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Cloning and Biochemical Characterization of LIMK-2, a Protein Kinase Containing Two LIM Domains

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We have isolated human and rat clones of the LIM motif-containing protein kinase, termed LIMK-2. LIMK-2 is related to the neuronally expressed LIM-kinase, whose hemizygous deletion appears to result in cognitive impairment in patients with Williams syndrome. The hallmark of this protein family is the presence of 1 or 2 N-terminal LIM motifs and an atypical C-terminal protein kinase domain. LIMK-2 mRNA was detected by Northern blot analysis in human tissues, most abundantly in placenta, lung, liver, and pancreas, and also in a variety of cell lines including neuronal, glioblastoma, and mammary carcinoma lines. The LIMK-2 transcript was also induced upon neuroectodermal differentiation of mouse P19 embryonal carcinoma cells. A 65 kDa recombinant LIMK-2 protein was identified in 293 cells stably transfected with a LIMK-2 expression vector. An *in vitro* kinase assay demonstrates LIMK-2 is autophosphorylated and exhibits serine/threonine kinase activity towards the exogenous substrate MBP. The endogenous 65 kDa LIMK-2 protein was detected in a variety of cell lines, and coprecipitates with a 140 kDa tyrosine phosphorylated protein, but was not itself tyrosine phosphorylated. At the subcellular level, LIMK-2 is localized in both the nucleus and in a Triton X-100 soluble fraction.

Key words: autophosphorylation, LIM motif, protein kinase, tyrosine phosphorylation.

Protein kinases play a key role in the regulation of cellular growth and differentiation. These proteins are classified in terms of the sequence identity in their catalytic domains, the presence of additional protein motifs, as well as their substrate specificity (*1*). Sequence similarities between different classes of kinases allows for the design of PCR based methods to isolate novel members of known families, as well as previously unidentified families. Using this approach on primary rat astrocytes we have cloned a novel protein kinase containing a conserved LIM amino acid motif.

LIM motifs are found in an increasing number of proteins and consist of 50-60 amino acids characterized by cysteine and histidine residues which define a zinc binding pocket (*2-4*). LIM domain containing proteins are emerging as a new family of cellular regulatory proteins, and based upon the number of LIM motifs and the presence or absence of additional domains the LIM family can be divided into 5 classes. The first group, from which the acronym LIM is derived, contains transcription factors with 2 LIM domains and a DNA binding homeodomain. The original members of this class are the *Caenorhabditis elegans* proteins lin-11 and mec-3 (*5, 6*) and the rat Isl-1 (*7*). A second class contains proteins consisting of only 1 or 2 LIM domains with no other known structural motifs, and includes the cysteine-rich intestinal protein (CRIP) as well as rhombotin (RBTM) 1 and 2, oncoproteins associated with acute T-cell leukemia (*8-10*). A third class of LIM domain containing proteins exhibits 3 LIM domains and interacts with cytoskeletal proteins. For example zyxin, a class III LIM protein, interacts with α-actinin, and paxillin, another class III LIM protein, associates with the focal adhesion kinase FAK (*11-13*). The fourth subfamily of LIM domain containing proteins consists of only one member of unknown function, Lasp-1 (MLN 50), which contains a single LIM domain and a C-terminal SH3 domain (*14*).

Recently, a fifth class of LIM domain containing proteins has been described, consisting of two members thus far. One or two LIM domains are found at the amino terminus of these proteins, while a kinase domain is present at the C-terminus. The first member of this class, LIMK-1, was cloned from a human hepatoma cell cDNA library, and subsequently rat (LIMK-1) and mouse (Limk1/Kiz-1) homologs have been identified (*15-19*). Recently, visuospatial cognitive impairment in Williams syndrome patients has been attributed to LIMK-1 hemizygosity (*20*). A second member of this LIM kinase class has been identified from rat and human sources (LIMK-2) (*18, 21*). While the predicted amino acid sequences of both family members clearly suggests the presence of a kinase domain, the catalytic specificity has been controversial, in part due to limited immunoreagents. We describe here the cloning, expression pattern and biochemical characterization of human and rat LIMK-2

MATERIALS AND METHODS

cDNA Cloning of Rat and Human LIMK-2—Degenerate PCR primers corresponding to the conserved protein kinase domains VIb and IX (*1*) were used to amplify cDNA

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fragments expressed in primary rat astrocytes. Total RNA was reverse transcribed using the BRL Superscript kit according to the manufacturer's instructions. Three novel clones were isolated, one of which was subsequently designated LIMK-2. A rat brain cDNA library and a human pancreas cDNA library were screened with the LIMK-2 insert. Several clones spanning the human LIMK-2 open reading frame were isolated and sequenced on both strands by dideoxy chain termination with Sequenase (US Biochemical).

