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

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

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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 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], p42MAP and p44MAP [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 p33CDK1 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 TFIH, 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-32P]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...
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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-32P]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
Table 1. Peptide substrate specificity of HeLa 90-kD nuclear kinase and recombinant RING3 proteins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HeLa (90 kD)</th>
<th>RING3 phosphorylated with NE</th>
<th>WT</th>
<th>K578A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kemptide</td>
<td>100 (±12)</td>
<td>100 (±6)</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>MLCK</td>
<td>135 (±7)</td>
<td>51 (±3)</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>tyrK</td>
<td>11 (±1)</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>S6K</td>
<td>7 (±1)</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>CaMKII</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>MAPK</td>
<td>&lt;5</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>CKII</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>&lt;5</td>
<td>N.D.</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>No substrate</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 90-kD kinase was renatured on nitrocellulose after partial purification from HeLa nuclear extract and used to phosphorylate peptides with [γ-32P]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), KKRQORATSNVFS; tyrK (tyrosine kinase), Raytide (an analog of gastrin, EGPWLEEEEAYG); S6K (S6 kinase), RRLSSLRA (S6 peptide); CaMKII (calmodulin-dependent kinase II), PLRTRLSVSS; MAPK (microtubule-associated protein kinase II), APRTPGGGR; CKII (casein kinase II), RRREEETTEE; PKC (protein kinase C), RFARKGSLQKNV. Duplicate assay; [N.D.] not determined.

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
chromatography [HPLC], and two peptides were microsequenced. The first of these [DSNPDEIEIDFE-TLKP Troll] 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.

**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
A novel, mitogen-activated nuclear kinase

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).

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
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.
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 reproped 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; Chevailler 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 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 (Fig. 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 I]; 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 "bled" primary sequence motifs. For example, lipases, serine proteases, and cholinesterases employ a similar

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 transacting 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 promyelocytic leukemia involves a gene with a *Krippel*-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-glycerol phosphate (50 mM) and centrifuged (100,000g, 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 polyvinylidene fluoride membranes, denatured, renatured, probed with γ-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 MgCl₂, 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-32P]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 32P into peptide (~100,000 dpm for MBP) was resolved by PAGE and visualized by autoradiography. 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 recombinant protein A (20 mM Tris at pH 7.0, 50 mM NaCl, 50 mM β-glycerol phosphate, 10% glycerol, 1 mM Na vanadate, 1 mM DTT, 0.2 mM EDTA, 0.02% NaN₃, 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 sodium ATP, 20 mM EDTA, and 0.5 mM 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 (10,000g, 45 min at 4°C). The pellet was recovered, dissolved in buffer C (buffer A supplemented with 10 mM MnCl₂, 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 mM 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 CDMS; Beck et al. 1992) was propagated in MC1061/P3 [Invitrogen]. A 1426-bp NcoI–EcoRI 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′-CCGCGCGGATCATGGTTCTCGGCGGCCGCTG3′ that engineered a 5′ BamHI site at the amino-terminal methionine of the coding sequence [nucleotide 1178] and a reverse primer 5′-GGTCGGGAATTCATAGGT3′ that was complementary to the native EcoRI site in the 3′-untranslated region [nucleotide 3640]. The 2467-bp BamHI–EcoRI fragment from PCR was ligated into pcDNA1[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 Sau3AI–EcoRI fragment that encodes a polyserine tail was removed from the carboxyl terminus [nucleotides 3156–3640, ΔSnaB1]. A 597-bp internal BspMI–BspM1 fragment that encodes a putative catalytic glutamate [nucleotides 2972, 6605] in addition to the polyserine tail [nucleotides 2914–3505, ΔBspM1] was removed. Overhanging ends were then filled-in with DNA polymerase I [Klenow], and the vector was bluntedligated. A site-directed mutant of RING3 was constructed from a 1508-bp SphII–EcoRI fragment with 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′-CC-CAAAAAAGCCCAAGCGAGCCGGCGCCACCTGCG-3′, where GCC [alanine] replaced AAG [lysine] and ACC is a silent site replacement of ACC. A 391-bp Espl–Espl fragment [nucleotides 2592–2983] of the M13 RF DNA was used to shuttle the mutated site [K578A] 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 Ni²⁺–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 MgCl₂, and protease inhibitors but no Na vanadate. As a negative control for ATP-dependent modification, HeLa NE was incubated with 460 U/ml of bcrxokinase and 250 mM d-glucose (25°C for 30 min) to deplete ATP before recombinant protein was added. Phosphorylated protein was repurified on Ni²⁺–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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References


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References
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