Multiple pathways mediated by Ras for the anchorage-independent growth

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一、中文摘要

從人類肝癌細胞中找到了一個新穎的蛋白質激　,稱為 SLK,這個激　在胺基酸端是激　催化區,leucine zipper 區。這個 cDNA 全長有 2179bp,其中含有全 A 的尾端其可轉譯區(open reading frame)為全長 2079bp。為了得到全長,於是設計了 5 端的 RACE 的實驗,得到了一個 250bp 的 DNA 片段,經過定序,確定這個從肝癌細胞來的 cDNA 是包括了全長。這個基因有 699 胺基酸長約 80Kda,是屬於 Mixed lineage protein kinase (MLK)家族
關鍵詞：MLK、肝癌細胞

Abstract

A novel mixed lineage kinase protein, single leucine-zipper bearing protein kinase (SLK), was cloned. This cDNA has 2456 bp and encodes a protein of 800 amino acids and the molecular weight of this protein is 91kDa. The expression of this kinase specifically leads to the activation of JNK/SAPK pathway.
Keywords: SLK, JNK/SAPK

二、Introduction

Protein kinases play important roles in the regulation of many cellular processes, such as the transmission of signals from growth factor, control of cell growth and differentiation, regulation of cytoskeletal changes, gene expression, translation and metabolism pathways(1, 2, 3,4). Among of all protein kinases, MAP kinase plays an essential part of cell signal transduction machinery. Protein kinases of MAPK family are involved in a variety of cellular responses to cytokines, hormones, and stress-inducing reagents. Several mammalian MAP kinases have been cloned, including ERK, JNK/SAPK and p38/Mpk2(5,6). JNKs/SAPKs were first identified as c-jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), both of which are activated in response to UV irradiation, osmotic shock, heat shock, and treatment with tumor necrosis factor-α or antibiotics known to be inhibitors of eukaryotic protein synthesis. MEK family protein kinases, MKK4 and MKK7, and the MKK kinases may include TPL2, MLK/DLK, TAK, and ASK activate JNK/SAPK. The activated JNKs/SAPKs are then translocated into nucleus and activated several transcription factors such as c-jun, ATF2, ELK, and Sap-1. Phosphorylated c-jun, by JNKs/SAPKs, form homodimer or hetrodimer with c-fos has potent AP-1 activity and regulates the expression of a number of genes. Recently, there are reports indicating that a new era of protein kinases is involved in JNK signaling. This family of kinase has been named as Mixed Lineage protein kinase (MLK). MLKs are in turn activated by a number of agonists. For example, MLK-3 has been found to mediated signals from the ste20 homologues germinal center kinase, and hematopoetic kinase, the guanine-nucleotide exchange protein C3G, and is a target of the small GTPases Rac and Cdc42.　MLKs bear a protein-protein interaction domains in which containing two leucine-zippers carboxyl-terminal to the catalytic domain. Not only MLK might participate in the JNK signaling cascade but might also mediate p38/Mpk2 cascade. And then MLK has been implicated as MAP kinase kinase kinase(7,8,9,10).

三、Material and Method

Rat6 mRNA was used as templates by RT-PCR using degenerate oligonucleotides based on the sequences encoding transmembranes domains (P1 5* GT(G/C)ATGAG(T/C)G(T/C)(G/A/C)GAC CG(C/A)TA and P2 5* GGGGTT(G/C)AGGCA(G/C)(G/C)(T/A)GT

