is essential for localization to the plasma membrane (*18, 19, 25*). The resulting RafCAAX and Raf6QCAAX cDNAs were cloned into the mammalian expression vector EXV3 and sequenced. Catalytically inactive Raf (KDRaf) has the catalytic lysine residue (K375) mutated to alanine. (**A**) Raf expressed in the absence of Ras. (**B**) Raf expressed with H-Ras G12V. (**C**) Raf expressed with cytosolic H-ras G12V, C186S. (**D**) KDRaf expressed with H-ras G12V. (**E**) RafCAAX and (**F**) Raf6QCAAX expressed alone. MDCK cells were microjected in the nucleus and fixed and stained as described (*26*). The Raf constructs in (A through D) are Glu-Glu–tagged; the Raf constructs in (E and F) are Myc-tagged. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

(*18, 19*). The RafCAAX protein localized to the plasma membrane in the absence of Ras (Fig. 1). By contrast, Raf6QCAAX, which is farnesylated but has the polylysine domain replaced with polyglutamine, did not localize to the plasma membrane.

The biological activities of RafCAAX and Raf6QCAAX were examined (Fig. 2). Coexpression of Raf and RasG12V in COS cells increased cytosolic MAP kinase activity to 17 times that in COS cells transfected with empty vector. Expression of Raf alone had minimal effect on MAP kinase activity but expression of RafCAAX alone increased MAP kinase activity 20-fold (Fig. 2A). Thus, plasma membrane–associated RafCAAX is activated to the same extent as Raf translocated to the plasma membrane by oncogenic Ras. Coexpression of either dominant negative RasS17N (*20*) or plasma membrane–targeted catalytic domain of Ras guanosine triphosphatase activating protein (GAP) [a potent downregulator of endogenous Ras (*21*)] did not abrogate the activa-

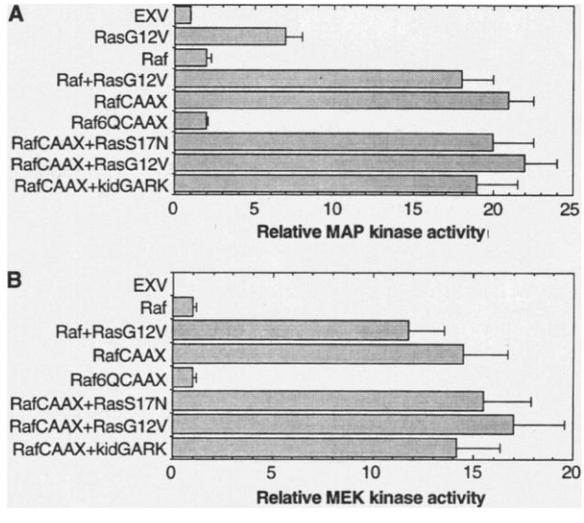

Fig. 2. Activation of plasma membrane–targeted Raf independent of Ras. (**A**) MAP kinase activity in the cytosol of COS cells transfected with combinations of plasmids encoding Raf and Ras, expressed relative to the MAP kinase activity in the cytosol of COS cells transfected with empty vector (EXV). The results are the means ± SEM of 3 to 4 independent experiments. The plasma membrane–targeted catalytic domain of RasGAP is denoted kid GARK. The expression of all proteins was verified by protein immunoblotting. MAP kinase was assayed as described (*22*). (**B**) MEK kinase activity of Raf solubilized with NP40 from P100 fractions of COS cells electroporated with combinations of plasmids encoding Ras, Raf, or RafCAAX. Raf was immunoprecipitated from the S100 fractions or from P100 fractions solubilized in 1% NP40. The immunoprecipitates were assayed for MEK kinase activity with catalytically inactive MEK as substrate (*28*). No MEK kinase activity was associated with immunoprecipitates from the S100 fractions. The graph shows MEK kinase activity present in Raf immunoprecipitates from P100 fractions. The results (mean ± SEM, $n = 3$) are expressed relative to the MEK kinase activity present in Raf immunoprecipitates from COS cells expressing wild-type Raf alone. The expression of all proteins was verified by protein immunoblotting aliquots of the lysates.

