Regulation of Cell Death Protease Caspase-9 by Phosphorylation

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Caspases are intracellular proteases that function as initiators and effectors of apoptosis. The kinase Akt and p21-Ras, an Akt activator, induced phosphorylation of pro–caspase-9 (pro-Casp9) in cells. Cytochrome c–induced proteolytic processing of pro-Casp9 was defective in cytosolic extracts from cells expressing either active Ras or Akt. Akt phosphorylated recombinant Casp9 in vitro on serine-196 and inhibited its protease activity. Mutant pro-Casp9(Ser196Ala) was resistant to Akt-mediated phosphorylation and inhibition in vitro and in cells, resulting in Akt-resistant induction of apoptosis. Thus, caspases can be directly regulated by protein phosphorylation.

Many apoptotic stimuli induce release of cytochrome c (cyto c) from mitochondria into the cytosol, where it binds to the CED-4 homolog Apaf-1, inducing binding to pro-Casp9 and resulting in proteolytic processing and activation of pro-Casp9. Active Casp9 then directly cleaves to and activates pro-Casp3, initiating a cascade of additional caspase activation that culminates in apoptosis. Cyto c induces caspase activation when added to cytosolic extracts in vitro with deoxyadenosine triphosphate (dATP) (*1*). We noticed that epithelial cell lines 267 (prostate) and MCDK (kidney) transfected with transforming Ki-Ras(Val12) or Ha-Ras(Val12) displayed resistance to apoptotic stimuli that are known to cause cyto c release (*2*), such as staurosporine and etoposide (VP16) (Fig. 1, A and B), and that cytosolic extracts derived from these cells were resistant to cyto c–induced caspase activation, as measured by cleavage of Ac-DEVD-AFC (*3*) (Fig. 1C) (*4*). Reduced caspase activity was not due to lower concentrations of pro-Casp3 but correlated with inhibition of proteolytic processing of pro-Casp3 (Fig. 1B) (*4*), implying a defect at or upstream of this caspase. Ras extracts, however, were not resistant to caspase activation induced by granzyme B (GraB) (*5*), which implies that other routes of protease activation were intact (Fig. 1D).

A farnesyl transferase inhibitor (FTI) reversed the resistance of Ras cytosolic extracts to cyto c (Fig. 1E), which suggests that the phenomenon is not due to secondary genetic changes in these cells. Moreover, resistance to cyto c–mediated activation of caspases was not an artifact of over-expressing oncogenic Ras, because extracts from DLD-1 colon cancer cells, which contain an endogenous activated Ki-Ras gene, displayed similar resistance to cyto c. Resistance was reverted by targeted disruption of the mutant Ras allele in two independent clones, DKO3 and DKS8 (Fig. 1F) (*6*).

The only proteins known to be required for cyto c–induced processing of pro-Casp3 are Apaf-1 and pro-Casp9 (*7*). Examination of Apaf-1 and pro-Casp9 mRNA and protein levels in control and Ras(V12)-expressing cells revealed no differences in their expression (*4*). Additionally, no difference in expression of Bcl-X_L or products of inhibitor of apoptosis (IAP) family genes (cIAP-1, cIAP-2, XIAP, NAIP, and survivin) was detected, excluding elevations in the concentration of these proteins that can directly or indirectly bind and inhibit some caspases within the cyto c pathway (*8*). Although pro-Casp9 protein was present at normal concentrations, its proteolytic processing in response to cyto c was impaired in cytosolic extracts derived from Ras(V12)-expressing cells (Fig. 2A) but not by GraB (*4*). Further, treatment of the extracts with a protein phosphatase (CIP) restored cyto c–induced processing of pro-Casp9 and accumulation of Ac-DEVD-AFC–cleaving caspases (Fig. 2, A and B), which implies that protein phosphorylation is required for Ras(V12)-mediated resistance to cyto c–induced processing of pro-Casp9.

