LOK Is a Novel Mouse STE20-like Protein Kinase That Is Expressed Predominantly in Lymphocytes*  

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We have identified a new gene, designated lok (lymphocyte-oriented kinase), that encodes a 966-amino acid protein kinase whose catalytic domain at the N terminus shows homology to that of the STE20 family members involved in mitogen-activated protein (MAP) kinase cascades. The non-catalytic domain of LOK does not have any similarity to that of other known members of the family. There is a proline-rich motif with Src homology region 3 binding potential, followed by a long coiled-coil structure at the C terminus. LOK is expressed as a 130-kDa protein, which was detected predominantly in lymphoid organs such as spleen, thymus, and bone marrow, in contrast to other mammalian members of the STE20 family. LOK phosphorylated itself as well as substrates such as myelin basic protein and histone IIA on serine and threonine residues but not on tyrosine residues, establishing LOK as a novel serine/threonine kinase. When coexpressed in COS7 cells with the known MAP kinase isoforms (ERK, JNK, and p38), LOK activated none of them in contrast to PAK- and GCK-related kinases. These results suggest that LOK could be involved in a novel signaling pathway in lymphocytes, which is distinct from the known MAP kinase cascades.
rabbits. Polyclonal antibody against LOK was affinity-purified using the GST and GST-LOK proteins bound to CNBr-activated Sepharose 4B (Pharmacia). The antibody was used to detect LOK by immunoprecipitation and Western blotting.

Cell Cultures—NFS5.3 (pre-B cell line) (12) was cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. WEHI 231 (B cell line) (13) and EL-4 (T cell line) (14) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Neuro2a (neuroblastoma line) (15), L929 (fibroblast cell line) (16), ES (embryonic stem cell) (17), and COS7 cells (18) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Transient Expression and Immune Complex Kinase Assays—For the kinase assay of LOK (Fig. 5), each plasmid DNA was transfected into COS7 cells by the calcium phosphate method. The cells were lysed after 48 h of transfection in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 20 mM EDTA, 1 mM Na3VO4, 10 mM NaF, and 50 units/ml aprotinin. For the co-transfection experiment (Fig. 6), COS7 cells were transiently transfected using LipofectAMINE (Life Technologies Inc.) and, after 24 h of transfection, cells were lysed as described previously (19). Cell lysates were precleared with protein A-Sepharose 4B (Pharmacia) for 30 min and incubated with anti-FLAG (M2, Eastman Kodak Co.), anti-LOK antibody, or anti-HA (12CA5) for 2 h at 4 °C. The immune complexes were precipitated with protein A-Sepharose, washed with lysis buffer, and then washed with kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, and 3 mM MnCl2). To assay the kinase activity of LOK, the immunoprecipitates were incubated with 20 μM cold ATP and [γ-32P]ATP with/without substrates for 20 min at 30 °C. The substrates include casein, histone IIA, and myelin basic protein (MBP). To assay the activity of MAPK, JNK/SAKP, or p38, samples were incubated for 30 min at 30 °C with 3 μg of MBP, c-Jun, or ATF2 as described previously (20). The reaction products were subjected to SDS-polyacrylamide gel electrophoresis. The gels were fixed, dried, and autoradiographed.

Phosphoamino Acid Analysis—Radioactive bands excited from dried gels were hydrolyzed in 6 N HCl for 1 h at 110 °C. The supernatant was lyophilized and dissolved in pH 1.9 buffer containing cold phosphoamino acids as markers. The phosphoamino acids were resolved electrophoretically in two dimensions using a thin layer cellulose (TLC) plate with two pH systems (pH 1.9 and 3.5). The markers were visualized by staining with ninhydrin (21).

RESULTS

Cloning and Sequencing of lok—To identify protein kinases expressed in lymphocyte precursor cells, degenerate oligonucleotide primers corresponding to conserved regions of subdomains VI and IX in the catalytic domain of protein kinases were used to amplify cDNA from a mouse precursor B lymphocyte line NFS5.3. The sequence determination of randomly chosen clones of the amplified products revealed that 6 of them had an identical sequence that could encode a novel kinase, most likely a serine/threonine kinase. We named this molecule lymphocyte-oriented kinase (LOK) according to its expression pattern as shown below.