Antibodies—A rabbit polyclonal antibody was raised against LIMK-2 peptide residues 186-203 in human LIMK-2 (NRMHISPNNRNAIHPGDR) (*21*), corresponding to a region between the LIM motifs and the kinase domain. The peptide was provided by Jim Schilling and Tom Yu (SUGEN Protein Chemistry group), and coupled to Keyhole limpet hemocyanin *via* a terminal cysteine residue and injected into rabbits. The antibody directed against hemagglutinin (HA) (clone 12CA5) was purchased from Boehringer Mannheim, and the antibody directed against phosphotyrosine, PY-20, was purchased from ICN Biochemicals.

Cell Lines and Transfections—The 293 (human embryonic kidney) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Murine P19 cells were cultured and treated with retinoic acid as described (*22*). Cath.a (CATH) and Path2 (PATH) cells were obtained from Dona Chikaraishi (Tufts University) and maintained in RPMI 1640 supplemented with 4% fetal calf serum and 8% horse serum. Additional cell lines used for RNA and protein expression analysis are PC12 rat pheochromocytoma cell line, rat Schwann cells, BT474 and MDA453 human breast carcinoma cell lines treated with TPA, U373 mg human glioblastoma cell line, Neuro2A mouse neuroblastoma cell line, and SY5Y human neuroblastoma cell line.

The human LIMK-2 isoform used for recombinant studies contained one and one-half N-terminal LIM motifs, as this form was most frequently seen in our cloning sources. To generate stable cell lines, 293 cells were transfected with DNA expression vectors for either wild type or kinase defective (lys at position 360 to arg substitution at the ATP binding domain) LIMK-2 using the calcium phosphate transfection system from GIBCO BRL, according to the manufacturer's specifications. Both constructs were tagged with a HA epitope on the C-terminus. Cells were selected in growth media containing 400 μg/ml geneticin (G418: GIBCO, BRL) and individual G418 resistant clones were analyzed for LIMK-2 expression by immunoblot. For transient expression, COS cells were transfected with an expression vector for the wild type LIMK-2 DNA using the lipofectamine reagent from GIBCO BRL according to the manufacturer's instructions.

Northern Blotting—Total RNA from cell lines was obtained either by acid-phenol extraction according to Ref. *23* or by solubilization and extraction using RNAzol B according to the manufacturer's instructions (BIOTECX). Poly-(A)$^+$RNA was selected using a Magnasphere kit from Promega. Human tissue northern blots were purchased from Clontech. RNA extracted from cell lines was fractionated by using 1.2% agarose/formaldehyde gels and transfered to Hybond-N (Amersham) paper. The resultant filters were hybridized with a ^{32}PdCTP labeled probe for rat or human LIMK-2 generated using a random prime kit from Stratagene. The human probe was derived from the N-terminal non-catalytic region of LIMK-2, whereas the rat probe was from the kinase domain. Filters were hybridized at 42°C overnight and washed in 2×SSC/0.1% SDS at 65°C as described (*22*).

Immunoprecipitation, Immunoblotting, and Gel Electrophoresis—293 cells expressing recombinant LIMK-2 were extracted with 1 ml HNTG buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) per 10 cm dish. All other cell lines were extracted with 1 ml RIPA buffer (10 mM Tris pH 7.0, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) per 10 cm dish. Both buffers contained a cocktail of protease and phosphatase inhibitors. Monolayers of cells were extracted for 15 min on ice, the extracts clarified by centrifugation in a microfuge for 10 min, and antibodies against either LIMK-2 or the HA epitope were added for 1 h at 4°C. The immune complexes were collected by addition of protein A sepharose (Pharmacia) for an additonal hour, and the immunoprecipitates were washed 3 times in solubilization buffer. The samples were analyzed on SDS-15% polyacrylamide gels and transfered to Immobilon-P (Millipore) filters by electroblotting. The filters were incubated first with a blocking solution of 5% nonfat dry milk in Tris buffered saline (TBS) for LIMK-2 and HA immunoblots, or 5% BSA fraction V in TBS for phosphotyrosine immunoblots. Filters were next incubated with primary antibodies diluted in blocking solution for 1 h at room temperature, washed 3 times in TBS with 0.1% Tween-20 (TBS-T), and subsequently incubated with either horseradish peroxidase conjugated protein A (Amersham) (LIMK-2 immunoblots) or horseradish peroxidase conjugated goat anti-mouse (Amersham) (HA and p-tyr immunoblots) for 30 min. Filters were then washed extensively in TBS-T and developed using an enhanced chemiluminescence (ECL) detection system (Amersham).

