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Gerald V. Denis
University of Massachusetts Medical School

Et al.

A novel, mitogen-activated nuclear kinase is related to a *Drosophila* developmental regulator

Gerald V. Denis and Michael R. Green[1]

Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605 USA

Although the ultimate targets of many signal transduction pathways are nuclear transcription factors, the vast majority of known protein kinases are cytosolic. Here, we report on a novel human kinase that is present exclusively in the nucleus. Kinase activity is increased upon cellular proliferation and is markedly elevated in patients with acute and chronic lymphocytic leukemias. We have identified a human gene that encodes this nuclear kinase and find that it is closely related to Drosophila *female sterile homeotic* (*fsh*), a developmental regulator with no known biochemical activity. Collectively, these results suggest that this nuclear kinase is a component of a signal transduction pathway that plays a role in *Drosophila* development and human growth control.

[Key Words: Signal transduction; kinases; *Drosophila*; leukemia; trithorax]

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A variety of extracellular signals can regulate the transcriptional activity of particular structural genes. Specific nuclear-localized transcription factors are believed to be the ultimate targets of diverse[1] signal transduction pathways (for reviews, see Hunter and Karin 1992; Jackson 1992; Karin 1994). Two mechanisms have been described by which signals that regulate transcription reach the nucleus. In the first mechanism, in response to an appropriate signal, the transcription factor itself translocates from the cytosol to the nucleus. The prototype transcription factor that uses this mechanism is NF-κB (Baeuerle and Baltimore 1988a,b), but other examples include members of the STAT family (Darnell et al. 1994), SV40 T antigen (Rihs et al. 1991), SWI5 (Moll et al. 1991), Dorsal (Rushlow and Warrior 1992), v-Jun (Chida and Vogt 1992), ISGF3 (Kilgour and Anderson 1994), steroid hormone receptors (Kuiper and Brinkmann 1994), and NFAT (Liu 1993) (for review, see Whiteside and Goodbourn 1993). A second mechanism involves the nuclear translocation of a cytosolic kinase, which in some cases directly phosphorylates the nuclear transcription factor target. For example, nuclear forms of protein kinase A (Nigg et al. 1985), protein kinase C (Leach et al. 1989), $p42^{mapk}$ and $p44^{mapk}$ (MAP) kinases (Lenormand et al. 1993), and ribosomal S6 (RSK) kinase (Chen et al. 1992) can be detected after mitogenic stimulation.

It remains possible, however, that there are kinases that are downstream targets of cytosolic signaling pathways but that are principally nuclear in localization. Little is known about such nuclear kinases, although some candidates include the c-Abl protein tyrosine kinase (Kipreos and Wang 1992), a DNA-dependent protein kinase (Jackson et al. 1990; Finnie et al. 1993), and a cell cycle-dependent complex containing $p33^{cdk2}$ kinase (Devoto et al. 1992; Faha et al. 1992). Furthermore, kinases that phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II, including the general transcription factor TFIIH, have been characterized (Feaver et al. 1991; Lu et al. 1992). However, a role for these kinases in signal transduction has not been established.

In this paper we describe a novel nuclear kinase that has significant homology with fsh, a *Drosophila* protein known to be important in embryonic pattern formation (Haynes et al. 1989). The autophosphorylation activity of this kinase was correlated with cellular proliferation and was elevated in patients with acute and chronic lymphocytic leukemias.

Results

Identification of a 90-kD nuclear kinase

To identify nuclear kinases, polypeptides in HeLa nuclear or cytosolic extracts were separated by polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to nitrocellulose, denatured, renatured, and incubated with [γ-^{32}P]ATP. This assay detects autologous phosphorylation, which is frequently correlated with kinase autoactivation (Hunter 1987). Figure 1 shows that one major and several minor polypeptides were autophosphorylated. The major multiplet, which had an apparent mobility of ~90 kD, was present in nuclear extract but absent from cytosolic extract. Several experiments suggest

[1]Corresponding author.

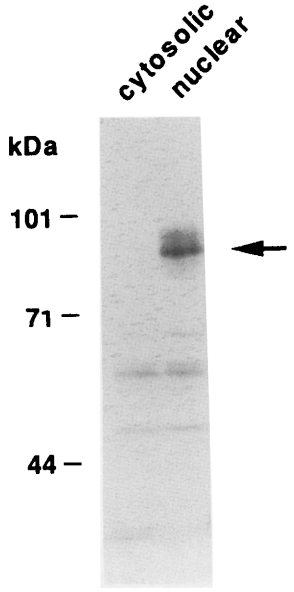

Figure 1. Identification of a 90-kD nuclear kinase. Proteins (50 μg) from HeLa nuclear and cytosolic extracts were prepared, then subjected to SDS-PAGE and renaturation on nitrocellulose. Renatured autophosphorylation activities are shown, with an arrow indicating the 90-kD multiplet. Blots probed with [α-^{32}P]ATP showed background radioactivity but no discrete bands (data not shown).

that these various ~90 kD polypeptides are differentially phosphorylated and, as a result, can be chromatographically separated (data not shown; see below). For convenience of discussion, we will refer to these multiple bands as "the 90-kD nuclear kinase". Several other autophosphorylation activities were detected, but these were equally present in nuclear and cytosolic fractions. In addition to HeLa cells, we have detected the 90-kD nuclear kinase in stimulated human peripheral blood lymphocytes (PBLs) and cell lines from humans [A431, Jurkat, EBV-transformed human B cell (32D), HUT78, and CEM], rodents (Mv1Lu, 3T3-L1, and CHO), and monkeys (CV-1 and COS-7) (data not shown).

The 90-kD nuclear kinase has a unique specificity

To determine whether the 90-kD autophosphorylation activity could phosphorylate an exogenous protein substrate, the kinase was partially purified (see below), resolved by SDS-PAGE, transferred to nitrocellulose, and renatured. A nitrocellulose strip containing the renatured kinase was then added to a reaction mixture containing myelin basic protein (MBP) and [γ-^{32}P]ATP. Figure 2A shows that the immobilized 90-kD nuclear kinase phosphorylated MBP. We then used this assay to determine the specificity of the kinase. Of several common peptide substrates tested, the 90-kD kinase phosphorylated only peptide substrates for smooth muscle myosin light-chain kinase (KKRPQRATSNVFS) and cAMP-dependent protein kinase (LRRASLG) (Table 1). This pattern distinguishes the 90-kD nuclear kinase from other kinases described to date (Pearson and Kemp 1991 and references therein).

The results of these peptide phosphorylation experiments suggested that the 90-kD nuclear kinase used serine and threonine as phosphoacceptors. To confirm this supposition, we performed phosphoamino acid analysis. Figure 2B shows that the immobilized 90-kD nuclear kinase phosphorylated MBP only on serine. Figure 2C shows that autophosphorylation occurred equally on serine and threonine. We conclude that the 90-kD enzyme is a serine–threonine kinase.