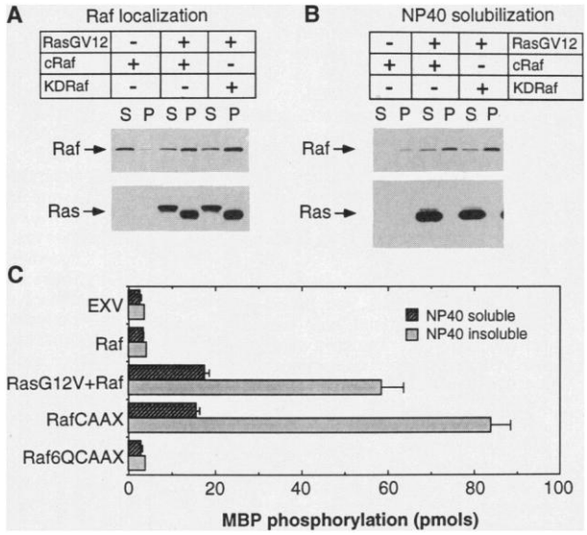

Fig. 3. Association of active Raf predominantly with cytoskeletal proteins. (**A**) S100 (S) and P100 (P) fractions of COS cells transfected with combinations of plasmids encoding RasG12V, Raf, and catalytically inactive Raf (KDRaf) were immunoblotted with monoclonal antibody Y13-259 to detect RAS and monoclonal antibody to Glu-Glu to detect Glu-Glu–tagged Raf proteins, respectively. Ras in the P100 fraction has undergone posttranslational processing and has a faster mobility during electrophoresis than unprocessed Ras in the S100 fraction. (**B**) P100 fractions from (A) were adjusted to 1% NP40, mixed, incubated on ice for 10 min, and centrifuged at 100,000*g*. The supernatant (S) (containing NP40-soluble proteins) and the sedimented material (P) (containing NP40-insoluble proteins) were immunoblotted as (A). (**C**) S100 and P100 fractions were prepared from COS cells expressing Raf, RasG12V and Raf, RafCAAX, Raf6QCAAX, or cells transfected with empty EXV vector, and Raf activity was measured with a linked MEK-MAP kinase assay (*29*). No activity was detected in the S100 fractions. The NP40-soluble and NP40-insoluble Raf activities present in the P100 fraction were determined in identical assays to allow a direct comparison to be made (*29*). Data are expressed as means ± SEM, $n = 3$. We estimate from this assay that RafCAAX is 40-fold activated over Raf.

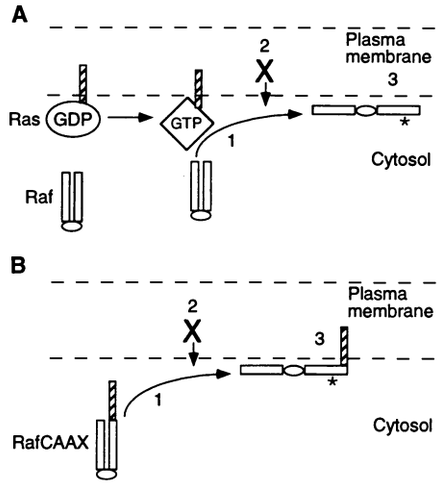

Fig. 4. (**A**) Cytosolic Raf is inactive, probably because the NH_2-terminal domain masks the COOH-terminal catalytic domain. RasGTP recruits Raf to the plasma membrane (event 1). A Ras-independent activation event (event 2) occurs mediated by X the unknown factor, shown as the unmasking of the kinase domain (denoted by the star) coincident with the association of Raf with the membrane. Raf remains attached to the membrane (event 3), predominantly with membrane cytoskeletal elements, independently of Ras. (**B**) RafCAAX, with K-Ras COOH-terminal membrane localization motifs, is constitutively recruited to the plasma membrane in the absence of Ras. Events 2 and 3 occur as in (A) and result in the activation of RafCAAX and its association with the membrane cytoskeleton.

tion of MAP kinase induced by RafCAAX (Fig. 2A). Moreover, coexpression of activated RasG12V did not further potentiate the activity of RafCAAX (Fig. 2A), indicating that RafCAAX activates MAP kinase in a Ras-independent manner. Raf6QCAAX expression had no effect on MAP kinase activity (Fig. 2A).

