Mutations and impaired function of LKB1 in familial and non-familial Peutz–Jeghers syndrome and a sporadic testicular cancer

Antti Ylikorkala*, Egle Avizienyte†, Ian P. M. Tomlinson‡, Marianne Tiainen, Stina Roth, Anu Loukola, Akseli Hemminki, Marie Johansson, Pertti Sistonen, David Markie, Kay Neale, Robin Phillips, Peter Zauber, Takeo Twama, Julian Sampson, Heikki Järvinen, Tomi P. Mäkelä* and Lauri A. Aaltonen1,*

Hartman Institute and Biocentrum Helsinki and 1 Department of Medical Genetics, Haartman Institute, PO Box 21, University of Helsinki, 00014 Helsinki, Finland, 2 Molecular and Population Genetics Laboratory, Imperial Cancer Research Fund, London, UK, 3 Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, 4 Laboratoire de Génétique Moléculaire, Department of Pathology, Dunedin School of Medicine, Dunedin, New Zealand, 5 Polyposis Registry, St Mark’s Hospital, Watford Road, Harrow, UK, 6 Old Hills Road, Livingston, NJ, USA, 7 Centre for Polyposis and Intestinal Diseases, 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113, Japan, 8 Department of Clinical Genetics, University Hospital of Wales, Heath Park, Cardiff, UK and 9 Second Department of Surgery, Helsinki University Central Hospital, Helsinki, Finland

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Germline mutations in LKB1 have been reported to underlie familial Peutz–Jeghers syndrome (PJS) with intestinal hamartomatous polyps and an elevated risk of various neoplasms. To investigate the prevalence of LKB1 germline mutations in PJS more generally, we studied samples from 33 unrelated PJS patients including eight non-familial sporadic patients, 20 familial patients and five patients with unknown family history. Nineteen germline mutations were identified, 12 (60%) in familial and four (50%) in sporadic cases. LKB1 mutations were not detected in 14 (42%) patients, indicating that the existence of additional minor PJS loci cannot be excluded. LKB1 is predicted to encode a serine/threonine kinase. To demonstrate the putative Lkb1 kinase function and to study the consequences of LKB1 mutations in PJS and sporadic tumors, we have analyzed the kinase activity of wild-type and mutant Lkb1 proteins. Interestingly, while most of the small deletions or missense mutations resulted in loss-of-function alleles, one missense mutation (G163D) previously identified in a sporadic testicular tumor demonstrated severely impaired but detectable kinase activity.

INTRODUCTION

Peutz–Jeghers syndrome (PJS) is a dominantly inherited disease characterized by predisposition to hamartomatous intestinal polyps, mucocutaneous melanin pigmentation and various neoplasms (1,2). Although multiple hamartomatous polyps of the gastrointestinal tract with a prominent stromal smooth muscle component are pathognomonic of PJS, patients commonly present with lesions of different histological types, such as adenomatous and hyperplastic polyps. Mucocutaneous pigmentation is expressed most frequently on the lips and oral area, although marked differences in localization and intensity of pigmentation between and even within families have been reported (3). In advanced age, pigmentation may diminish or disappear.

The incidence of cancer among PJS patients has been estimated to be 18-fold higher than in the general population (2). Moreover, the prognosis of a given neoplasm in PJS patients may be worse than in the general population (4). Most frequently, cancer affects the gastrointestinal tract (stomach, small intestine, colon), pancreas, breast, testis and ovary (1,2,4). A recent study showed a 5-fold risk of early-onset breast cancer associated with PJS (5).

Truncating germline mutations in the LKB1 gene have been reported in PJS patients (6), and polyps from a PJS patient with a germline LKB1 mutation demonstrate loss of the wild-type allele (7). These observations suggest that LKB1 is a tumor suppressor gene. Our previous extensive haplotype analyses of 12 PJS kindreds has shown an almost complete penetrance, as none of 17 unaffected at-risk individuals showed the disease-associated haplotype (7; unpublished data).