90-kD nuclear kinase activity is stimulated by agents that promote cellular proliferation

We then asked whether the 90-kD nuclear kinase was activated by particular signal transduction pathways. To

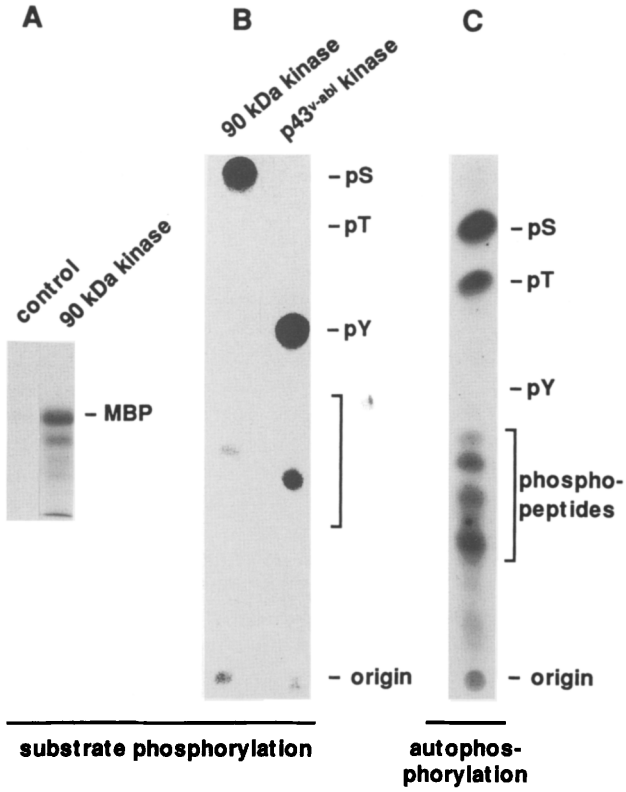

Figure 2. The 90-kD nuclear kinase phosphorylates serine and threonine residues. (*A*) Purified 90-kD nuclear kinase was resolved by SDS-PAGE, renatured on nitrocellulose, and used to phosphorylate MBP. As a negative control, MBP was incubated under the same conditions with nitrocellulose that did not have immobilized kinase. Activity was Mg^{2+}-dependent; Mn^{2+} did not substitute (data not shown). (*B*) Phosphoaminoacids of radiolabeled MBP (*A,right*) were determined. The mobilities of authentic phosphoaminoacid standards are indicated. (*B,left*) MBP phosphorylated with renatured 90-kD kinase. (*B,right*) MBP phosphorylated with a tyrosine kinase (recombinant p43$^{v\text{-}abl}$). (*C*) Phosphoamino acids of kinase renatured on a polyvinyldifluoride membrane and autophosphorylated with [γ-^{32}P]ATP.

Table 1. *Peptide substrate specificity of HeLa 90–kD nuclear kinase and recombinant RING3 proteins*

	HeLa (90 kD)	RING3 phosphorylated with NE	
		WT	K578A
Substrate	kinase activity (% of Kemptide control)		
Kemptide	100 (±12)	100 (±6)	<5
MLCK	135 (±7)	51 (±3)	<5
tyrK	11 (±1)	N.D.	N.D.
S6K	7 (±1)	<5	<5
CaMKII	<5	<5	<5
MAPK	<5	N.D.	N.D.
CKII	<5	<5	<5
PKC	<5	N.D.	<5
No substrate	<5	<5	<5

The 90–kD kinase was renatured on nitrocellulose after partial purification from HeLa nuclear extract and used to phosphorylate peptides with [γ-^{32}P]ATP. Recombinant proteins were renatured in polyacrylamide gels. (WT) Wild type; (K578A) site-directed point mutant where catalytic Lys578 is changed to alanine. Phosphate incorporation was linear through at least 60 min and 2 mg/ml of peptide. 100% represents ~100,000 dpm. Peptide substrate sequences are Kemptide (cAMP-dependent protein kinase), LRRASLG; MLCK (myosin light-chain kinase from smooth muscle), KKRPQRATSNVFS; tyrK (tyrosine kinase), Raytide (an analog of gastrin, EGPWLEEEEEAYG); S6K (S6 kinase), RRLSSLRA (S6 peptide); CaMKII (calmodulin-dependent kinase II), PLSRTLSVSS; MAPK (microtubule-associated protein kinase II), APRTPGGRR; CKII (casein kinase II), RRREEETEEE; PKC (protein kinase C), RFARKGSLRQKNV. Duplicate assay; (N.D.) not determined.

determine whether the activity was correlated with cellular proliferation, a serum-responsive cell line, A431, was starved of serum overnight and then provided with 20% serum for 15 min. Figure 3A shows that autophosphorylation activity was significantly increased in extracts prepared from serum-stimulated cells. In a related experiment, PBLs that were normal and quiescent were cultured in the presence of phytohemagglutinin (PHA) to induce proliferation. Figure 3B shows that the 90-kD nuclear kinase activity was significantly increased in response to PHA. We tested several components of serum to determine which, if any, caused this increase. Only interleukin-1α (IL-1) treatment resulted in significant stimulation (Fig. 3C). Substances that did not increase 90-kD kinase activity included epidermal growth factor, platelet-derived growth factor-β, acidic fibroblast growth factor, insulin, glucagon, transforming growth factor-β, and interleukin-2, each of which was tested in an appropriately responsive cell line. Other potential stimuli of 90-kD autophosphorylation, such as herpesvirus infection, heat shock, ultraviolet light, hydrogen peroxide, sodium periodate, sodium vanadate, or cadmium chloride, were similarly ineffective (data not shown). Interestingly, we also found that forskolin, an activator of cAMP-dependent protein kinase pathways, transiently increased kinase activity in the mammalian T-cell line Jurkat (Fig. 3D).

Autophosphorylation of the 90-kD kinase increased sharply ~5 min after stimulation in CHO (Fig. 3C) or Jurkat (Fig. 3D) cells and declined slightly through 30 min. The rapid time course suggested that a post-translational modification was responsible for the increased activity. Consistent with this idea, autophosphorylation was dramatically reduced when extracts were prepared in the absence of phosphatase inhibitors or were briefly warmed to 37°C in the presence of 5 mM magnesium chloride (data not shown). These results suggest that the 90-kD nuclear kinase is reversibly phosphorylated.