Among the effectors of Ras is phosphatidylinositol 3-kinase (PI3K), which generates phosphoinositol phospholipid second messengers that activate Akt, a serine-threonine protein kinase previously implicated in apoptosis suppression (*9*). In vitro kinase assays revealed elevated Akt activity in Ras(V12)-transfected 267 cells as compared to control 267 cells (*4*). Treatment of Ras(V12)-expressing cells with the PI3K inhibitor Ly294002 (Fig. 2C) or with wortmannin (*4*) before (but not after) preparation of cytosolic extracts restored in vitro sensitivity to cyto c, resulting in pro-Casp9 processing and accumulation of Ac-DEVD-AFC–cleaving caspase activity. Further evidence of involvement of the PI3K/Akt pathway was obtained by expressing active v-akt in cells (*10*), resulting in suppression of cyto c–induced processing of pro-Casp9 and reduced caspase activity (Fig. 2D).

We therefore explored whether active Ras(V12) and active Akt induced phosphorylation of pro-Casp9 in cells. Immunoprecipitation of pro-Casp9 from cells metabolically labeled with $^{32}PO_4$ revealed increased radio incorporation into this protein [but not Apaf-1 (*4*)] in cells transfected with Ki-Ras(V12) or active Akt as compared to control cells (Fig. 3, A and B) (*11, 12*). Conversely, in cells transfected with a dominant negative (dn) kinase-inactive mutant of Akt(K178M), serum- and Ki-Ras(V12)–induced phosphorylation of pro-Casp9 was reduced (Fig. 3, C and D). The observation that Akt(K178M) only partially suppressed ^{32}P labeling of pro-Casp9 may indicate that it only partly blocks endogenous Akt or that other kinases can also phosphorylate pro-Casp9.

Pro-Casp9 contains sites that conform to the consensus Akt phosphorylation motif RXRXXS/T at Ser^{183} (RTRTGS) and Ser^{196} (RRRFSS) (*3, 13, 14*). To explore whether Akt can directly phosphorylate Casp9, active Akt was immunoprecipitated from Akt-transfected 293T human epithelial kidney (HEK) cells (*15*), and in vitro kinase assays were performed with [$\gamma^{32}P$]ATP and recombinant, purified, unprocessed Casp9 or processed Casp9 as candidate substrates (*16, 17*). As controls, Akt immune complexes were also incubated with a known substrate histone 2B (positive control) or with an irrelevant protein (negative control) that is not phosphorylated by Akt. Akt immune complexes induced phosphorylation of both unprocessed recombinant Casp9 and the large subunit of processed Casp9 in vitro (Fig. 4A). In contrast, a variety of control kinase complexes did not cause in vitro phosphorylation of either pro-Casp9 or processed Casp9 (*4*). Similar results were obtained with baculovirus-produced, recom-

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binant active GST-Akt (*18*), resulting in phosphorylation in vitro of Casp9 but not of other recombinant caspases such as Casp3 or Casp8 (Fig. 4B). Thus, Akt can directly phosphorylate both unprocessed and processed Casp9 in vitro. Moreover, the protease activity of bacteria-produced processed Casp9 was consistently reduced after in vitro treatment with Akt (Fig. 4, A and B) (*19*).

To explore whether Ser^{183} or Ser^{196} was phosphorylated by Akt in vitro, these sites were individually mutated to alanine. His_6-tagged versions of these mutant pro-Casp9 proteins were produced in bacteria and affinity purified for use as in vitro substrates of GST-Akt (*20*). Casp9(S196A) was not phosphorylated when treated with GST-Akt and its protease activity was not inhibited (Fig. 4C), which suggests that Ser^{196} is the predominant Akt phosphorylation site. In contrast, the Casp9(S183A) protein was still phosphorylated by Akt and its protease activity was reduced.