LKB1 is predicted to encode a serine/threonine kinase of unknown function. The apparent Lkb1 homolog (87% identity) in Xenopus laevis was characterized as an embryonically expressed kinase Xeek1 (8), and an uncharacterized homolog has been...
identified in mouse (unpublished data). Lkb1 is less related (35% identity) to the Snf1/AMPK kinase family involved in cellular stress responses (9). LKB1 is expressed in all tissues examined, with the highest expression in testis (unpublished data).

Many LKB1 germline mutations are small deletions or point mutations predicted to severely truncate the Lkb1 protein, but a few exceptions with missense mutations or small in-frame deletions have been detected (6). A search for somatic mutations in LKB1 in colorectal adenocarcinomas, testicular tumors, and primary breast and ovarian cancers has revealed a very low frequency of mutations (10,11; unpublished data).

Although the major, if not the only, locus for PJS is on chromosome 19p13.3 (7,12–14), three families incompatible with linkage to chromosome 19p have been reported (14), and a locus in 19q has been proposed (13). The results indicate that minor PJS loci may exist.

To investigate these questions, we have analyzed a series of unrelated PJS patients for germline LKB1 mutations. We have also extended LKB1 mutation analyses to studies on the Lkb1 kinase, and to the functional consequences the observed mutations have on Lkb1 activity.

RESULTS
To investigate the prevalence and nature of germline mutations in PJS families and non-familial PJS cases, we performed germline mutation analyses of LKB1 in 33 unrelated PJS patients (Table 1). Nineteen mutations were identified, 18 of these were detected in sequence analysis and one through Southern blotting as a large genomic rearrangement. As two unrelated individuals (P11 and P22) had an identical mutation, 18 different mutations were identified.

Table 1. LKB1 germline mutations and clinical characteristics of Peutz–Jeghers polyposis patients

<table>
<thead>
<tr>
<th>No.</th>
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Fam, familial; Sp, sporadic; U, unknown; Pigm, pigmentation; No pigm, no pigmentation; No, no associated cancer in the patient or family.

1, multiple lipomas; 2, pancreatic cancer in the family; 3, colon cancer in the family; 4, colorectal cancer; 5, breast carcinoma (age 40) and cervix carcinoma (age 45), multiple myeloma in family; 6, ovarian carcinoma (age 12), thyroid papillary carcinoma; 7, pancreatic and small intestine cancer in the family; 8, breast and pancreatic cancer in the family; 9, breast cancer (age 39); 10, gastric cancer in the family; 11, colon adenoma, sex cord tumor, breast carcinoma and cervix adenocarcinoma in P33, and brain tumor in the family.
Of the 18 different mutations, three (P11, P14 and P22) changed splice donor or acceptor sites, and are thus predicted to affect mRNA levels. The G→C change at −1 in intron 7 in P11 and P22 segregated with the disease in the familial case, and in LKB1 RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected. Another splice site mutation (a 1 bp insertion in the +3 position of the splice donor site of intron 5) was detected in one sporadic PJS case (P14), and again RT–PCR did not reveal any aberrant bands. RT–PCR performed from P11 mRNA no aberrant bands were detected.

Figure 1. Germline mutations in familial and sporadic Peutz–Jeghers syndrome. Mutations are indicated by arrows. (A) A 4 bp deletion (codons 264–265) in the P20 DNA sample (reverse sequence). (B) A C256→T change (Arg86→stop) in the P28 sample (reverse sequence). (C) A 1 bp deletion (codon 281) in the P8 DNA sample (reverse sequence). (D) A C658→T change (Gln→stop) in the P12 DNA sample. (E) Genomic rearrangement (*) of EcoRI-digested P9 DNA detected with a 1172 bp cDNA probe (nucleotides 66–1199). (F) Genomic rearrangement (*) of TaqI-digested P9 DNA detected with a 1172 bp LKB1 probe.