Purification and microsequence analysis of 90-kD nuclear kinase

We purified the kinase from HeLa nuclear extract according to the scheme diagramed in Figure 4A. The partially purified kinase was resolved by SDS-PAGE, transferred to nitrocellulose, and digested with trypsin. Tryptic peptides were separated by high-pressure liquid

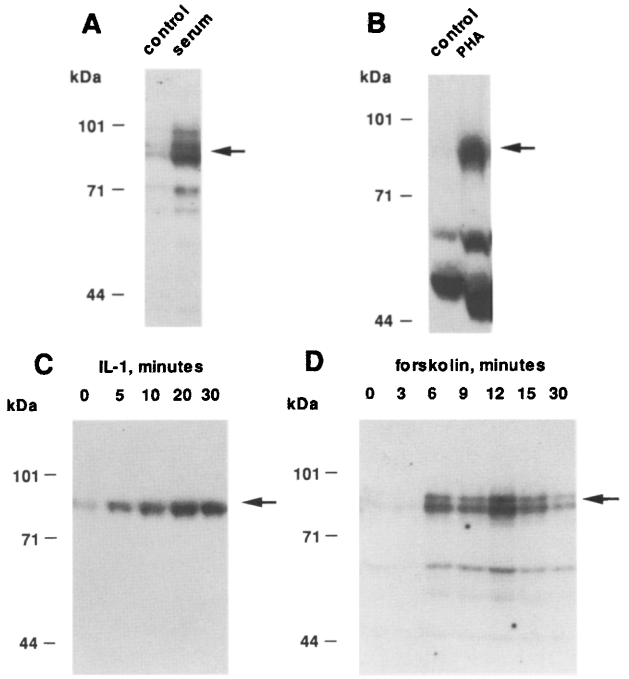

Figure 3. Stimulation of 90-kD autophosphorylation. (*A*) A431 cells were starved of serum, then treated with control buffer or calf serum (20%) for 15 min at 37°C. Kinases in nuclear extracts were assayed by renaturation assay. An arrow indicates the 90-kD multiplet. (*B*) Total PBLs (~10^7) were isolated from normal, healthy volunteers, suspended in RPMI1640 with 10% autologous serum, and cultured for 3 days, either unstimulated or stimulated with 2 μg/ml of PHA. (*C*) CHO cells were starved of serum, then treated with IL-1α (10 ng/ml) for the indicated times. (*D*) Jurkat cells were stimulated with 50 μM forskolin for the indicated times. Stimulated 90-kD kinase activity in Jurkat and CHO extracts was enriched by application to phosphocellulose and elution with 0.3 M NaCl. Increased autophosphorylation was correlated with increased MBP-directed substrate phosphorylation (results not shown).

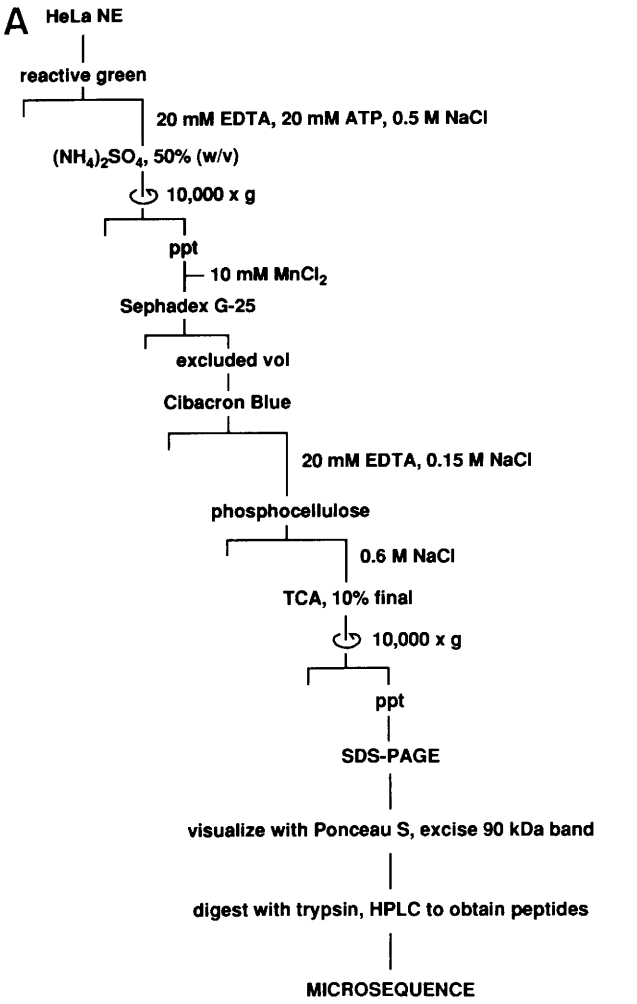

Figure 4. Purification and microsequence analysis. (*A*) Chromatographic scheme. (*B*) Microsequence Alignment. Homologous sequences are indicated with boldface type. Bromodomains are indicated with a heavy solid line. PEST scores are indicated in boldface above a broken line that defines a PEST sequence (analyzed with the BASICA-supported program PESTFIND). The putative NLS is indicated with a box. Microsequenced peptides are aligned as pept1 and pept2. Numbering is according to the translated cDNA for *fsh*. Percent conservation of sequence between RING3 and fsh has been reported (Beck et al. 1992).

chromatography (HPLC), and two peptides were microsequenced. The first of these (DSNPDEIEIDFETLKPTTL) was used to probe the GenBank data base; a match was obtained to the *Drosophila* homeotic gene, *female sterile homeotic* (*fsh*) (DROFSHB) (Haynes et al. 1989); there were 18 of 19 identities and a conservative T to S substitution. A match was also obtained with the RING3 gene of humans (HUMRING3) (Beck et al. 1992); there were 17 of 19 identities with a conservative D to E substitution and the same T to S substitution as in fsh. RING3, an acronym for "really interesting new gene", was serendipitously discovered during sequencing of the class II region of the human major histocompatibility complex (Beck et al. 1992). The second microsequenced peptide (AVHEQLAALSQAP) matched RING3; there were 12 of 13 identities with a conservative A to G substitution at position 12. This peptide did not match fsh. Figure 4B presents an alignment of fsh and RING3. The calculated molecular mass of RING3 is 83 kD, which correlates well with the apparent mobility of the 90-kD phosphoprotein as measured by SDS-PAGE.

B

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                10                                                  60
RING3  .......MAS VPALQLTPAN PPPPEVSNPK KPGRVTNQLQ YLHKVVMKAL WKHQFAWPFR
 fsh   MSSSEPPPRY EPPVEPVNGI VQPPVIPPAE RPGRNTNQLQ YLIKTVMKVI WKHHFSWPFQ

               70 bromodomain I                                     120
RING3  QPVDAVKLGL PDYHKIIKQP MDMGTIKRRL ENNYYWAASE CMQDFNTMFT NCYIYNKPTD
 fsh   QPVDAKKLNL PDYHKIIKQP MDMGTIKKRL ENNYYWSAKE TIQDFNTMFN NCYVYNKPGE

              130                 150  384                         410
RING3  DIVLMAQTLE KIFLQKVASM PQEEQELVVT ...TAAPPAQ PLAKKKGVKR KADTTTPTPT
 fsh   DVVVMAQTLE KVFLQKIESM PKEELELEPV ...AVIPPQQ PAKIKKGVKR KADTTTPTAN