We directly compared the activity of RafCAAX with that of Raf activated by RasG12V in immune complex kinase assays. Supernatant (S100) and sedimented (P100) fractions (*22*) were made from homogenates of COS cells expressing RafCAAX or coexpressing Raf and RasG12V, and Raf was immunoprecipitated from each fraction. MEK kinase activity associated with the Raf immunoprecipitates was measured with catalytically inactive MEK as substrate. No MEK kinase activity was detected in the S100 fractions but the MEK kinase activity in the NP40-solubilized P100 fraction from cells transfected with either RafCAAX alone, or Raf and RasG12V, was 15 times greater than that in the equivalent fraction from cells transfected with Raf alone. Consistent with the data on MAP kinase activity in cells expressing RafCAAX, the MEK kinase activity of RafCAAX was unaffected by coexpression of dominant negative RasS17N, plasma membrane–targeted RasGAP catalytic domain, or oncogenic activated H-Ras (Fig. 2B). Immunoprecipitates of Raf6QCAAX had no more MEK kinase activity than immunoprecipitates of Raf (Fig. 2B). Thus, the additional amino acids and the farnesyl group at the COOH-terminus of Raf do not themselves activate RafCAAX. We conclude from these data that association with the plasma membrane is sufficient to activate Raf. Because the activation of plasma membrane–targeted RafCAAX was independent of Ras, the contribution of Ras to the activation of wild type Raf may be simply to locate Raf at the plasma membrane.

We determined whether Raf is anchored in the membrane by Ras or whether, once recruited to the plasma membrane by Ras, Raf remains attached through other mechanisms. S100 and P100 fractions were prepared from COS cells coexpressing Raf and RasG12V, and the presence of Ras and Raf was detected by protein immunoblotting. Much of the Raf protein translocated to the P100 fraction when coexpressed with RasG12V (Fig. 3A). The P100 fractions were solubilized in NP40 and centrifuged at 100,000*g*. The detergent soluble and insoluble fractions were analyzed by protein immunoblotting. Whereas all of the Ras was extracted from the sedimented membrane by NP40, the majority of the Raf was not (Fig. 3B). Thus, Ras, having recruited Raf to the membrane, does not continue to function as a membrane anchor for most of the recruited Raf. The Raf in the P100 fraction that was insoluble in buffer containing NP40 may be associated with cytoskeletal elements, rather than with the lipid bilayer. An anchoring role for Ras cannot, however, be excluded for the smaller fraction of Raf that was extracted in buffer containing NP40. Identical results were obtained with catalytically inactive Raf (Fig. 3, A and B) indicating that attachment of Raf to the membrane or cytoskeleton is not dependent on its activation.

We measured Raf activity in cytosol and in NP40-soluble and NP40-insoluble membrane fractions with a linked MEK-MAPK assay. No Raf activity was detected in the S100 fractions from COS cells expressing Raf or Raf with RasG12V or RafCAAX, consistent with the lack of MEK kinase activity associated with Raf immunoprecipitates from these fractions. Thus, cytosolic Raf was not active, even in Ras transformed cells. We directly compared the relative amount of Raf activity in the NP40-soluble and NP40-insoluble membrane fractions (Fig. 3C). Up to 80% of total Raf activity from cells expressing Raf with RasG12V and from cells expressing RafCAAX was in the NP40-insoluble fraction. This result reiterates that activation of Raf results from plasma membrane localization but that up to 80% of the Raf activity is apparently not actually anchored to the membrane by Ras because Ras is not present in NP40-insolu-

ble fraction. No Raf activity was detected in the cytosol or cell membranes of cells expressing Raf6QCAAX (Fig. 3C). Assays that measure Raf activity in immunoprecipitates from cell lysates subjected to high speed centrifugation may detect <20% of the total Raf activity in the cell.