Mass spectrometry (MS) analysis of in vitro phosphorylated Casp9 was also undertaken as an alternative to site-directed mutagenesis, using a modified form mass spectroscopy: surface-enhanced laser desorption/ionization (SELDI) (*21, 22*). SELDI analysis of V8 protease digests of recombinant Casp9 after treatment with GST-Akt (but not GST control) revealed the presence of one 80-dalton mass-shifted peptide (*4*), which is consistent with the presence of a single Akt phosphorylation site in Casp9. The relevant phosphopeptide corresponded to amino acids 187 to 200 of Casp9 (as deduced from the V8 protease map of C9 and the MS profile), which contains Ser^{196} but not Ser^{183}. To confirm that Akt could phosphorylate this site, a peptide representing the deduced V8 fragment KLRRRFSSLHFMVE (*3*) was synthesized and used as a substrate for in vitro kinase assays employing GST-Akt. This Ser^{196}-containing peptide was phosphoryl-

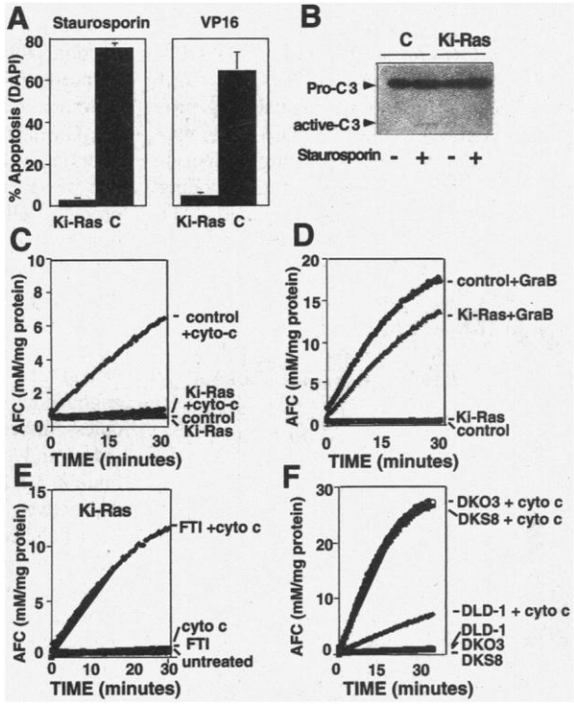

Fig. 1. Cytosolic extracts from Ras(V12)-expressing cells are refractory to cyto c–induced caspase activation. 267 and 267-Ki-Ras(V12) cells (*28*) were cultured with (+) or without (−) 1 μM staurosporin for 5 hours or 10 μM VP16 for ~18 hours before (**A**) determination of the percent of apoptotic cells by staining with 4′,6′-diamidino-2-phenylidole (DAPI) (Sigma) (mean ± SE; $n = 3$) (*4*) or (**B**) preparation of lysates for SDS-PAGE and immunoblot analysis (50 μg per lane) using antiserum to Casp3 with enhanced chemiluminescence (ECL) detection (*29*). Untreated cells were >95% nonapoptotic. In (**C**) through (**F**), cytosolic extracts were prepared (*30*) from 267 (control) and 267-Ki-Ras cells, with or without prior culture with 2 μM FTIase inhibitor [*N*-(amino-3-mercaptopropylamino-3-methyl-butyl)-Phe-Met-OH (Alexis, San Diego, California)] for 48 hours, or from DLD-1 cells, which contain an endogenous Ki-Ras(V12) allele and two clones, DKO3 and DKS8, in which the Ki-Ras(V12) allele was interrupted by homologous gene recombination (*6*). Extracts were normalized for protein concentration and incubated at 30°C with or without 10 μM cyto c (and 1 mM dATP) or with 10 μM GraB (*5*). Ac-DEVD-AFC hydrolysis was measured with continuous-reading instruments (*31*).

