An LKB1-Interacting Protein Negatively Regulates TNFα-Induced NF-κB Activation

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Abstract
The Peutz-Jeghers syndrome (PJS) is a hereditary disorder that predisposes an individual to benign and malignant tumors in multiple organ systems. Recently, the locus responsible for PJS was mapped genetically to the LKB1 gene, with a subsequent investigation proving that it is responsible for most cases of PJS. LKB1 encodes a nuclear serine/threonine protein kinase, and potential tumor-suppressing activity has been attributed to LKB1 kinase. However, how LKB1 exerts its tumor-suppressing function remains to be determined. In this report, we describe the identification of a putative human LKB1-interacting protein, FLIP1, using the yeast two-hybrid system. Two regions of the LKB1 sequence have been determined to be crucial for the interaction with FLIP1. FLIP1 encodes a protein of 429 amino acids with a predicted molecular weight of 47 kd. In contrast to LKB1, which is mainly nuclear, FLIP1 is a cytoplasmic protein, and its expression is ubiquitous in all human tissues examined to date. Interestingly, deletion of the 195 N-terminal amino acids allows FLIP1 to enter the nucleus, suggesting the presence of a regulatory mechanism through its N-terminus for nuclear entry. In addition, we found that ectopic expression of FLIP1 selectively blocks cytokine-induced NF-κB activation. The involvement of FLIP1 in the regulation of NF-κB activity may shed new light on the role of LKB1 in tumor suppression.

Introduction

The Peutz-Jeghers syndrome (PJS) is an inherited autosomal-dominant disease characterized by hamartomatous polyps in the gastrointestinal tract and by mucocutaneous melanin pigmentation [12, 35]. One of the most significant clinical aspects of PJS is the predisposition to benign and malignant tumors of various organs beyond the gastrointestinal tract [12, 35]. Recently, a locus for PJS was mapped to human chromosome 19p13 by genetic approaches [15]. The use of polymorphic markers in this region has demonstrated that a presumptive wild-type allele was lost in hamartomatous polyps from PJS patients, which led to the conclusion that the target of the deletion was a PJS tumor-suppressor gene [14, 17]. Subse-
quent sequence analysis of the mutated gene in PJS revealed that it corresponded to a serine/threonine protein kinase gene, LKB1 [14, 17]. Since the finding that LKB1 defects are responsible for the PJS, several studies have attempted to describe the function of the gene [2, 4, 28, 34], but the physiological function of this protein kinase and its regulation are still unclear.

One approach to unraveling the biological function of LKB1 is to identify its interacting protein. In this study, we provide an initial characterization of an LKB1-interacting protein, FLIP1, using yeast two-hybrid screening, and provide evidence that FLIP1 blocks the action of nuclear factor κB (NF-κB) induced by tumor necrosis factor α (TNFα). NF-κB regulates expression of a plethora of genes in response to extracellular factors that promote inflammation, cell proliferation, and apoptosis [5, 6, 10, 11]. Various studies have already indicated that regulation of NF-κB activation may play a role in tumorigenesis [5, 6, 10, 11]. The fact that FLIP1 negatively regulates NF-κB may thus provide a novel insight about how LKB1 acts as a tumor suppressor.