We suggest the following model for the role of Ras in Raf activation (Fig. 4). Nucleotide exchange catalysed by Sos activates Ras and RasGTP recruits Raf to the plasma membrane by binding to the NH_2-terminal CR1 domain. Ras does not appear to anchor Raf to the membrane. The interaction of Ras and Raf is transient and Raf remains attached to the membrane independently of Ras. In this model, Ras catalytically recruits Raf to the plasma membrane until guanosine triphosphatase activating proteins (GAP or NF1) bind and inactivate Ras. Because normal Raf and catalytically inactive Raf can both be recruited to the membrane, the mechanism by which Raf associates with the membrane is separable from Raf activation. Coincident with membrane attachment, Raf is activated by an unknown mechanism (denoted X in Fig. 4). The role of Ras may be limited to recruiting Raf to the plasma membrane because RafCAAX is constitutively active and is insensitive to both dominant negative Ras or activated Ras. The interaction of Raf with the lipid bilayer might result in exposure of the kinase domain, which may normally be complexed with the NH_2-terminal regulatory domain when Raf is inactive in the cytosol. Alternatively, X may be a kinase, a lipid, or some other protein. A related issue is whether the superactivation of Raf that is seen when Ras and Src are coexpressed with Raf (*30*) is mediated through the X activation pathway or is a separate phenomenon. This question will be simpler to address now that the role of Ras in Raf activation has been defined.