The PJS patient samples in which no mutations were identified by direct genomic sequencing subsequently were scrutinized for LKB1 rearrangements by RT–PCR or Southern blotting analysis in the cases where RNA (RT–PCR) or sufficient amount of DNA (Southern) was available. RT–PCR with primers LKBF1 and LKBR5 spanning nucleotides 66–1199 of the ORF (87%) was performed with samples P1, P2, P4, P5, P6, P7, P9, P30 and P31; no additional bands were detected. Southern analysis of EcoRI- and TaqI-digested genomic DNA from P1, P2, P4, P5, P6, P7, P9, P12, P18, P24, P26, P30, P31 and P33 revealed aberrant bands in P9 with both EcoRI (Fig. 1E) and TaqI (Fig. 1F). The hybridization pattern of TaqI suggests that an ~2 kb deletion may have occurred in one LKB1 allele. No abnormalities were detected in RT–PCR of nucleotides 66–1199 of the ORF, suggesting that the promoter or 3′ part of LKB1 might be deleted. One TaqI polymorphism was detected in four of 15 patients and two of four controls using a 561 bp probe derived from the 3′-untranslated region of LKB1 (data not shown).

Microsatellite markers D19S180, D19S880, D19S891 and D19S254 (13) were used to test linkage to 19q13.4 in a set of six families (P17, P18, P19, P26, P30 and P33) in which no LKB1 genetic defect was found. Multipoint linkage analysis when all families were considered together excluded almost the whole 19q13.4 region as a disease-predisposing locus. Multipoint analysis of markers D19S180, D19S880, D19S891 and D19S254 resulted in lod scores of −2.71 (θ = 0.0 cM), −10.16 (θ = 7.31 cM), −2.56 (θ = −8.01 cM) and 0.54 (θ = 13.62 cM), given at the locus map positions, respectively.

In summary, the prevalence of observed LKB1 mutations in familial cases was 12/20 (60%), and 4/8 in sporadic cases (50%). Three of the five (60%) PJS cases with no available family documentation had LKB1 mutations. In the whole series, 19 mutations in 33 PJS individuals (58%) were observed.

The structural consequences of the various mutations affecting LKB1 identified above and in our previous reports (6,10) are summarized in Figure 2. While most mutant LKB1 alleles are predicted to encode severely truncated polypeptides, a number of mutations are small in-frame deletions, missense mutations or small C-terminal truncations. Some of these mutations target residues identified as necessary among well-characterized serine/threonine kinases (such as N181→Y in P15), and are likely to result in loss-of-function alleles. In other cases, such predictions are unreliable due to low homology of Lkb1 to structurally characterized kinases, and thus it was of interest to characterize these mutants functionally.

To characterize initially the protein encoded by LKB1, we transiently expressed hemagglutinin (HA) epitope-tagged Lkb1 in a human osteosarcoma cell line U2OS. The cells were subjected to a metabolic labeling using [35 S]cysteine and [35 S]methionine for 2 h, and subsequently the labeled Lkb1 and associated proteins were immunoprecipitated specifically with HA-Lkb1, and could represent an endogenous protein associated with the overexpressed HA-Lkb1.

The Xeek1 protein was reported to have autocatalytic activity when expressed in a recombinant form in bacteria (8). However,
Figure 2. Schematic representation of predicted proteins encoded by mutant LKB1 alleles detected in ref. 7 (*), ref. 10 (**), and this study. The gray area denotes the kinase domain, and black areas denote nonsense proteins encoded following frameshifts. Alleles analyzed functionally in this report are underlined. P, patient; SL, cell line derived from patient; testis ca., testis carcinoma with a somatic sporadic LKB1 mutation (10).

this activity was not observed when Xeek1 was immunoprecipitated from frog cell lysates, and instead a presumed substrate of 155 kDa was phosphorylated in these preparations. Thus, it was of interest to analyze whether human Lkb1 has autocatalytic activity in vivo and whether p115 could represent a substrate of Lkb1. To this end, we immunopurified unlabeled Lkb1 followed by an in vitro kinase reaction (Fig. 3B). Lkb1 was initially tested for its ability to phosphorylate widely used kinase substrates such as myelin basic protein, histone H1 and the C-terminal domain of RNA polymerase II, but no activity was observed (data not shown). We also did not observe specific phosphorylation of p115 or other co-immunoprecipitated bands, but instead noted a strong phosphorylation of a 60kDa polypeptide only seen in the HA-Lkb1-transfected sample, and thus presumed to represent autocatalytic activity of Lkb1.