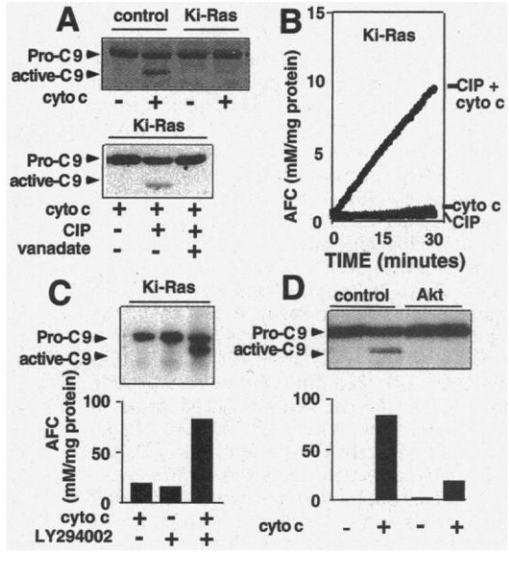

Fig. 2. Inhibition of cyto c–induced processing of pro-Casp9 in cytosolic extracts from Ras(V12)- or v-akt-expressing cells. (**A**) Cytosolic extracts from 267B or 267B-Ki-Ras(V12) cells were normalized for total protein content, then pretreated at 37°C for 1 hour with (+) or without (−) 1 U of PBS-exchanged calf intestinal alkaline phosphatase (CIP) (Boehringer-Mannheim) or with CIP and 2 mM Na-orthovanadate. In vitro translated (IVT) [^{35}S]pro-Casp9 in reticulocyte lysates (10% v:v) was added, and the extracts were treated with (+) or without (−) 10 μM cyto c and 1 mM dATP at 30°C for 0.5 hour before analysis by SDS-PAGE and autoradiography. (**B**) Cytosolic extracts (normalized for total protein) from 267B-Ki-Ras cells were pretreated at 37°C for 1 hour with nothing or with 1 U of CIP, then incubated at 30°C in the presence of 10 μM Ac-DEVD-AFC with (+) or without (−) 10 μM cyto c and 1 mM dATP. Substrate cleavage was monitored by AFC fluorescence. (**C** and **D**) Cytosolic extracts were prepared from either (C) Ki-Ras 267 cells that had been cultured with or without 10 μM Ly294002 for 1 hour or (D) NIH-3T3 cells that had been stably infected with control or v-akt–encoding retroviruses (*10*). IVT [^{35}S]pro-Casp9 was added (top panels) or Ac-DEVD-AFC cleavage was monitored (bottom panels) for 0.5 hour as above.

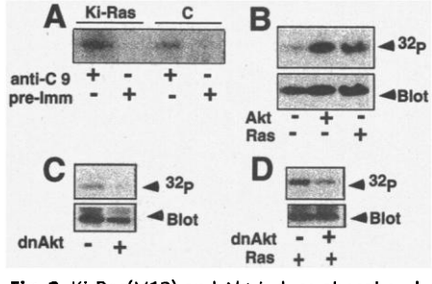

Fig. 3. Ki-Ras(V12) and Akt induce phosphorylation of pro-Casp9. (**A**) 267B and 267B-Ki-Ras(V12) cells were cultured in $^{32}PO_4$-containing medium (*11*). Immunoprecipitations were performed with either antibody to Casp9 or preimmune serum (*12*). Immune complexes were analyzed by SDS-PAGE and autoradiography. Immunoblotting confirmed equivalent amounts of endogenous pro-Casp9 in 267B and 267B-Ki-Ras(V12) cell lysates (*4*). (**B** through **D**) 293T cells were transfected with plasmids encoding FLAG-pro-Casp9 (Cys287Ala) alone or together with pCMV6-Akt(E40K), pCMV-dn-Akt (*15*), pZipNeo-Ha-Ras(V12) (gift of C. Hauser), or combinations of these plasmids. Cells were ^{32}P-labeled (*11*) and pro-Casp9 was immunoprecipitated with an antibody to FLAG (Kodak). Immune complexes were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for autoradiography (top) or anti-FLAG immunoblot (bottom) analysis using ECL-based detection to verify immunoprecipitation of equivalent amounts of FLAG–pro-Casp9.

ated in vitro by Akt (Fig. 4D), as judged by a characteristic phosphatase-sensitive 80-dalton shift in peptide mass.