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22. COS cells were electroporated (*21*) with various plasmid combinations and plated onto 10-cm dishes. Sixty hours later, cells were deprived of serum for 18 hours. The cells were washed twice with PBS and scraped on ice into 0.5 ml of buffer A [10 mM tris (pH 7.5), 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μM $NaVO_4$]. After 10 min on ice, the cells were homogenized with 30 strokes in a Dounce homogenizer. Cell nuclei were removed by centrifugation and the supernatant was centrifuged at 100,000*g*. The supernatant (S100) was removed and the sedimented fraction (P100) was rinsed briefly and resuspended in 100 μl of buffer A. To measure MAP kinase activity 40 to 50 μg of the S100 fraction was used. The volume was adjusted to 75 μl with buffer A and 1.5 μl of 100 mM $MgCl_2$, 0.5 μl of 10 mM adenosine triphosphate (ATP), and 10 μCi of γ-[^{32}P]ATP (NEN, NEG-002H, 3000Ci/nmol) added together with 25 μl of a 1:5 suspension of MEKB beads (MEKB, catalytically inactive MEK with a COOH-terminal Glu-Glu epitope tag; the MEKB was immobilized on protein G–Sepharose beads through a covalently coupled monoclonal antibody to Glu-Glu). More than 90% of MEK kinase activity in COS cell cytosol is accounted for by MAP kinase and we therefore now routinely use phosphorylation of MEKB as a readout for MAP kinase activity [K. Cadwallader, H. Paterson, S. G. Macdonald, J. F. Hancock, *Mol. Cell. Biol.*, in press; (*27*)]. The reactions were mixed at 30°C for 30 min and placed on ice. The beads were washed with 1 ml of buffer B [50 mM tris (pH 7.5), 75 mM NaCl, 25 mM NaF, 5 mM $MgCl_2$, 5 mM EGTA, 100 μM $NaVO_4$, 1% NP40] and 1 ml of buffer C [50 mM tris (pH 7.5), 75 mM NaCl, 25 mM NaF, 5 mM $MgCl_2$, 5 mM EGTA, 100 μM $NaVO_4$] and resuspended in 40 μl of 2× Laemmli sample buffer. After separation by SDS–polyacrylamide gel electrophoresis (PAGE) (10% gel), the amount of radioactivity incorporated into MEKB was measured by scanning the gels in an Ambis β-scanner.
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25. The 17 COOH-terminal amino acids of K-Ras contain two signals that are required for plasma membrane localization, a CAAX motif and an adjacent polybasic domain comprising six lysines. These sequences, when added to the COOH-terminus of heterologous proteins (*18, 19, 21*), are sufficient to localize them to the plasma membrane. When the K-Ras polybasic domain is mutated to polyglutamine (6Q), K-Ras localizes predominantly to the cytosol. Similarly, the 17 COOH-terminal amino acids of K-Ras6Q when added to the COOH-terminus of heterologous proteins localize <10% of the protein to the membrane (*18, 19, 21*). K-Ras6Q, G12V and H-RasC181S,C184S, G12V (the equivalent H-Ras mutant of K-Ras6Q) some retain transforming activity in focus assays on NIH 3T3 cells, despite being localized predominantly to the cytosol (*17, 18*). The focus assay may overestimate the inherent biological activity of K-Ras6Q, G12V and H-RasC181S,C184S, G12V because transformed cell lines derived from the focus assays express much greater amounts of K-RasK6Q, G12V or H-RasC181S,C184S, G12V than all lines expressing wild-type Ras (K. Cadwallader, H. Paterson, S. G. Macdonald, J. F. Hancock, *Mol. Cell Biol.*, in press). The small proportion of farnesylated K-Ras6Q,G12V that associates with the membrane may be responsible for its biological activity and the decreased efficiency of membrane association of K-Ras6Q may be compensated by increased amounts of expression.
26. MDCK cells growing on coverslips in minimal essential medium (MEM) containing donor calf serum (5%) were microinjected in the nucleus with EXV expression plasmids (100 μg/ml). Fifteen hours later, cells were fixed in paraformaldehyde (4%) and permeabilized in buffer containing Triton X100 (0.1%). After blocking with 5% milk in phosphate-buffered saline (M-PBS), cells were incubated with antibody to Myc or antibody to Glu-Glu monoclonal antibodies in M-PBS, washed and incubated with donkey antibodies to mouse IgG coupled to Texas Red (Jackson) in M-PBS, washed and mounted in FITC-Guard (Testog). Fluorescence was observed in an Axiovert 100 inverted microscope with a 100× Plan-Neofl. 1.3NA objective. Image collection was done with a Peltier-cooled charge coupled device camera (Photometrics) provided with a Kodak-Videk, 1,340×1,037 pixel chip used in bin-2 mode and Biological Detection Systems software. Pictures were obtained with a Personal LFR slide writer (Lasergraphics).
27. We validated the MAP kinase assay by performing the following experiments: (i) Cytosol from COS cells expressing oncogenic mutant Ras was fractionated on a MonoQ anion exchange column and the fractions were analyzed for MEKB kinase activity and MBP kinase activity. The activity profiles, which comprise a single major peak, were identical. (ii) MEKB kinase activity was detected only in fractions that contained immunoblottable MAP kinase. (iii) The amount of MEKB kinase activity present in each fraction was directly proportional to the amount of MAP kinase detected by immunoblotting. (iv) No other peaks of MEKB kinase activity were present in the MonoQ chromatogram and no such activity was present in the flow-through. (v) No MEKB kinase activity was detected in fractions that contained immunoblottable MEK kinase [C. A. Lange-Carter, C. M. Pleiman, A. M. Gardner, K. J. Blumer, G. L. Johnson, *Science* **260**, 315 (1993)]. (vi) No MEKB kinase activity was detected in soluble fractions that contained immunoblottable Raf. (vii) When performed in parallel, immune complex MAP kinase assays with myelin basic protein (MBP) as substrate gave very similar estimates (±10%) of MAP kinase activity in COS cell lysates to those obtained from MEKB phosphorylation assay. Phosphorylation of MEK by MAP kinase has no effect on the activation of MEK by Raf nor on the activation of MAP kinase by Raf-activated MEK. Thus, MAP kinase phosphorylation does not appear to regulate MEK activity in vitro. MEK is nevertheless a good MAP kinase substrate in vitro and in vivo [Y. Saito *et al.*, *FEBS Lett.* **341**, 119 (1994)].
28. S100 and P100 fractions were prepared from COS cells electroporated with combinations of plasmids encoding Ras, Raf, or RafCAAX. Raf was immunoprecipitated from S100 fractions and from P100 fractions solubilized in NP40 (1%). Immunoprecipitated Raf was washed extensively and incubated in Buffer C with catalytically inactive MEK (MEKB and MAPK 2 μg) and 0.75 μl of 100 mM $MgCl_2$, 0.25 μl of 10 mM ATP, and 5 μCi of γ-[^{32}P]ATP in 50 μl at 30°C for 30 min. After separation of protein by SDS-PAGE the amount of radioactivity incorporated into MEKB was measured by scanning the gels in Ambis β-scanner. There was no evidence that MAP kinase contaminated these Raf immunoprecipitates because the immunoprecipitates were unable to phosphorylate MBP.
29. S100 and P100 fractions were prepared from COS cells expressing Raf, RasG12V+Raf, Raf-CAAX, or Raf6QCAAX, or transfected with empty vector. After normalizing for protein, portions of the P100 fractions were washed twice with buffer