          11.1     427  491                bromodomain II      530
RING3  AILAPGSPAS PPGSLEP... CNGILKELLS KKHAAYAWPF YKPVDASALG LHDYHDIIKH
 fsh   AFESPYTQMD SKSAKIA... CNEILKELFS KKHSGYAWPF YKPVDAEMLG LHDYHDIIKK
                5.8
               540                                                  590
RING3  PMDLSTVKRK MENRDYRDAQ EFAADVRLMF SNCYKYNPPD HDVVAMARKL QDVFEFRYAK
 fsh   PMDLGTVKRK MDNREYKSAP EFAADVRLIF TNCYKYNPPD HDVVAMGRKL QDVFEMRYAN

pept2    595  670   AVHEQLAA LSQAP  690          NLS    938          950
RING3  MPDEP...EQ LRAVHEQLAA LSQGPISKPK RKREKKEKKK KRKA...KTA PPALPTCYDS
 fsh   IPDEP...SK LLGLQEEIRK LSEEASAKKK AKKKLKEKKK SIGG...SKK KPSQVMNFDS

pept1  17.2  960                                  5.8 DSNPDE IEIDFETLKP
RING3  EEEEESRPMS YDEKRQLSLD INKLPGEKLG RVVHIIQARE PSLRDSNPEE IEIDFETLKP
 fsh   EEEDTAKPMS YDEKRQLSLD INKLPGDKLG RVVHIIQNRE PSLRDSNPDE IEIDFETLKP
        5.7                                  5.4
pept1  TTL  1020                                                  1070
RING3  STLRELERYV LSCLRKKPRK PYTIKKPVGK TKEELALEKK RELEKRLQDV SGQLNSTKKP
 fsh   STLRELESYV ASCLRKKTRK PYY.KKPSGK SKDEQMAEKK QELEKRLQDV TGQLGASKKT

              1080                          1110
RING3  PKKANEKTES SSAQQVAVSR LSASSSSSDS SSSSSSSSSS DTSDSDSG
 fsh   AKKDESASSK VEAVQPANPV SSSSSSSDSS SSSSSDSSSS DSSDSEAG
                                  20.1
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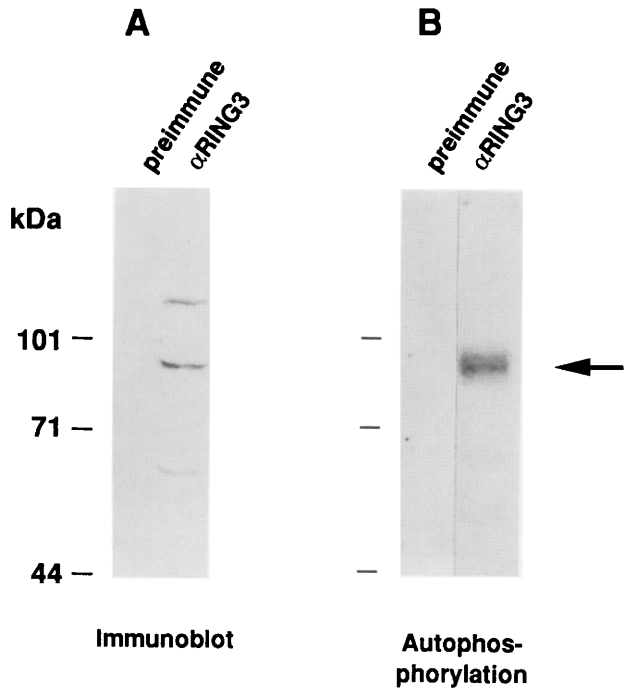
Immunoprecipitation and immunofluorescence analysis

We performed a series of experiments to test whether human RING3 encodes a kinase. First, we raised antisera to recombinant RING3 and asked whether it could immunoprecipitate 90-kD nuclear kinase activity. Figure 5A characterizes the affinity-purified αRING3 polyclonal antibody by immunoblot analysis. The results show that in a HeLa nuclear extract the αRING3 antibody principally detected a 90-kD polypeptide, whose size is consistent with that of the RING3 open reading frame (ORF) (Fig. 4B) and the nuclear autophosphorylation activity. Two other polypeptides of unknown identity were also detected, and, as discussed below, these additional polypeptides could be members of a protein family. Figure 5B shows the immunoprecipitation analysis. Immune complexes were resolved by SDS-PAGE, and activity was determined by renaturation/autophosphorylation. The results show that the 90-kD kinase was immunoprecipitated by the αRING3 antibody but not by preimmune serum. Significantly, the size of the autophosphorylation activity was identical to that of the major polypeptide detected in the immunoblot experiment of Figure 5A. These data strongly support the interpretation that the nuclear autophosphorylation activity results from RING3.

The αRING3 antibody was also used in the indirect immunofluorescence experiment of Figure 5C. RING3 is



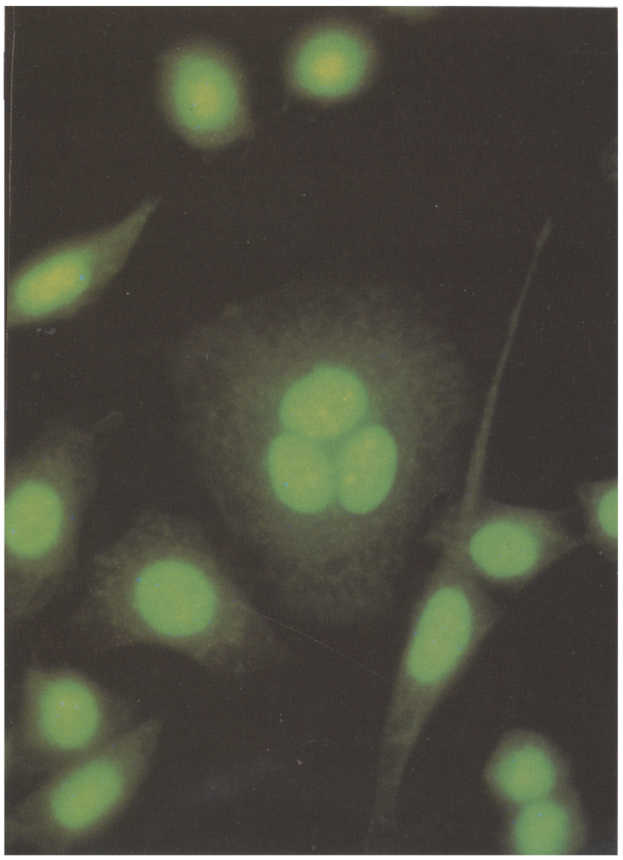


Figure 5. Immunoblot, immunoprecipitation, and immunofluorescence. (*A*) Immunoblot analysis. HeLa nuclear extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit preimmune serum or purified αRING3 antibody. (*B*) Immunoprecipitation analysis. HeLa nuclear extract was immunoprecipitated with rabbit preimmune serum (1:50) or αRING3 antibody (1:50) by recombinant protein A–agarose. The immune complexes were resolved by SDS-PAGE and assayed for renaturable autophosphorylation activity as in Fig. 1. (*C*) Nuclear immunofluorescence of αRING3. HeLa cells were fixed and incubated with αRING3 antibody. Primary antibody was visualized with FITC conjugated to goat antirabbit secondary antibody. αRING3 immunofluorescence colocalized with 4′,6-diamidino-2-phenylindole (DAPI) stain and with immunofluorescence from rabbit antibody to TATA box-binding protein (αTBP), both of which are authentically nuclear (data not shown). Rabbit preimmune serum did not exhibit significant immunofluorescence (data not shown).

clearly nuclear localized, in agreement with the biochemical fractionation data of Figure 1. We note that the RING3 ORF contains a putative nuclear localization signal (NLS) (see Fig. 4B).