Using the functional assay described above, we compared wild-type Lkb1 kinase activity with three mutants with minor predicted changes identified in PJS (Fig. 4A). The mutants used were SL8 (C-terminal truncation), SL26 (four amino acid deletion) and SL25 (Leu67 → Pro) shown underlined in Figure 2. HA-tagged mutant or wild-type Lkb1 was expressed in U2OS osteosarcoma cells and subjected to immunoprecipitation and kinase assays as described in Figure 3. The only prominent phosphorylated band was observed in the wild-type Lkb1-transfected sample at 60 kDa (Fig. 4A). To control the expression of the apparently inactive mutant alleles, transfected lysates were analyzed for mutant Lkb1 proteins by western blotting (Fig. 4B). The wild-type HA-Lkb1 co-migrated exactly with the phosphorylated band, and the mutant proteins were expressed at levels comparable with wild-type, although the SL8-encoded Lkb1 was truncated as expected and migrated at 50 kDa.

In addition to PJS, LKB1 variants have been identified in a few sporadic tumors (10, unpublished data). One somatic change was identified in a testicular carcinoma with a G163D missense variant (10; Fig. 2) of unknown functional consequence. Using the assay described above, we tested the activity of Lkb1-G163D (Fig. 4C) using in this case Myc epitope-tagged LKB1 expression vectors due to their lower background activity. In contrast to the mutants described above, Lkb1-G163D had retained autocatalytic activity, although the specific activity was dramatically reduced when compared with wild-type Lkb1. Both proteins were found to be expressed at comparable levels in the transfection (Fig. 4D). This result demonstrates that mutant alleles of LKB1 (at least G163D) can retain low kinase activity, while having apparently lost their growth-suppressive function.

DISCUSSION

To evaluate better the genetic defects underlying PJS, we performed LKB1 germline mutation analysis in 33 PJS patients. The diagnoses were based on the presence of histopathologically confirmed intestinal Peutz–Jeghers polyposis, and the series included familial and sporadic cases. We did not detect germline mutations in 14/33 (42%) of the families included in this study. However, four of these were compatible with 19p linkage in a previous study; added two-point lod score 1.14 at D19S886 (7).

In this study, utilizing these four families and two additional
Figure 4. Functional analysis of \textit{LKB1} mutant alleles. (A) The indicated HA-tagged Lkb1 mutants were analyzed for autocatalytic activity with wild-type and mock controls as described in Figure 3. (B) Western blotting analysis of the indicated HA-tagged Lkb1 mutants. (C) The testis tumor-derived missense mutant allele (testis ca.) was analyzed for autocatalytic activity as in (A) using Myc epitope-tagged plasmids. (D) Western blotting analysis of the indicated Myc-tagged Lkb1 proteins.

families with no detected \textit{LKB1} defect, evidence for linkage to a suggested second PJS locus at marker \textit{D19S880} (13) was not obtained. The results suggest that our analysis did not identify all \textit{LKB1} mutations, some of which could reside, for example, in the non-coding regions of the transcript, in the promoter region, or could involve large genomic deletions or other rearrangements not detected by our analyses.

The proportion of mutation-positive PJS individuals in this study (19/33, 58\%) was substantially lower than in our initial study of 12 selected 19p-linked PJS kindreds (6) in which as many as 11 were shown to display \textit{LKB1} changes. In addition to chance, the difference may reflect patient selection. Especially in the sporadic cases, the diagnosis of PJS may be uncertain, despite the histological evaluation of the lesions. Although PJS seems to be a genetically homogeneous disease, the existence of minor loci has been suggested. A potential second locus on 19q13.4 has been identified in a large Indian family (13). Three families have been reported to be unlinked to 19p13.3, and none of the available PJS polyps from these families showed allelic loss at \textit{D19S886} (14). Whether minor PJS loci exist remains to be determined.