Evidence that Ser^{196} is phosphorylated by Akt in vivo was also obtained by mass spectroscopy and ^{32}P metabolic labeling comparisons of pro-Casp9 and pro-Casp9(S196A) recovered by immunoprecipitation from transfected 293 HEK cells (*23, 24*) (Fig. 4, E and F). For these experiments, however, it was necessary to mutate the active site cysteine (Cys^{287}) of Casp9 and Casp9(S196A) to avoid inducing apoptosis. MS analysis of V8 digests of pro-Casp9 immunoprecipitated from cells that had been transfected with active Akt revealed an 80-dalton mass-shifted form of the peptide fragment containing Ser^{196}. In contrast, the corresponding peptide fragment from pro-Casp9(S196A) recovered from Akt-transfected cells was not phosphorylated (Fig. 4E). Similarly, when coexpressed with active Akt in ^{32}P-labeled 293 cells and then recovered by immunoprecipitation (*11*), less ^{32}P incorporation into the pro-Casp9(S196A) protein was observed than in the wild-type pro-Casp9 (Fig. 4F).

Overexpressing pro-Casp9 in 293T HEK cells induces apoptosis (*24*). Thus, Akt should suppress apoptosis induced by pro-Casp9 but not by the pro-Casp9(S196A) mutant that lacks the identified Akt phosphorylation site. Transfection of plasmids encoding pro-Casp9 or pro-Casp9(S196A) into 293T HEK cells resulted in apoptosis of most of the successfully transfected cells (Fig. 4G) (*4*). Coexpression of active Akt with wild-type pro-Casp9 rescued ~40% of the cells from apoptosis. In contrast, Akt rescued approximately half the cells expressing pro-Casp9(S196A), which is consistent with the failure of the Ser^{196} mutant to serve as a substrate for Akt in vitro and in vivo.

Akt has been implicated in signal transduction pathways for apoptosis suppression induced by Ras, growth factor receptors, neurotrophin receptors, and some oncoproteins (*9*). The data presented here suggest that phosphorylation and inactivation of Casp9 may be one of several mechanisms used by Akt to promote cell survival (*25*). Pro-Casp9 activation by Apaf-1/cyto c oligomeric complexes reportedly involves both autocatalytic self-processing and trans-processing of inactive pro-Casp9 molecules by active Casp9 molecules held within the same complex (*26*). Occupation of binding sites on Apaf-1 by endogenous phosphorylated Casp9 molecules, therefore, presumably explains why cyto c–induced processing of pro-Casp9 was inhibited in extracts from cells with elevated Akt activity. Ser^{196} in Casp9 is predicted to be distal from the substrate binding pocket, based on the three-dimensional structure of the homolog Casp3 (*27*). Thus, inhibition by phosphorylation may involve an allosteric mechanism that affects subunit dimerization or that alters the catalytic machinery of the substrate cleft through conformational changes. Though further studies are required to delineate the enzymological and structural details of how Akt-mediated phosphorylation of Ser^{196} in Casp9 inhibits its proteolytic activity, the findings reported here elucidate a mechanism for regulating caspases.