T) that are conserved among G-protein coupled receptors. The PCR conditions were as follows: denaturation at 930C for 1 min, annealing at 500C for 2 min, and extension at 720C for 3min, for 35 cycles, followed by a 7-min extension at 720C. A band in the 500-base pair range was excised, labeled with [32P]dCTP and used to screen human HepG2 cDNA library. The hybridization condition for screening this library were 5 X SSC and 40% formamide at 420C for 16 h and the final washing condition was 1 X SSC at 680C. A marathon cDNA mix (Liver, Plancenta, Thyroid, Fetus, and Stomach) was employed to amplify 5* end of this gene. Antisense gene-specific primers (AGSPs), AGSP1 5'GAGAGGCACCAAAGTCACAGAT and AGSP2 5'CACCTTGACAGGAGCCTCCATATGTA A, were designed and adaptor primers (AP1 and AP2) (Clontech) to perform the 5*-end RACE. The first-PCR was performed by using the cDNA mix and AGSP1 and AP1 and conditions were as follows: denaturation at 940C for 1 min, annealing at 650C for 1 min, and extension at 720C for 1min, for 35 cycles, followed by a 7-min extension at 720C. The resultant of first PCR was then used as template to perform secondary-PCR with AGSP2 and AP2 and PCR conditions as previously. A band about 400 bp was amplified and subcloned into pGEM-T vector and sequenced. The additional 5*-end DNA fragment of 303bp was then added to the original clone to make up the full-length of this gene with a putative open reading frame of 800 amino acids.

Cell lysates were prepared in IP buffer (40 mM Tris-Hcl (pH 7.5), 1% NP40, 150 mM NaCl, 5 mMEGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, proteinase inhibitors, and 1 mM sodium vanadate). Cell extracts (600 mg) were incubated with 5 mg of anti GFP mAb (Clontech) for 6 h at 40C, mixed with 20 ml protein-A sepharose suspension, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, wash three times with IP buffer plus 0.5% deoxycholate, five times with IP buffer along, and subjected to SDS-PAGE. Immunoblot analysis was performed with the anti-HA mAb 12CA5 (Roche).

SLK- or empty vector-expressed cells were harvested in lysis buffer (50 mM Tris-HCl, pH 8.0/250 mM NaCl/1% NP-40, 2 mM EDTA) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated on SDS-PAGE and transferred to nitrocellulose membranes (Amersham) and the membranes were probed with specific antibodies. After extensive washing (with 40 mM Tris-Hcl, pH 8.0/50 mM NaCl/1 mM EDTA), the blots were reprobed with horseradish peroxidase-conjugated secondary antibody and specific protein bands visualized with the ECL chemiluminescent detection system (Amersham).

Protein kinase assays were carried out using a fusion protein between glutathione S-transferase (GST) and c-Jun (amino acids 1-79) as a substrate. The GST-Jun fusion proteins were bound to glutathione Sepharose beads and incubated for 15 min on ice with the cellular extract that contains JNK, in the presence of kinase buffer (20 mM Hepes, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl, 2 mM MnCl, 5 mM NaF, 1 mM NaVO, 50 mM NaCl). The beads were pelleted and thoroughly washed with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% -MeOH, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine), before they were incubated with [g-32P]ATP (50 cpm/fmol) in the presence of kinase buffer. These steps were undertaken to ensure that c-Jun phosphorylation is carried out by JNK which is known to exhibit high affinity to this portion of c-Jun under these conditions. Following extensive washing, the phosphorylated GST-Jun was boiled in SDS sample buffer, and the eluted proteins were run on a 15% SDS-PAGE. The gel was dried, and phosphorylation of the Jun substrate was determined by autoradiography.

四、Results and Discussion

From the previous study, a novel purinoceptor might be existed. Two degenerated oligo-nucleotide primers were designed based on the homologue region of published purinoceptors. Low stringency RT-PCR technique starting from mRNA of Rat6 cells and these two primers obtained a 517-bp DNA fragment. This fragment was then used as probe to screen HepG2 cDNA library (Stratagene Cat.# 935202) by a low stringency hybridization method. A positive clone was found and sequenced. This cDNA extend 2179 nucleotide bases with a poly-A tail and contains a continuous open reading frame of 2097 bp. In order to obtain the 5'-end of this gene, a mixture (liver, placenta, thyroid, fetus, and stomach; Stratagene) of 5'-RACE cDNA library was employed and a further upstream DNA fragment of 303bp was found (data not shown). Moreover, this DNA fragment was sequenced and ligated to the previous cDNA clone to make up the full-length of this gene. The open reading frame of this cDNA encodes a putative polypeptide of 800 amino acids, with a calculated molecular mass of 91kDa (fig. 1). The amino acid sequence was compared in genbank and this cDNA encodes protein turn out to be a novel gene and belong to the family of MLK based on their characteristic sequences.