Materials and Methods

Yeast Two-Hybrid Screening

The yeast GAL4 two-hybrid system used in this study has been described previously [7]. The cDNA was synthesized from human fetal-liver poly(A)+ RNA (Clontech, Palo Alto, Calif., USA) by SuperScript RT reverse transcriptase using the SuperScript Plasmid System, according to the manufacturer's instructions (Life Technologies, Rockville, Md., USA). The entire coding region of LKB1 was obtained by PCR amplification using fetal-liver cDNA as a template, which was then fused in frame to the GAL4 DNA-binding domain of the pPC62 vector to create pPC62-LKB1. For the prey, a human fetal-liver cDNA library was fused to the GAL4-activation domain of the pPC62 vector to create pPC62-FLIP1. For deleted mutants of LKB1, the entire coding sequence of LKB1 on the plasmid pBluescript II SK+ (Stratagene, La Jolla, Calif., USA) was digested by BamHI, SalI, and NotI, SphI, or SalI, and NotI, or SalI and NotI, or SphI and SalI to remove the corresponding regions of NA87, NA135, and CA46, respectively. The resultant DNA fragments were purified and ligated into the yeast two-hybrid, bacterial, or mammalian expression vectors as needed. LKB1-K78M was created by site-directed mutagenesis using a PCR protocol to introduce a lysine-to-methionine nine change at residue 78 of LKB1. The truncated FLIP1 mutants were obtained by designing specific primers according to the truncation sites indicated and using PCR amplification.

For bacterial glutathione-S-transferase (GST)-fusion protein expression, pGST-FLIP1 was constructed by subcloning the original DNA fragments amplified by PCR into pGEX4T-2 (Amersham Biosciences, Piscataway, N.J., USA). The bacterial expression constructs were then transformed into Escherichia coli strain BL21 (DE3; Novagen, Madison, Wisc., USA) for protein expression. The induction and purification of the recombinant proteins were performed according to the manufacturers' recommendations [30]. The mammalian expression vectors, pCMV and pFLAG-CMV-2, were obtained from Invitrogen (Carlsbad, Calif., USA) and Kodak (Rochester, N.Y., USA), respectively.

Northern Blot Hybridization

Human multiple-tissue Northern RNA blots (Clontech) were hybridized with a cDNA probe containing the entire coding region of FLIP1. The 1.0-kb probe was then purified, labeled with α-32P-dCTP, and hybridized to the RNA blots using established protocols [30]. A probe of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) clone included in the kit was used as a control in the hybridization.

Cell Culture and Transfection

Human embryonic kidney cells, HEK 293T, were maintained in DMEM (Life Technologies) supplemented with 10% fetal calf serum. Transfection of 293T cells was performed using the standard calcium phosphate precipitation method [30].

Immunoprecipitation and Western Blotting

The FLIP1 antiserum was raised in mice against purified recombinant-FLIP1 proteins. The antibodies were affinity purified on nitrocellulose blots of recombinant proteins, as has been described previously [33].

For the immunoprecipitation experiment, 293T cells were cotransfected with pFLAG-CMV-LKB1 and pCMV-FLIP1. Cell lysates were prepared 24 h after transfection in a lysis buffer (50 mM Heps, pH 7.0, 0.5% Nonidet P-40, 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 mM EGTA, 2 μg/mL of each aprotinin, pepstatin, and leupeptin, 0.5 mM sodium orthovanadate, 5 mM sodium fluoride, and 50 mM β-glycerophosphate). The cell lysate was then incubated with the anti-FLIP1 antibody and immobilized onto protein A/G-Sepharose (Amersham Biosciences). Immunoprecipitates were then resolved by SDS-PAGE, followed by immunoblotting with anti-FLAG to check for the presence of LKB1.

Two anti-FLAG monoclonal antibodies, M5 and M2, were obtained from Sigma. Western blotting was performed as has been described previously [13] with the reacted proteins detected by horseradish-conjugated anti-mouse or anti-rabbit IgG antibodies (Jackson ImmunoResearch, Boston, Mass., USA) and an ECL system (Amersham Biosciences).

In vitro Pull-Down Assay

Cell lysates were prepared from 293T cells transfected with either pCMV-FLAG-LKB1 or an empty vector as a control. The cell lysates were precleared with GST-glutathione-agarose beads, incubated with soluble GST-FLIP1 or GST proteins in the binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA), and then mixed with reduced glutathione-agarose beads (Sigma, St. Louis, Mo., USA). The protein-bound agarose beads were subsequently collected and washed thoroughly with the binding buffer. This was followed by SDS-PAGE analysis. The FLIP1-bound LKB1 was detected by immunoblotting with an anti-FLAG antibody.