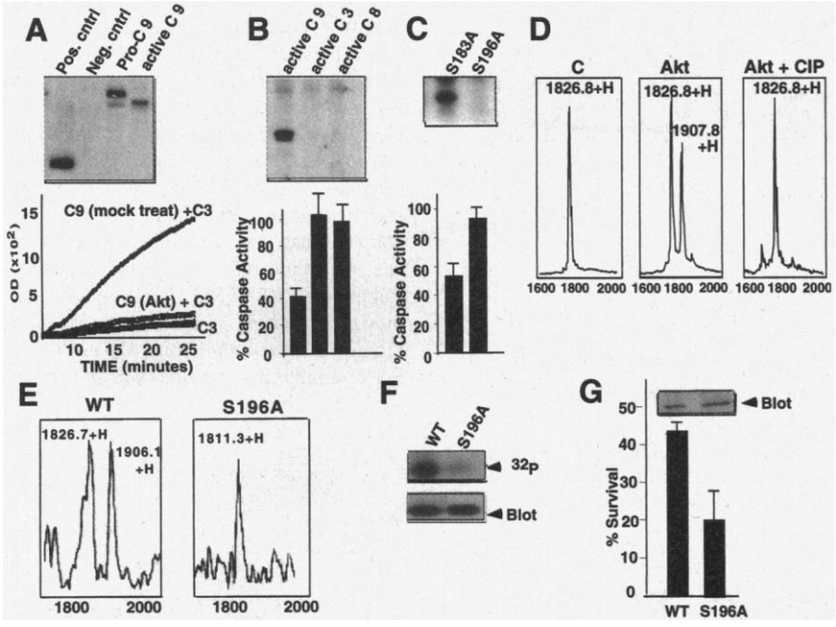

Fig. 4. Akt phosphorylates and inactivates Casp9. (**A**) Akt-containing immune complexes (*15*) were used to phosphorylate histone 2B as a positive control (Pos. cntrl), calmodulin as a negative control (Neg. cntrl), or recombinant unprocessed pro-Casp9(C287A) and active processed Casp9 in vitro in the presence of [γ^{32}P]ATP (*17*), followed by SDS-PAGE and autoradiography analysis (upper panel). Alternatively, active Casp9 was treated with Akt or control immune complexes using unlabeled ATP, and Casp9 activity was measured on the basis of its ability to activate pro-Casp3, which then cleaves the colorimetric substrate AC-DEVD-pNA (lower panel) (*19*). (**B** and **C**) Purified GST-Akt (*18*) was incubated with recombinant active Casp3, Casp8, Casp9, Casp9(S183A), or Casp9(S196A) (*20*) in the presence of [γ-^{32}P]ATP for SDS-PAGE and autoradiography analysis (upper panel) (*17*) or unlabeled ATP for caspase activity assays (lower panel) (mean ± SE; $n = 3$) (*19*). (**D**) A synthetic peptide KLRRRFSSLHFMVE (*3*) corresponding to residues 187 to 200 of pro-Casp9 was used for in vitro kinase reactions with either control protein (left) or GST-Akt (middle). A portion of the Akt-kinased peptide (50 pmol) was subsequently treated with 0.2 U of CIP for 4 hours at 37°C (right). Peptides were analyzed by MS. (**E**) V8 digests of FLAG-Casp9 (WT) and FLAG-Casp9(S196A) immunoprecipitated from 293 cells and analyzed by SELDI (*21, 22*). The V8-produced peptide corresponding to Casp9(187–200) is shown, demonstrating the ~80-dalton mass-shifted species in Casp9 but not Casp9(S196A). (**F**) 293T cells were transfected with pCMV6myrAkt and FLAG-tagged C287A mutants of Casp9 (WT) or Casp9 (S196A). The next day, cells were labeled with ^{32}P, and pro-Casp9 proteins were recovered by anti-FLAG immunoprecipitation, subjected to SDS-PAGE, and transferred to PVDF membranes for analysis by autoradiography (top panel) and anti-Casp9 immunoblot (ECL) (bottom panel) analysis. (**G**) 293T cells were transfected with 3 μg of pcDNA3-FLAG plasmids encoding pro-Casp9, or pro-Casp9(S196A), together with either 5 μg of control plasmid or pCMV6-myrAkt-HA and 1 μg of pEGFP. Sixteen hours after transfection, cells were deprived of serum and adherent and floating cells were collected 5 hours later and stained with DAPI. The percent of green fluorescent protein–positive cells with nonapoptotic nuclei was determined (mean ± SE; $n = 3$) (*30*). Lysates were analyzed by anti-FLAG immunoblotting to confirm expression of equivalent amounts of the wild-type and S196A FLAG-Casp9 proteins (inset).