Our study demonstrates that, unlike in Bannayan–Riley–Ruvalcaba hamartomatous polyposis syndrome (15), sporadic PJS patients often display similar germline defects to familial cases. This is important in view of genetic counseling and follow-up of such patients.

Homology searches using the \textit{LKB1} cDNA sequence suggested that it encodes a serine/threonine kinase related to \textit{X. laevis} Xeek1 (6,16). Here biochemical evidence demonstrated that Lkb1 indeed is a functional kinase with autocatalytic activity. Auto-phosphorylation of Xeek1 was only detected with recombinant GST–Xeek1; when purified from cell extracts, Xeek1 only phosphorylated a co-immunoprecipitating band of 155 kDa (8). This is in contrast to Lkb1, which did show autocatalytic activity when purified from mammalian cells and which did not phosphorylate other substrates detectably. Although we have no evidence regarding the autophosphorylation site, it is interesting to note that the autophosphorylation site of Xeek1 (T192; 8) is conserved in both mouse and human Lkb1 (T189).

Moreover, the results demonstrate that all analyzed mutant alleles of \textit{LKB1}, whether identified in PJS or in a sporadic testis tumor, exhibit severely compromised activity. In the case of the testicular cancer, the result is of particular importance, providing conclusive evidence of somatic impairment of Lkb1 function in sporadic tumorigenesis. Interestingly, the partially functional Lkb1-G163D mutation suggests that this and other mutant \textit{LKB1} alleles may provide useful tools for elucidating the cellular function(s) of Lkb1.

\section*{MATERIALS AND METHODS}

\subsection*{Patient selection}

Thirty three PJS patients were available for the mutation screening in \textit{LKB1}. The diagnosis was based on the presence of histopathologically confirmed intestinal Peutz–Jeghers polyposis. Other clinical data, such as information on mucocutaneous pigmentation, and clinical features of family members were not available for all patients. Of the 33 patients, 20 had a family history of PJS and eight were sporadic. In five cases, family data was not available. Mucocutaneous pigmentation was documented in almost all cases (16 of 17) where information about the pigmentation was available (Table 1).

\subsection*{Isolation of DNA and RNA}

Isolation of DNA was performed using standard procedures. Cell lines were prepared from PJS patients’ blood samples using standard methods. RNA was extracted using the RNaseasy kit (Qiagen).

\subsection*{Sequencing}

Mutation screening was performed by genomic sequencing of the nine coding \textit{LKB1} exons. Primers were designed to cover all exonic coding sequences as well as splice acceptor and donor sites. The nine \textit{LKB1} exons (accession nos AF055320–AF055327) were amplified using primers and conditions that have been described previously (10). The PCRs were performed in DNA Engine
thermal cyclers (MJ Research). Aliquots of 5 µl of PCR products were run in 3% agarose (NuSieve) gel to verify the specificity of the PCR reaction. The rest of the reaction product was purified using QIAquick PCR purification kit (Qiagen). Direct sequencing of PCR products was performed using the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing ready reaction kit (Perkin-Elmer). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC Bioproducts) and analyzed on an Applied Biosystems model 373A or 377 automated DNA sequencer (Perkin-Elmer).

RT–PCR

A 0.8 µg aliquot of RNA was used in the reverse transcription reaction. The standard random priming method with M-MLV reverse transcripts (Promega) and RNase inhibitor (Promega) was used to obtain 20 µl of cDNA. The following primers were used to amplify most of the coding region of LKB1: LKBF1, 5′-GAG GT-3′; LKB5, 5′-GCC CTG GAT TTG GTG CTC-3′. PCR was carried out in a volume of 50 µl containing 3 µl of cDNA, 1× PCR buffer (Life Technologies), 2.5 mM MgCl₂, 200 µM of each dNTP (Life Technologies), each primer at 0.8 µM, 10% dimethylsulfoxide (DMSO) and 2 U of AmpliTaqGOLD polymerase (Life Technologies). Cycling conditions were as follows: 10 min at 95°C, 40 cycles of 45 s at 95°C, 1 min at 56°C, 2 min at 72°C, final extension 10 min at 72°C.