Since SLK contained a leucine-zipper motif, which has been implicated for the protein-protein interaction, it is rational to test if SLK can form homodimer in cells. The interaction was investigated using in vivo co-precipitation. Hep G2 cells were co-transfected with plasmids that express a GFP-tagged SLK construct and an HA-tagged SLK construct. Protein complexes were immunoprecipitated by using anti-GFP antibody and co-precipitated HA-SLK was probed by an anti-HA monoclonal antibody. As shown in figure 2, HA-SLK can be detected only when cells expressed both HA-SLK and GFP-SLK plasmids. This result demonstrated that SLK proteins do form homodimer in mammalian cells. However, it is still possible that SLK might form complexes with other proteins in cells.

Recently, reports have been accumulated that MLK family proteins activate the JNK/SAPK pathway raises the intriguing possibility that SLK might activate JNK/SAPK pathway or other MAPK pathways. It is, therefore, to test if the transient expression of SLK will activate MAPK pathways. Rat6, HepG2, and Hep3B cells over-expressed SLK and cell lysates were prepared and then the phosphorylation status of the p44/42ERK, p54/46JNK, and p38/Mpk2 were determined by immunoblot analysis with antibodies that recognized the activated phosphorylated forms of these kinases. The increasing of phosphorylated form of p54/46JNK (fig. 3) could be detected in all three-cell lines when cells transiently expressed SLK. Furthermore, there was no significant change in phosphorylation status of p44/42ERK and p38/Mpk2 (fig. 3). This result suggested that SLK may specifically lead to the activation of JNK/SAPK pathway. To test the phosphorylated state of JNK/SAPK is correlated to the activities of such kinase. A recombinant protein, GST-c-Jun (1-79), was produced in bacteria and used for the *in vitro* kinase assay of JNK/SAPK. As shown in figure 4, SLK strongly induce the activation of JNK/SAPK in indicated cells. This result correlated with the study on the phosphorylation status of JNK/SAPK. Thus, SLK specifically activates JNK/SAPK pathway in mammalian cells.

In summary, a novel kinase, SLK, was cloned and this kinase is belonging to the mixed lineage kinase family and it is expressed in most of tissue. This kinase bearing a kinase domain at the beginning of N-terminal and followed the kinase domain is a single leucine-zipper motif, which is distinct from most of the two leucine-zipper bearing MLK proteins. A long stretch of C-terminal portion is existed, however, the function of this region is not clear yet. When SLK proteins expressed in cells, they form protein complexes and most likely as a homodimer. The functional study of SLK indicates that it can specifically activate JNK/SAPK pathway.

五、參考文獻

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[1] 五、**Figure**

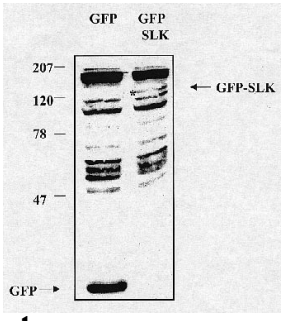

Figure 1

Figure2

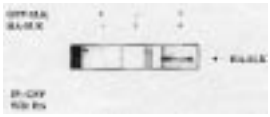

Figure3

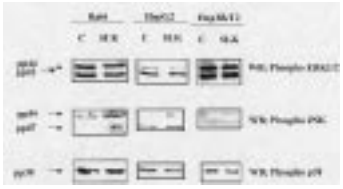

Figure4