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3. Single-letter abbreviations for the amino acid residues are as follows: A, 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; X, any amino acid; and Y, Tyr.
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10. NIH 3T3 cells expressing activated oncogenic Akt were generated by viral infection of NIH 3T3 cells with a retrovirus expressing v-akt [T. Franke *et al.*, *Cell* **81**, 727 (1995)].
11. For labeling experiments, 4×10^5 cells in 35-mm dishes were cultured 1 day after transfection for 3 hours in 1 ml of phosphate-free Dulbecco's modified Eagle's medium containing 1 mCi/ml ortho-^{32}P (New England Nuclear) with or without 5% dialyzed serum. Cells were lysed in 20 mM Hepes, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 20 mM NaF, 2 mM Na_3Va0_4, 10 mM β-glycerophosphate, and protease inhibitors. Lysates were precleared with protein A– or protein G–Sepharose with preimmune serum. Casp9 was immunoprecipitated with a monoclonal antibody (mAb) to FLAG or a polyclonal antibody to Casp9, washed, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography or by phosphoimager analysis.
12. A rabbit antiserum to Casp9 was raised against purified recombinant His_6-active Casp9 and verified to be specific for Casp9 by immunoblotting experiments using a panel of recombinant caspases, including Casp3, Casp6, Casp7, Casp8, and Casp10.
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15. 293T cells (10^7) were transiently transfected with 25 μg of pCMV6-myrAkt-HA or pcDNA (control) DNA. The activated form of Akt was generated by adding the NH_2-terminal Src myristoylation sequence to a pre-existing construct expressing Akt-HA in pCMV-6 (*19*). Cells were lysed 1 day later in 1.5 ml of 20 mM tris-HCl (pH 7.4), 140 mM NaCl, 1% NP-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, and protease inhibitors. After normalizing for protein concentration, lysates were precleared with protein G–Sepharose and preimmune serum for 1 hour and incubated at 4°C with 0.5 μg of rat high-affinity mAb to hemagglutinin (HA) (Boehringer-Mannheim), followed by addition of 10 μl of protein G–Sepharose (Pharmacia) for 1 hour. Alternatively, endogenous Akt was immunoprecipitated from 267 or 267-Ki-Ras cells with antibody to Akt (Santa Cruz Biotech), producing similar results (*4*). Immunoprecipitates were washed three times in lysis solution and two times in kinase solution [20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, and 3 μM ATP].
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18. GST-Akt was expressed from a recombinant baculovirus in Sf9 cells with activated forms of PI3K to achieve kinase activation. GST-Akt was purified from Sf9 lysates by glutathione-Sepharose affinity chromatography.
19. To determine the effects of Akt-mediated phosphorylation on caspase activity, in vitro kinase reactions were performed as described (*17*), except that 0.1 mM ATP was substituted for [γ-^{32}P]ATP. Immobilized Akt was removed by centrifugation, and half the sample (20 μl) was incubated with 10 μM Ac-DEVD-pNA (Alexis) and 2 μM purified pro-Casp3 in a final volume of 0.1 ml of caspase buffer (50 mM Hepes, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 5 mM dithiothreitol). Caspase activity was based on cleavage of the colorimetric substrate Ac-DEVD-pNA (*5*) and was normalized relative to Akt-untreated (mock) material. For Casp9 measurements, the addition of pro-Casp3 created a coupled Casp9 → Casp3 → DEVD-pNA reaction, because Casp9 does not efficiently cleave DEVD (*16*). Activity percent was measured and normalized to mock-treated samples. Anti-HA immune complexes prepared from control-transfected cells and immobilized GST control protein resulted in no significant alterations of caspase activity (*4*).
20. Pro-Casp9 and Pro-Casp9(C287A) cDNAs, as well as S183A and S196A mutants of these, were expressed with NH_2-terminal His_6-tags from pET23b in BL21 cells for production of processed Casp9 and unprocessed Casp9, respectively (*16*). Expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at $OD_{600} \cong 0.6$ to 0.8 and ~25°C for 4 hours for the S183A mutant and for 1 hour for the S196A mutant. Proteins were affinity purified by Ni-chelate Sepharose (Pharmacia).
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22. For MS analysis, 1 pmol of a 1.826-kD synthetic peptide corresponding to a V8 fragment containing the Akt phosphorylation site in Casp9 was kinased in vitro or mock treated and spotted onto a SELDI chip (Ciphergen Biosystems, Palo Alto, CA) and imbedded with cinamininic acid matrix. Alternatively, 293T cells were transiently transfected with pCMV6-myrAktHA and pcDNA3-FLAG constructs encoding C287A mutants of either pro-Casp9 or pro-Casp9(S196A). Casp9 (wild type) and Casp9(S196A) were isolated by immunoprecipitation using antibody to FLAG, eluted from beads with glycine (pH 3.0), and digested with 0.05 U of V8 protease for 8 hours in 50 mM NH_4OAc (pH 4.0) at room temperature. The samples were then analyzed by SELDI as described above. An 80-dalton increase in mass indicated that the peptide fragment was phosphorylated.
23. Casp9 mutants were generated by site-directed polymerase chain reaction (PCR) mutagenesis from a human pro-Casp9 cDNA (V. Dixit) and subcloned into pcDNA3-FLAG, pCMV2-FLAG, or pET23b plasmids. The primer pairs used to generate the S183A and S196A mutants were 5′-CCGCACCCGCACTGGCGCGAACATCGACTGTGAG-3′ plus 5′-CTCACAGTCGATGTTCGCGCCAGTGCGGGTGCGG-3′; and 5′-CGGCGTCGCTTCTCCGCGCTGCATTTCCTGGTGG-3′ plus 5′-CCACCATGAAATGCAGCGCGGAGAAGCGACGCCG-3′, respectively. PCR was performed for 16 cycles at 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min. Twenty microliters of the reactions was digested with Dpn I (10 U) for subsequent subcloning into plasmids.
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Dual Requirement for Gephyrin in Glycine Receptor Clustering and Molybdoenzyme Activity