Recombinant RING3 has kinase activity

We then asked whether expression of RING3 cDNA in bacteria and mammalian cells gave rise to a protein with kinase activity. For bacterial expression, RING3 was tagged with six histidines at the amino terminus, expressed in *Escherichia coli*. and purified to homogeneity by Ni^+–agarose chromatography (Fig. 6A; B, Coomassie, WT). Figure 6B (lane 2) shows that this *E. coli*-derived protein lacked kinase activity. In several other cases, recombinant kinases are inactive in the absence of specific post-translational phosphorylation (e.g., see Williams et al. 1992; Kozma et al. 1993). Therefore, we incubated the *E. coli*-derived RING3 with HeLa nuclear extract and ATP and repurified it according to the scheme shown in Figure 6A, which included a stringent 6 M guanidine hydrochloride wash of the Ni^+ column to remove any contaminating kinase activities (see Fig. 6B, lanes 1,8). The modified recombinant protein now possessed kinase activity (Fig. 6B, lane 3); as expected, this modification was ATP dependent (Fig. 6B, lane 8). These results also imply the existence of a RING3-activating kinase in HeLa nuclear extract.

To provide further evidence that the observed kinase activity was from recombinant RING3, we constructed two RING3 carboxy-terminal deletion mutants (Fig. 6B, bottom). The first deletion removed a polyserine tail (see Fig. 4B), which slightly reduced its size (Fig. 6B, Coomassie, ΔSnaBI), but did not abolish kinase activity (Fig. 6B, lane 5). The reduced size of the autophosphorylation activity observed with the ΔSnaBI mutant strongly argues that recombinant RING3 is the kinase. (Like wild-type RING3, the unmodified ΔSnaBI protein lacked kinase activity; Fig. 6B, lane 4.) A more extensive deletion (ΔBspMI), which removed a putative catalytic glutamate (see below), abolished kinase activity (Fig. 6B, lane 6).

To confirm that recombinant RING3 possessed the same specificity as the HeLa 90-kD nuclear kinase, we compared the ability of the two enzymes to phosphorylate peptide substrates. Table 1 shows that, like the HeLa

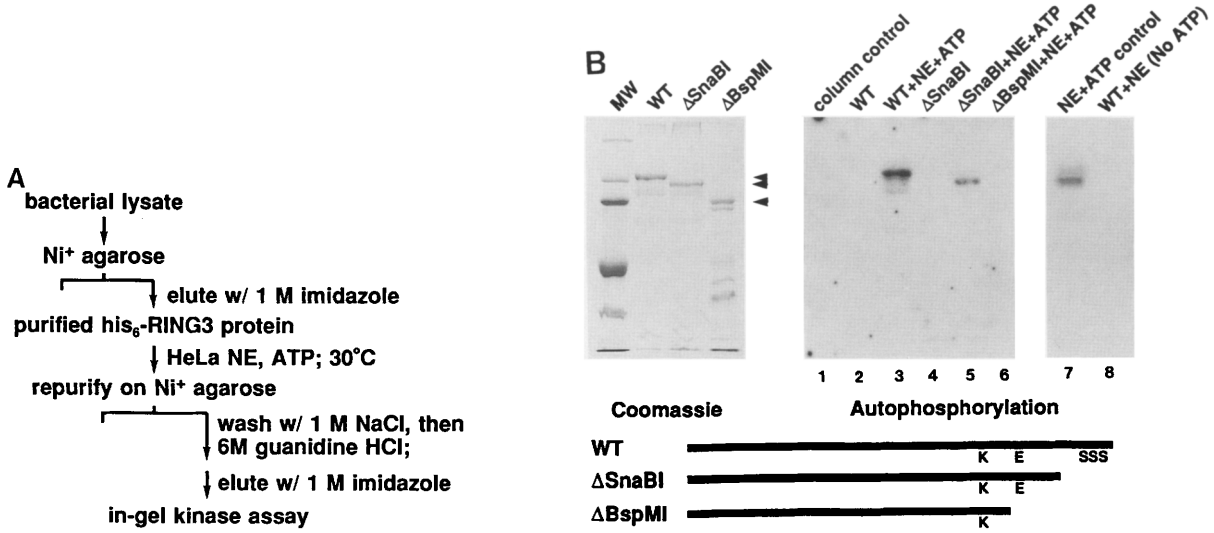


Figure 6. Autophosphorylation activity of recombinant wild-type and mutant RING3 derivatives. (*A*) Purification of recombinant RING3. (*B*) Activity of recombinant RING3. Bacterially expressed wild type (WT) and two deletion mutants (ΔSnaBI and ΔBspMI) of histidine-tagged RING3 were visualized by Coomassie stain and assayed by in-gel kinase assay. (Lane *1*) NE incubated with ATP in the absence of recombinant enzyme, then processed (*A*) as a negative control (column control); (lane *2*) unmodified WT enzyme; (lane *3*) WT enzyme after modification with NE and ATP; (lane *4*) unmodified ΔSnaBI enzyme; (lane *5*) ΔSnaBI enzyme after modification; (lane *6*) ΔBspMI enzyme after modification; (lane *7*) positive control for NE alone; (lane *8*) WT enzyme after modification with NE in the absence of ATP.

90-kD nuclear kinase, RING3 phosphorylated only peptide substrates for both cAMP-dependent protein kinase (Kemptide) and smooth muscle myosin light-chain kinase. Finally, we used site-directed mutagenesis to change a putative catalytic lysine (see below) to alanine (K578A). When expressed as a histidine-tagged protein and modified as above, this RING3 amino acid substitution mutant was unable to phosphorylate any of these peptides (Table 1).

For mammalian overexpression, COS cells were transiently transfected with a CMV vector that directed expression of RING3 cDNA (CMV–RING3). Nuclear extracts were prepared from these transfected cells and fractionated by phosphocellulose chromatography, which we found could resolve ectopically expressed from endogenous kinase activity (Fig. 7; data not shown). Figure 7 shows that 90-kD nuclear kinase activity was greatly increased in COS cells transfected with CMV–RING3. These data and those of Figures 5, B and C, 6B, and Table 1 indicate that RING3 is a nuclear kinase.