Southern blotting analysis

Genomic DNA (8 µg) was digested with EcoRI and TaqI restriction enzymes, resolved by 0.8% agarose gel electrophoresis and blotted onto Hybond-N membrane (Amer sham). The first probe, 1172 bp in length, was amplified with primers LKB1F1 and LKB5R5 from cDNA. The second probe, 561 bp in length, was created from genomic DNA by PCR. The primers for the PCR were: 3UTRF, 5′-CTG GA T TTG GTG CTC-3′; 3UTRR, 5′-GGA AGG AAG ACG GAA GAG GT-3′. PCR was performed in a 50 µl volume reaction containing: 1× PCR buffer (Life Technologies), 2 mM MgCl₂, 200 µM of each dNTP (Life Technologies), each primer at 0.8 µM, 10% dM SO and 2 U of AmpliTaqGOLD polymerase (Life Technologies). Cycling conditions were as follows: 10 min at 95°C, 40 cycles of 45 s at 95°C, 1 min at 56°C, 2 min at 72°C, final extension 10 min at 72°C.

Lkb1 expression constructs

The coding region of wild-type or mutant LKB1 was PCR amplified under the following conditions: 3 µl of each cDNA (see RT–PCR) in 50 µl of 1× PCR buffer (Perkin-Elmer) with 200 µM of primers 10175, 5′-ACG AAT TCT CCA GCA TGG AGG TGG TG-3′, and 10185, 5′-GGG TCC ACG CCT CAC TGC TGC TGG CA-3′, and 2 U of AmpliTaq polymerase (Perkin-Elmer). PCR was for 30 cycles of 95°C 30 s, 55°C 45 s, 72°C 1 min. Subsequently the PCR fragment was digested with EcoRI and SalI and subcloned into the EcoRI and SalI sites of pC1-Neo (Promega) based vectors containing N-terminal HA or Myc epitope tags. All LKB1 alleles were confirmed by sequencing.

Cell culture and transfections

U2OS human osteosarcoma cell line was grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin and streptomycin. U2OS cells were transfected using the calcium phosphate transfection method essentially as described (17).

Metabolic labeling of cellular proteins

At 48 h after transfection, cells were starved for 1 h in media lacking cysteine and methionine followed by a metabolic labeling with 200 µCi/ml of a mixture of [35S]cysteine and [35S]methionine (Promix; Amersham) for 2 h. Subsequently cells were lysed in lysis buffer [ELB; 150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM diithiothreitol (DTT), 2.5 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol-phosphate, 50 mM NaF, 1 µg/ml leupeptin] and subjected to immunoprecipitation. Immunoprecipitates were washed four times with ELB and subjected to SDS–PAGE analysis followed by fluorography.

Immunoprecipitation and kinase assays

At 48 h after transfection, cells were collected into phosphate-buffered saline (PBS) and lysed in 500 µl of ELB lysis buffer. Then 50 µl of 25% protein A–Sepharose beads (Pharmacia) and 1 µl of 12CA5 anti-HA or 9E10 anti-Myc monoclonal antibody were added into cleared cell lysates followed by an overnight immunoprecipitation. Subsequently immunoprecipitates were washed four times in lysis buffer and once with kinase buffer (20 mM Tris pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT) and then incubated at 30°C for 30 min in 30 µl of kinase buffer containing 10 µCi of [γ-32P]ATP. The reaction was stopped by adding boiling SDS–PAGE sample buffer and loaded on a 10% SDS–PAGE gel.
Western blotting

A 20 µg aliquot of protein from transfected lysates was analyzed by SDS–PAGE and western blotting according to standard techniques (17) using anti-HA (1:1000) or anti-Myc (1:1000) antibodies, and detected by enhanced chemiluminescence.

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