Since Northern blot analysis using the lok cDNA fragment as a probe detected the expression of a 5 kilobases of mRNA in NFS5.3 cell line as well as in thymus and spleen (data not shown), a cDNA library was prepared from mouse thymus and spleen (Fig. 1).

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screened with the cDNA fragment to isolate full-length lok cDNAs. The nucleotide sequence of the obtained full-length cDNA (5033 base pairs) showed an open reading frame of 966 amino acids with the Kozak consensus (22) at the initiation codon (Fig. 1). The 3′- untranslated region contains 20 repeats of dinucleotide CA, representing a microsatellite sequence (Fig. 1, thin underlined).

The kinase domain is located at the N terminus of the protein and contains all 11 subdomains of serine/threonine kinases (Fig. 1, boxed, and Fig. 3B) (23). Within the kinase domain, LOK displays the highest homology to members of the STE20 family such as PAK and GCK with 38–46% identity (Fig. 2). Moreover, the subdomain VIII of LOK contains the sequence GTPY/FWMAPEV characteristic for the STE20 family, indicating that LOK is a new member of the family. However, the C-terminal non-catalytic domain of LOK does not show any homology to known members of the family. Homology search of the non-catalytic domain of LOK failed to find any homologous proteins except rat AT1–46 carrying no kinase domain (see "Discussion"). The C-terminal region of LOK is predominately α-helical and supposed to form a coiled-coil structure (Fig. 3B, as predicted by the Pepcoil algorithm (Fig. 3A). Between the catalytic domain and the coiled-coil region, there is a proline-rich region containing putative SH3-binding motifs, PXXP (Fig. 1, thickly underlined, and Fig. 3B).

LOK Is Expressed Predominantly in Lymphocytes—When FLAG epitope-tagged cDNA of lok was transfected into COS7 cells, the gene product was detected as a 130-kDa protein by Western blotting with anti-FLAG antibody (data not shown). The polyclonal antibody generated against LOK-GST fusion proteins could detect the same protein in lok cDNA-transfected COS7 cells (Fig. 4C). The expression of LOK in various mouse tissues was then examined by Western blotting with this antibody. Surprisingly, LOK expression was found not ubiquitous and rather restricted to lymphoid organs such as spleen, thymus and bone marrow (Fig. 4, A and B). Both T and B lymphocytes in spleen expressed LOK (Fig. 4B). Analysis of LOK in a panel of in vitro cell lines confirmed the predominant expression of the kinase in lymphocytes (Fig. 4C, not all data shown). Eventually this pattern of expression led us to designate this kinase lymphocyte-oriented kinase, LOK.

LOK Possesses the Activity of Serine/Threonine Kinase—Since the nucleotide sequence predicted LOK as a novel serine/threonine kinase, we next analyzed whether the LOK protein was enzymatically active (Fig. 5). FLAG epitope-tagged LOK immunoprecipitated from COS7 transfectants autophosphorylated itself and phosphorylated substrates such as myelin basic protein and histone IIA when analyzed by the in vitro kinase assay. Furthermore, native LOK immunoprecipitated from the B cell line WEHI 231 also showed the kinase activity (Fig. 5, lane 10). The phosphoamino acid analysis revealed that serine and threonine residues but not tyrosine residues were phosphorylated in LOK and histone IIA (Fig. 5B). The substitution of lysine at position 65 in the ATP-binding site of the catalytic domain with arginine (KR mutant) abolished the kinase activity of LOK (Fig. 5A, lane 9), indicating that the observed kinase activity was directly from LOK and not from other molecules.