Potential relevance to leukemia

The stimulation of 90-kD autophosphorylation activity by serum (Fig. 3A) and mitogenic lectins (Fig. 3B) suggested a relationship with cellular proliferation. Furthermore, as discussed below, several pieces of evidence suggested that RING3 may be in a signal transduction pathway involved in human leukemias. To test whether there is an association between 90-kD kinase activity and leukemic proliferation, PBLs from normal individuals were compared with an equivalent number of PBLs from individuals with chronic lymphocytic leukemia (CLL) or acute lymphocytic leukemia (ALL). Figure 8A shows that leukemic PBLs (blasts or mature T cells) had very high levels of 90-kD autophosphorylation activity

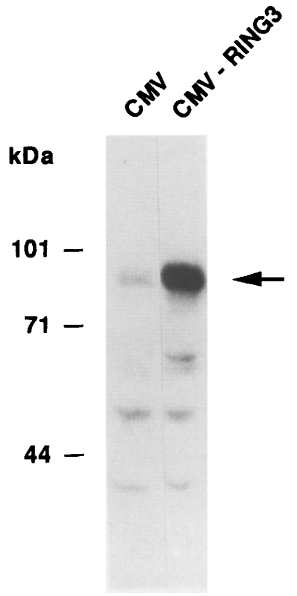


Figure 7. Mammalian expression of 90-kD nuclear kinase activity from RING3 cDNA. COS cells were transfected by calcium phosphate and harvested 48 hr later. Nuclear extracts were applied to phosphocellulose and eluted with 0.2 M NaCl, which separated ectopically expressed activity from endogenous activity. Transfections were with empty vector (CMV) or with vector containing RING3 cDNA (CMV–RING3). An arrow indicates the 90-kD multiplet.

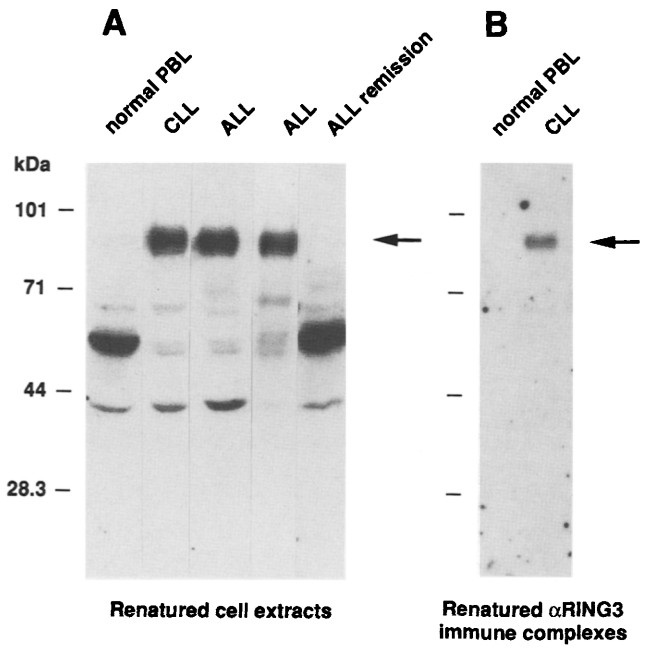


Figure 8. Elevated 90-kD nuclear kinase activity in acute and chronic leukemic cells. (*A*) Cell extracts were prepared from 10^7 PBLs. Proteins were separated by SDS-PAGE, renatured, and autophosphorylated. (Normal PBL) Normal 34-year-old male; (CLL) 69-year-old male diagnosed with chronic lymphocytic leukemia (>10^6 mature T cells/μl, lymphostasis, immune suppression); (ALL) 4-year-old female diagnosed with acute lymphocytic leukemia in proliferative blast stage; (ALL) 15-year-old male with same diagnosis; (ALL remission) same 15-year-old male after chemotherapy. Results were representative for 10 normal controls and 10 ALL patients (>5×10^4 blasts/μl in each case). Immunoblot with αRING3 confirmed no significant differences in 90-kD protein expression between ALL cases and controls (results not shown). (*B*) Two PBL extracts from *A* were immunoprecipitated with αRING3 antibody. Immune complexes were washed, solubilized with SDS, and assayed as in *A*. The 90-kD kinase activity is indicated by an arrow.

compared with normal PBLs. Interestingly, there was a renaturable autophosphorylation activity of ~50 kD that was prominent only in normal PBLs. One of the ALL patients entered remission after chemotherapy, and the same number of PBLs were assayed again. In this case (ALL remission), the 90-kD autophosphorylation signal was comparable with normal controls.

To confirm that the increased 90-kD autophosphorylation activity in leukemic cells was related to RING3, we performed an immunoprecipitation experiment. αRING3 immune complexes were resolved by SDS-PAGE and assayed by renaturation and autophosphorylation. Autophosphorylation (90 kD) was recovered only from leukemic PBLs (Fig. 8B). Taken together, these data reveal a correlation between 90-kD autophosphorylation activity and leukemia.

Discussion

We sought to identify nuclear kinases that might be important in signal transduction and growth control. We found a novel, renaturable kinase of an apparent molecular mass of 90 kD. Three lines of evidence indicate that the kinase is encoded by human gene RING3: First, two microsequenced peptides of the purified 90-kD nuclear kinase matched RING3 in the GenBank data base (Fig. 4B); second, antibody raised against recombinant RING3 specifically immunoprecipitated the 90-kD nuclear kinase (Fig. 5B); and third, RING3 cDNA encoded 90-kD autophosphorylation and transphosphorylation activities with properties similar to the native enzyme (Figs. 6B and 7; Table 1).

RING3 is a nonconsensus kinase

Tryptic peptides of the purified kinase were highly homologous to two proteins that share regions of homology: fsh and RING3. While this manuscript was in preparation, we reprobed the GenBank data base with the two peptide sequences and found a new match to an unpublished open reading frame, accession number D26362, that is highly homologous to RING3. These results suggest that the 90-kD kinase may be part of a protein family. There was no prior information regarding the cellular localization, biochemical activity, or expression of these proteins. For convenience of discussion, we will refer to the mammalian proteins as "RING3 kinase." The homologous regions of fsh, RING3, and D26362 include kinase motifs (see below), bromodomains (found in certain regulatory genes; Haynes et al. 1992; Tamkun et al. 1992), and PEST sequences (linked to high turnover rates; Dice 1987; Rechsteiner et al. 1987; Rechsteiner 1988, 1990; Chevaillier 1993) (Fig. 4B).

Most protein kinases conform to a consensus comprising a set of relatively short amino acid motifs (Hunter 1987). RING3 and fsh appear to deviate from this consensus, which explains in part why these proteins were not previously suspected to be kinases. Whereas certain kinases, for example, $p160^{c\text{-}BCR}$ (Maru and Witte 1991) lack the canonical serine/threonine sequence motifs, careful inspection of RING3 reveals the presence of consensus kinase motifs that appear to be "out of order" (Fig. 9). For example, RING3 contains a putative ATP binding motif (GXGXXG; subdomain I; Hanks et al. 1988; Hanks 1991) at amino acid 558 and a putative catalytic lysine (AXK; subdomain II) at amino acid 578 (Figure 9). When compared with a data base of all kinases, these sequences and spacing are most similar to the corresponding subdomains of c-*mos*. A putative subdomain III, which contains a catalytic glutamate at amino acid 605, is not closely related to c-*mos* but falls into a different class of kinases characterized by an EKR motif at that position. Therefore, RING3 is not a close relative of any existing protein kinase. Other putative subdomains, for example, kinase consensus sequences YHRDLK (subdomain VIB) and APE (subdomain VIII) (Hanks et al. 1988; Hanks 1991), are found in the expected order in two amino-terminal domains of both RING3 and fsh. Kinases with multiple catalytic domains (Jones et al. 1988; Banerjee et al. 1990) or mosaics of serine/threonine and tyrosine kinase subdomains (Levin et al. 1987)

have been described. We conclude that RING3 defines a new class of kinase, possessing several serine/threonine kinase motifs, but with rearranged subdomains.