A containing 0.5 M NaCl and once with buffer A to remove any MAP kinase and MEK activity associated with the P100 fraction (*15*). The washed membranes were rinsed with buffer A, resuspended in 50 μl buffer A containing NP40 (1%), mixed, incubated on ice for 10 min, and centrifuged. The supernatant was removed and the sedimented NP40-insoluble material was resuspended in 50 μl of buffer A. Portions (10 μl) of the resuspended material and supernatant were placed into a reaction with 5 μl (1 μg) of MEK, 5 μl (1 μg) of MAP kinase [as a glutathione-S-transferase (GST) fusion protein] and 5 μl of Mg^{2+}-ATP (40 mM and 0.5 mM, respectively). Portions (10 μl) of each sample were also incubated in identical reactions omitting either MEK or MAP kinase and MEK. After 30 min at 30°C, 10 μl of each reaction was diluted into 40 μl of ice-cold buffer C and 10 μl of the diluted reaction was incubated with 5 μl (16 μg) of MBP, 25 μl of buffer C, 10 μl of Mg^{2+}-ATP (50 mM and 0.5 mM, respectively), with γ-[^{32}P]ATP (specific activity, 1,200 cpm/pmol). After 10 min at 30°C, portions (40 μl) were removed and spotted on 2 cm × 2 cm squares of phosphocellulose paper and immersed in 75 mM of orthophosphoric acid. After washing 4 times in phosphoric acid and once in acetone, the papers were dried and counted in scintillation fluid (1 ml). The control incubations omitting either MEK or MAP kinase and MEK showed that only small amounts of MAP kinase and MEK remained associated with the P100 fraction after washing. These counts (<4000 cpm) were subtracted from those incorporated into MBP in the incubation with MEK and ERK to arrive at a measure of Raf activity present in the samples. Because the NP40-soluble and insoluble fractions were taken through identical assays, the Raf activities are directly comparable.

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Reversal of Terminal Differentiation Mediated by p107 in $Rb^{-/-}$ Muscle Cells

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The terminal differentiation of mammalian muscle cells requires the tumor suppressor retinoblastoma protein (Rb). Unlike their wild-type counterparts, multinucleated myotubes from mouse cells deficient in Rb ($Rb^{-/-}$) were induced by serum to reenter the cell cycle. Development of the myogenic phenotype in $Rb^{-/-}$ cells correlated with increased expression of p107, which interacted with myogenic transcription factors. Serum-induced cell cycle reentry, on the other hand, correlated with decreased p107 expression. Thus, although p107 could substitute for Rb as a cofactor for differentiation, it could not maintain the terminally differentiated state in $Rb^{-/-}$ myotubes.