The polyclonal antibody generated against LOK-GST fusion proteins could detect the same protein in lok cDNA-transfected COS7 cells (Fig. 4C). The expression of LOK in various mouse tissues was then examined by Western blotting with this antibody. Surprisingly, LOK expression was found not ubiquitous and rather restricted to lymphoid organs such as spleen, thymus and bone marrow (Fig. 4, A and B). Both T and B lymphocytes in spleen expressed LOK (Fig. 4B). Analysis of LOK in a panel of in vitro cell lines confirmed the predominant expression of the kinase in lymphocytes (Fig. 4C, not all data shown). Eventually this pattern of expression led us to designate this kinase lymphocyte-oriented kinase, LOK.
The present study has revealed that LOK is a new member of the STE20 family and has unique features among the family. First of all, LOK was found to be expressed predominantly in lymphocytes. GCK (germinial center kinase) is expressed ubiquitously, despite its original naming, like many of other family members (31). A few members show the expression pattern relatively restricted to a certain tissue: PAK3/PAKβ in brain (32), and HPK1 in hematopoietic cells (25, 26). Those tissue-specific kinases including LOK may play important roles in response to a certain external stimulus in a tissue-specific manner. LOK is expressed in both B and T lymphocytes throughout their life, from early stage of their development in bone marrow and thymus till the differentiated effector phase in the periphery. Since recent studies have clarified that pre-cursor cells of both B and T lymphocytes use the same or very similar mechanisms to regulate their differentiation (3, 4), LOK may be involved in such regulations.

Second, LOK has a unique non-catalytic domain, which does not have any similarity to that of other members of the STE20 family. The family can be divided into three subfamilies based on the overall structure (6): (a) the archetypal PAK subfamily, containing an N-terminal p21-binding domain and a C-terminal kinase domain; (b) the pleckstrin homology homology-PAK subfamily, having a structure similar to the archetypal PAK subfamily, except they contain a pleckstrin homology domain N-terminal to the p21-binding domain (this subfamily has been found so far only in yeast but not in higher eukaryotes); (c) the GCK subfamily, having a kinase domain at the N terminus and lacking a recognizable p21-binding domain. Among this subfamily GCK (31), KHS (27), HPK1 (25, 26), and NIK (28) have extensive homology with each other in their non-catalytic domain to make one subgroup, whereas Mst1 (33) and Mat2 (34) makes another subgroup. Although the overall structure of LOK indicates that LOK belongs to the GCK subfamily, the C-terminal non-catalytic domain displayed no resemblance to that of other members of the GCK subfamily including SOK1 (35) and Sps1 (36). Thus, LOK appears to be a distinct member of the subfamily. Homology search of the nucleotide sequence of LOK non-catalytic domain identified rat AT1–46 cDNA with extensive homology to that of Sps1. Homology search of the nucleotide sequence of LOK non-catalytic domain identified rat AT1–46 cDNA with extensive homology to that of Sps1.
LOK activated none of the known MAP kinase cascades, ERK, JNK/SAPK, and p38 (44), when expressed in COS7 cells together with the MAP kinases. In contrast, the archetypal PAK subclass family members and many of GCK subclass members have been shown to activate the JNK/SAPK MAP kinase cascade, most likely via MEK1 (or MLK3) and MKK4/SEK1 (24–30). At least two possibilities can be considered to explain why LOK failed to activate the known MAP kinase cascades. Ample of evidence indicates that the STE20 family resides near the top of the MAP kinase cascades and functions as a MAP kinase kinase kinase (6). Since the expression of LOK is most likely to be expressed predominantly in lymphocytes. If this is the case, LOK could not activate the MAP kinase cascades in the ectopic system with COS7 cells in the absence of these MAPKKK and/or MAPKK downstream of LOK is also expressed predominantly in lymphocytes. In this sense it is interesting to note that several “orphan” MAP kinase cascade modules such as ERK5 and its activator MK5 have been described, in which upstream activators and down-

FIG. 5. Catalytic activity of LOK. A, COS7 cells (1 × 10^6/10-cm dish) were transiently transfected with the empty expression vector (lanes 1–3 and 8), FLAG-LOK (lanes 4–6 and 7) or FLAG-LOK(KR) in which lysine-65 is replaced by arginine (lane 9). Lysates of the transfected COS7 cells were incubated with anti-FLAG antibody (lanes 1–9). In case of WEHI 231 B cells, lysates were incubated with anti-LOK antibody (lane 10). Immunoprecipitates were subjected to the in vitro kinase assay with casein, histone IIA or MBP as substrates. B, phosphoamino acid analysis of in vitro phosphorylated LOK and histone IIA. Radioactive bands excited from dried gels prepared as shown in A were assayed as described under “Experimental Procedures.” The relative positions of unlabeled phosphoamino acids are indicated. S, serine; T, threonine; Y, tyrosine.