There are several precedents for enzymes with "scrambled" primary sequence motifs. For example, lipases, serine proteases, and cholinesterases employ a similar catalytic triad of serine, histidine, and aspartate/glutamate in a conserved three-dimensional arrangement, but the individual members of the triad can be located on different loops or even on α-carbon backbones of opposite direction (Schrag et al. 1991; Dodson et al. 1992).

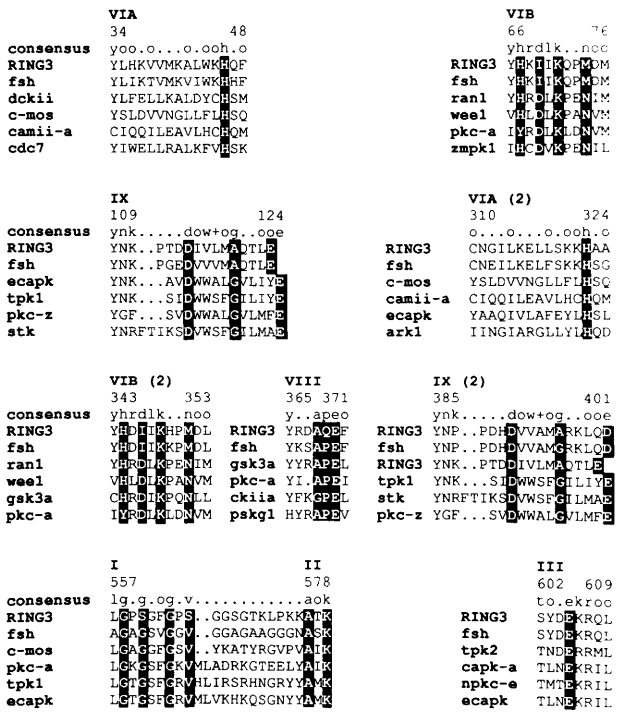


Figure 9. Comparison of putative subdomains of RING3 and fsh with known protein kinases. The authentic subdomains of known kinases are compared with fsh and RING3; the closest overall resemblances were with kinases of the cAMP-dependent protein kinase and protein kinase C families. For comparison, some kinases with unusual subdomain sequences are included. Numbering corresponds to the translated RING3 cDNA sequence. Conserved amino acids are identified above the RING3 sequence by lowercase letter. The most highly conserved amino acids are identified by a solid black box with white type. Hydrophobic amino acids (F, Y, L, V, W) are referred to as a class with an o, small side chain (A, S, T) with a +, and no consensus with a period. Where RING3 appears twice, internally duplicated domains are compared. The Hanks subdomains that are not obviously present in RING3 are IV, V, VII, and the conserved arginine of XI. (ark1) *Arabidopsis thaliana* receptor kinase; (camii-a) rat calcium/calmodulin-dependent protein kinase II-α; (capk-a) human cAMP-dependent protein kinase α; (cdc7) *Saccharomyces cerevisiae* wild-type CDC7; (ckiia) human casein kinase II-α; (c-mos) human cellular homolog of v-mos; (dckii) *Drosophila melanogaster* casein kinase II-α; (ecapk) *Caenorhabditis elegans* cAMP-dependent protein kinase *c*; (gsk3a) glycogen synthase kinase 3; (npkc-e) mouse protein kinase C-like protein; (pkc-a) rat protein kinase C-α; (pkc-z) rat protein kinase C-ζ; (pskg1) human putative protein–serine kinase; (ran1) *Schizosaccharomyces pombe* wild-type "meiotic bypass" protein; (stk) hydra src-related protein; (tpk1) *S. cerevisiae* cAMP-dependent protein kinase type 1; (tpk2) *S. cerevisiae* cAMP-dependent protein kinase type 2; (wee1) *S. pombe* wild-type "reduced size at division" protein; (zmpk1) maize receptor protein kinase.

Role of RING3 in development and leukemogenesis

The physiological substrates of RING3 kinase are unknown, but in light of its nuclear location and responsiveness to mitogenic signals, transcription factors are likely targets. Sequence homology strongly suggests that fsh, a *Drosophila* homeotic gene product that is highly related to RING3, is also a kinase (Figs. 4B and 9). Genetic studies in *Drosophila* suggest that *fsh* is a *trans*-acting effector of *trithorax*, another homeotic gene (Digan et al. 1986; Mozer and Dawid 1989; Breen and Harte 1991, 1993). For example, reduced levels of *fsh* can increase the severity of *trithorax* mutations (Gans et al. 1980; Forquignon 1981). The sequence of trithorax strongly suggests that it is a transcription factor (Mazo et al. 1990). On the basis of these considerations, we hypothesize that fsh activates trithorax through phosphorylation, and that these two proteins are components of a signal transduction pathway involved in *Drosophila* development.

A human homolog of *trithorax* has been identified and referred to as ALL-1 (also called HRX, MLL, and HTRX-1; Cimino et al. 1991; Djabali et al. 1992; Gu et al. 1992; Tkachuk et al. 1992; Ford et al. 1993). In certain leukemias, the gene encoding ALL-1 is interrupted by a reciprocal chromosomal translocation that results in an ALL-1 fusion protein, whose functional properties are presumably altered. In several other instances, human homologs of *Drosophila* homeotic genes are rearranged in leukemic cells: An 8q22 translocation associated with acute myelogenous leukemia interrupts a gene with homology to *runt* (Miyoshi et al. 1993), an 11q23 translocation associated with acute promyelogenous leukemia involves a gene with a *Krüppel*-like zinc finger (Chen et al. 1993), and an 11q23 translocation associated with T-lymphoblastic leukemia interrupts TAN-1, a human homolog of *notch* (Ellisen et al. 1991).

Intriguingly, our results have revealed a relationship between RING3 kinase activity and lymphocytic leukemia. These new data, in conjunction with previous studies on ALL-1, suggest that RING3 and ALL-1 are components of a signal transduction pathway that becomes deregulated in certain leukemias. The availability, for the first time, of an assayable biochemical activity for a component of this putative signal transduction pathway will facilitate more detailed study. Further analysis of the functional relationship between fsh and trithorax and between RING3 and ALL-1 will help illuminate the role of nuclear kinase activity in development, growth control, and leukemogenesis.

Materials and methods

Autophosphorylation assay

Extracts were prepared (Dignam et al. 1983) in the presence of

sodium vanadate (1 mM) and β-glycerolphosphate (50 mM) and centrifuged (100,000*g*, 60 min at 4°C) to remove particulate matter. The proteins in the supernatants were separated by SDS-PAGE (Laemmli 1970), electroblotted to nitrocellulose or polyvinyldifluoride membranes, denatured, renatured, probed with [γ-^{32}P]ATP for autophosphorylation (Ferrell and Martin 1989), and subjected to autoradiography.