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Glycine receptors are anchored at inhibitory chemical synapses by a cytoplasmic protein, gephyrin. Molecular cloning revealed the similarity of gephyrin to prokaryotic and invertebrate proteins essential for synthesizing a cofactor required for activity of molybdoenzymes. Gene targeting in mice showed that gephyrin is required both for synaptic clustering of glycine receptors in spinal cord and for molybdoenzyme activity in nonneural tissues. The mutant phenotype resembled that of humans with hereditary molybdenum cofactor deficiency and hyperekplexia (a failure of inhibitory neurotransmission), suggesting that gephyrin function may be impaired in both diseases.

The main inhibitory inputs to spinal cord and brain-stem motoneurons use glycine as a neurotransmitter (*1*). The α and β transmembrane subunits of glycine receptors (GlyRs) from spinal cord copurify with gephyrin, a 93-kD cytoplasmic protein (*2*). Gephyrin binds to the β subunit of the GlyR and to tubulin, thereby linking GlyRs to the cytoskeleton (*3*). This interaction appears to be important for the accumulation of GlyRs at synapses, because GlyRs are precisely colocalized with gephyrin at synapses in the brain and spinal cord, gephyrin aggregates GlyRs when coexpressed with them in heterologous cells, and attenuation of gephyrin synthesis with antisense oligonucleotides prevents clustering of GlyRs at synaptic sites on cultured spinal neurons (*4–6*). Molecular cloning of gephyrin (*7*) revealed unexpected similarity to three *Escherichia coli* proteins (moeA, moaB, and mog), a *Drosophila melanogaster* protein (cinnamon), and an *Arabidopsis thaliana* protein (cnx1), all of which are involved in the synthesis of a molybdenum-containing cofactor essential for the activity of molybdoenzymes (*8*). This conservation (Fig. 1A) suggested that genes of the

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