85% identity, and the C-terminal half of LOK shows 96% amino acid identity to AT1–46 protein (37). This gene was originally cloned from rat brain and predicted to encode a 472-amino acid protein with no kinase domain, which corresponds to the coiled-coil region of LOK. Even though the authors claimed to have obtained a full-length AT1–46 cDNA of 4320 base pairs, their Northern blot analysis revealed the presence of a single band of 5.2-kilobase transcripts hybridized with the cDNA. Moreover, the expression of the transcripts was highest in thymus and spleen and lowest in brain. Therefore, AT1–46 appears to be a part of the cDNA encoding rat homologue of mouse LOK.

Outside of the kinase domain, LOK possesses two intriguing regions that have potential to interact with other molecules. The proline-rich region with PXXP motifs found near the middle of the protein is a good candidate for the binding site of SH3-containing molecules (38, 39). PAK and NIK among the STE20 family members have been shown to associate through their proline-rich motifs with the adaptor protein Nck, which is composed of three SH3 domains and one SH2 domain and a common target for a variety of growth factor receptors (28, 40–42). It remains to be determined what kind of a SH3-containing molecule(s) is bound to the proline-rich region of LOK. The C-terminal half of LOK is predicted to form a ~400-amino acid coiled-coil structure. This region exhibits a 24% amino acid sequence identity with the intermediate filament-associated protein, trichohyalin, which associates with keratin intermediate filaments (43). Thus, the coiled-coil structure of LOK is indicative of its interaction with itself (homodimerization) or with other coiled-coil proteins (heterodimerization).
stream targets remain to be determined (45). Since at least five MAP kinase cascades have been identified in yeast (46), it would be reasonable to speculate the existence of more distinct MAP kinase cascades in mammals in addition to the known ones.

Some members of the STE20 family have been shown to become activated by stress such as inflammatory cytokines and H₂O₂ (9, 35). Therefore, we examined whether such stress can activate LOK in lymphocytes. The stimulation of lymphocytes with tumor necrosis factor α, H₂O₂, and mitogens such as lipopolysaccharide and anti-CD3 antibody failed to increase LOK kinase activity significantly, as determined by the immunocomplex kinase assay (unpublished observation). The problem in this in vitro kinase assay was that the basal activity of LOK isolated from unstimulated lymphocytes was already considerably high and might have obscured the activation of LOK. This high basal activity seems a common feature of the GCK subfamily in this assay (25, 26, 31, 33–35). It is not clear yet that this high basal activity is due to autophosphorylation and autoactivation of LOK in the presence of ATP in vitro or indeed reflects the constitutive activation of LOK in vivo. Since NIK (NFkB-B inducing kinase), a newly identified MAPKKK, has been shown to be involved in activation of NFkB (47), we explored the possibility of involvement of LOK in this activation. However, LOK did not activate NFkB, and the KR mutant of LOK did not interfere the activation of NFkB by tumor necrosis factor α, at least when analyzed in L929 fibroblast.²

Thus, possible elements upstream and downstream of LOK remain to be identified. Nevertheless, the predominant expression of LOK in lymphocytes and its distinctive non-catalytic domain make LOK very attractive among the STE20 family members. The expression of LOK not only in mature lymphocytes but also in their precursors in bone marrow and thymus may suggest an intriguing potential involvement in regulation of differentiation, proliferation, and activation of lymphocytes. To explore this possibility, we are in the process of establishing mice deficient for LOK. Furthermore, identification of molecules bound to the proline-rich motif and the coiled-coil structure of LOK should help us to understand the pathway of signal transduction through LOK.

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