In vitro phosphorylation

Synthetic peptides were incubated at 30°C for 1 hr in 10-μl reactions containing 30 mM $MgCl_2$, 30 mM HEPES (pH 8.0), 2 mM DTT, 0.5 mM EDTA, 0.1% NP-40, 10 μM ATP, 0.15 mCi/ml of [γ-^{32}P]ATP, and 1 mg/ml of peptide. Reaction mixtures were quenched with ice-cold 10% phosphoric acid and applied to 0.1-ml phosphocellulose columns or P-81 paper circles (Whatman), which were washed extensively with 0.5% phosphoric acid. Incorporation of ^{32}P into peptide (~100,000 dpm for Kemptide) was determined for duplicate reactions by Cerenkov counting. (Raytide and p43$^{v\text{-}abl}$ were from Oncogene Science.) For MBP phosphorylation, reactions contained 1 mg/ml of protein and were quenched with SDS sample buffer. Radiolabeled MBP was resolved by PAGE and visualized by autoradiography. Phosphoaminoacids were determined (Cooper et al. 1983; Lewis et al. 1990).

Kinase purification

HeLa nuclear extract (100 ml) was applied to a column of reactive green-19 resin (40-ml bed volume, equilibrated with buffer A (20 mM Tris at pH 7.0, 50 mM NaCl, 50 mM β-glycerolphosphate, 10% glycerol, 1 mM Na vanadate, 1 mM DTT, 0.2 mM EDTA, 0.02% NaN_3, and 0.1% NP-40). All steps were conducted on ice or at 4°C. The flowthrough was discarded, and the column was washed extensively with buffer A and then eluted in batch with buffer B (buffer A supplemented with 20 mM disodium ATP, 20 mM EDTA, and 0.5 M NaCl, at pH 7.0). Ammonium sulfate was gradually added to the eluate to 50% (wt/vol) over 60 min at 4°C, whereupon the suspension was centrifuged (10,000*g*, 45 min at 4°C). The pellet was recovered, dissolved in buffer C (buffer A supplemented with 10 mM $MnCl_2$, pH 8.0), and desalted on Sephadex G-25 that had been equilibrated with buffer C. The desalted protein was applied to a column of Cibacron blue 3GA agarose (type 3000) that had been equilibrated with buffer C. The flowthrough was discarded; the column was washed extensively with buffer C and eluted in batch with buffer D (buffer A supplemented with 20 mM EDTA and 0.15 M NaCl, at pH 8.0). The eluate was diluted 1:1 with buffer E (buffer A with no NaCl, at pH 8.0) and applied to phosphocellulose that had been equilibrated with buffer F (buffer A at pH 8.0). The phosphocellulose was washed extensively and eluted in batch with 0.6 M NaCl in buffer F. The eluate was precipitated with trichloroacetic acid (10% final), washed with acetone, and solubilized in SDS sample buffer. Proteins were resolved by SDS-PAGE in 8% polyacrylamide, blotted to nitrocellulose, and visualized with Ponceau S. The band corresponding to the autophosphorylation activity of the 90-kD kinase was excised and digested with trypsin. Tryptic peptides were resolved by HPLC and microsequenced.

Plasmid constructs and antibody production

RING3 cDNA (clone CEM32, a 4-kb insert in CDM8; Beck et al. 1992) was propagated in MC1061/P3 (Invitrogen). A 1426-bp *Nco*I–*Eco*RI fragment (nucleotides 2214–3640) of RING3 was ligated into pGEX-2T for bacterial overexpression (Lin and Green 1991). Rabbit polyclonal antibody was raised against purified glutathionine *S*-transferase (GST) fusion protein. For production of purified antibody, rabbit immune sera were incubated with GST–agarose to remove antibodies against GST epitopes. Polyclonal antibodies against RING3 epitopes were then purified by antigen affinity chromatography on Affigel columns that contained the purified GST fusion protein.

For construction of a eukaryotic overexpression vector, the full-length RING3 coding sequence was obtained by double-stranded polymerase chain reaction (PCR) amplification of clone CEM32 with a forward primer (5′-CGCCGCGGATCCATGGCTTCGGTGCCTGCT-3′) that engineered a 5′ *Bam*HI site at the amino-terminal methionine of the coding sequence (nucleotide 1178) and a reverse primer (5′-GGCTGGGAATTCAATGTT-3′) that was complementary to the native *Eco*RI site in the 3′-untranslated region (nucleotide 3640). The 2467-bp *Bam*HI–*Eco*RI fragment from PCR was ligated into pcDNA(I) (Invitrogen). For bacterial overexpression, this fragment was ligated into RSETA (Invitrogen), which fused six histidines to the amino terminus of RING3.

Deletion mutants and site-directed mutagenesis

Deletion mutants of RING3 were constructed as follows: a 481-bp *Sna*BI–*Eco*RI fragment that encodes a polyserine tail was removed from the carboxyl terminus (nucleotides 3156–3640, ΔSnaBI). A 597-bp internal *Bsp*MI–*Bsp*MI fragment that encodes a putative catalytic glutamate (nucleotide 2972, E605) in addition to the polyserine tail (nucleotides 2914–3505, ΔBspMI) was removed. Overhanging ends were then filled-in with DNA polymerase I (Klenow), and the vector was religated blunt.

A site-directed mutant of RING3 was constructed from a 1508-bp *Sph*I–*Eco*RI fragment (nucleotides 2128–3640) that contains the codon for a putative catalytic lysine (nucleotide 2897, K578). This fragment was subcloned into M13 mp18 and mutagenized (Kunkel 1985) with an oligonucleotide, 5′-CCCAAAAAGGCCACAGCGACGGCCCCACCTGCC-3′, where GCG (alanine) replaced AAG (lysine) and ACG is a silent site replacement of ACC. A 391-bp *Esp*I–*Esp*I fragment (nucleotides 2592–2983) of the M13 RF DNA was used to shuttle the mutated site (K578A) back into RSETA for overexpression of the full-length mutant protein. All clones were verified by dideoxy sequencing.

HeLa NE modification of RING3

Histidine-tagged recombinant protein was purified on Ni^+–agarose (Qiagen), and phosphorylated by incubation for 30 min at 30°C with HeLa nuclear extract in buffer A that contained 10 mM ATP, 5 mM $MgCl_2$, and protease inhibitors but no Na vanadate. As a negative control for ATP-dependent modification, HeLa NE was incubated with 460 U/ml of hexokinase and 250 mM D-glucose (25°C for 30 min) to deplete ATP before recombinant protein was added. Phosphorylated protein was repurified on Ni^+–agarose and assayed by in-gel kinase assay (Gotoh et al. 1990).

Sequence analysis

Sequence data bases were scanned with BLAST (Altschul et al. 1993) and Intelligenetics software. Putative subdomains of RING3 and fsh were aligned to a data base of the catalytic domains of all known protein kinases (quinn@salk-sc2.sdsc.edu). Separate alignments were performed for each subdomain and were optimized by visual inspection.

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G V Denis and M R Green

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