Terminal differentiation in mammalian cells comprises two interdependent and normally irreversible biological phenomena: withdrawal from the cell cycle and phenotypic differentiation. The tumor suppressor Rb, a member of the viral oncoprotein-binding pocket protein family, is a regulator of cell cycle progression (*1*). Rb accumulates during embryonic development and cell differentiation and thus may participate in the terminal differentiation of various cell lineages (*2*). The cell type–specific function of Rb may derive from interaction with cell-specific regulatory molecules (*3*). Skeletal myogenesis involves the direct interaction of Rb with muscle-specific basic helix-loop-helix (bHLH) factors of the MyoD family (*4*). Although the exact mechanism is unclear, interaction of Rb and myogenic bHLH factors mediates the permanent withdrawal of muscle cells from the cell cycle and the activation of myogenic differentiation.

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Mice that are genetically deficient in Rb ($Rb^{-/-}$) die in fetal life from defective development in the hematopoietic and central nervous systems but have histologically normal skeletal musculature (*5*). Cultured skeletal muscle cell lines derived from $Rb^{-/-}$ mouse cells developed mature multinucleated myotubes, but unlike their wild-type counterparts, these myotubes from $Rb^{-/-}$ cells could synthesize DNA after restimulation with serum growth factor–rich medium. Serum induced most (>75%) nuclei within $Rb^{-/-}$ myotubes to asynchronously reenter the cell cycle, which we documented by immunostaining for the nucleoside analog 5-bromodeoxyuridine (BrdU) incorporated into nascent genomic DNA (Fig. 1A). Within 24 hours, many of these nuclei had visibly condensed chromatin, suggestive of entry into mitotic prometaphase (Fig. 1B). Serum growth factors also induced the expression of the proliferating cell nuclear antigen (PCNA) (*6*) in the nuclei of $Rb^{-/-}$ (Fig. 1D) but not wild-type myotubes (Fig. 1C). Wild-type myotubes did not respond to growth factor stimulation with the induction of D-type cyclin synthesis (*7*) (Fig. 1E), but the $Rb^{-/-}$ myotubes did (Fig. 1F). Activity of the cyclin-dependent kinase $p34^{cdc2}$, which distinguishes cycling from quiescent cells, also reappeared in cellular extracts of serum-stimulated $Rb^{-/-}$ but not wild-type myotubes (*8*). Thus, distinct from wild-type myotubes, the terminally differentiated state in $Rb^{-/-}$ myotubes was reversible by restimulation with serum growth factors.

The molecular defect in the terminal differentiation of these cells could be rescued by expression of Rb from a single gene copy in myotubes derived from hemizygous ($Rb^{+/-}$) teratoma cells (Fig. 1G) or by reconstitution of $Rb^{-/-}$ muscle cells with wild-type human Rb (Table 1). The possibility that Rb might be required in other cell lineages that are destined to terminal differentiation was examined in heterogeneous populations of differentiated $Rb^{-/-}$ teratoma cells. Like the myotubes, phenotypically mature $Rb^{-/-}$ neuronal, cardiac muscle, and other cell types synthesized DNA when stimulated with growth factor–rich serum (Fig. 1H). This suggested that homozygous deletion of Rb in mouse cells might cause a global failure of authentic terminal differentiation.

The behavior of $Rb^{-/-}$ skeletal muscle cells suggested that a different molecule

Table 1. Reconstitution of Rb or constitutive expression of p107 rescues the defect in terminal differentiation in CC42 ($Rb^{-/-}$) muscle cells, and transfection of p107 induces growth arrest in RD rhabdomyosarcoma cells. CC42 or RD cells were transiently transfected with pCMV-p107 (*9*), pCMV-p107DE-HAtag (*9*), or pCMV-Rb (*16*), as in (*16*), then stimulated for 24 hours with 20% FCS in the presence of BrdU and double immunostained with BrdU and either p107, HA-tag (p107DE), or Rb mAbs (*21*). For each plasmid and a corresponding control, the number of transfected marker-positive cells that had incorporated BrdU was counted and the percent inhibition of DNA synthesis calculated as in (*22*). Each result is representative of transfections performed in triplicate.

Transfected protein	Cells counted (number)	Inhibition of DNA synthesis (%)
	CC42 ($Rb^{-/-}$) cells	
p107	276	88
p107DE	302	2
Rb	254	85
	RD cells	
p107	125	74
p107DE	141	0