To prepare Triton soluble and insoluble fractions, cells were extracted with CSK buffer (50 mM NaCl, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 30 mM sucrose) for 15 min on ice and the extracts clarified by centrifugation in a microfuge. The supernatant was collected as the Triton-soluble fraction. The Triton-insoluble pellet was resuspended in SDS buffer (15 mM Tris pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS), boiled for 10 min and diluted to 1 ml with CSK buffer. Both fractions were immunoprecipitated as described above.

To prepare nuclear and cytoplasmic fractions, cells were scraped off the dish in PBS, washed once in RSB (10 mM Tris pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$), and resuspended in RSB with 0.5% Triton X-100 added. The cells were homogenized with 20 strokes in a glass Dounce homogenizer and the nuclei pelleted by centrifugation in a microfuge at 2,000×*g* for 10 min. The supernatant was collected as the cytoplasmic fraction. The nuclear pellet was washed 2 times in RSB and the final pellet was solubilized in RIPA buffer. Both fractions were immunoprecipitated as described above.

In Vitro Kinase Assay—LIMK-2 was immunoprecipitated from cell lysates as described above. Washed immunoprecipitates were incubated in 50 μl kinase assay buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) containing 5 mM $MnCl_2$, 10 mM $MgCl_2$, and 10

μCi γ^{32}P-ATP. Myelin basic protein (MBP) was included as a substrate at 250 μg/ml. After incubation for 30 min at room temperature the immune complexes were washed twice with kinase assay buffer containing 0.2 mM Na-ortho-vanadate, resuspended in Laemmli sample buffer, boiled, and analyzed on SDS-15% polyacrylamide gels. The gel was dried and exposed to X-ray film with an intensifying screen.

RESULTS

Sequence and Structure of LIMK-2 cDNA—Degenerate PCR based on conserved protein kinase motifs VIb and IX (*1*) was performed on primary rat astrocytes, leading to the isolation of a fragment of rat LIMK-2. A human pancreas cDNA library was screened with this insert, resulting in the isolation of several independent clones containing full or partial length cDNA inserts.

The rat and human LIMK-2 sequences concur with those now in GenBank, accession numbers D31873 and D31874 for rat and D45906 for human (*18, 21*). In addition we identify both a 638 and 617 amino acid predicted open reading frame for human LIMK-2, presumably a result of an alternatively spliced exon at the 5′ end generating a human sequence analogous to the rat LIMK-2b isoform (*18*). This 617 amino acid form was found to be more adundant in our cDNA clones and was used for subsequent expression constructs.

The basic structural features of LIMK-2 have been described in detail (*18, 21*), and consist of one and one-half or two complete N-terminal LIM motifs most closely related to those present in LIMK-1 and in the LIM homeobox domain class of proteins. The C-terminal catalytic domain is distinct among protein kinases, having features of both serine/threonine and tyrosine kinases. Within the region between the LIM and kinase domains of LIMK-2 is a 43 amino acid motif (aa 280-322) with similarity to the C-terminal half of the PDZ or DRG/DHR motif present in a variety of cytoplasmic proteins (*24*).

Expression of LIMK-2 mRNA in Human Tissues and Cell Lines—The expression pattern of LIMK-2 mRNA was analyzed by northern blot using a probe derived from the N-terminal non-catalytic domain of the human LIMK-2 cDNA. A 4 kb transcript was detected from most human tissues, with highest expression in placenta, lung, liver and pancreas and lowest expression in peripheral blood leukocytes and spleen (Fig. 1A). LIMK-2 RNA is also expressed in a variety of cell lines of mouse, rat, and human origin, including mammary carcinoma, glioblastoma, and neuronal cell lines (Fig. 1B). Furthermore, LIMK-2 expression is increased in mouse P19 embryonal carcinoma cells induced to differentiate along the neuroectodermal lineage with retinoic acid. A second transcript of approximately 1.6 kb is also detected in untreated P19 cells, and is increased upon retinoic acid treatment.

Identification of Recombinant LIMK-2 and Analysis of Protein Kinase Activity—Since the major LIMK-2 transcript observed contained one and one-half N-terminal LIM domains, we elected to characterize the biochemical activity of this isoform. An expression plasmid was constructed containing the region encoding this isoform with a C-terminal HA epitope tag. This construct was transfected into 293 cells to generate stable LIMK-2 expressing lines. Similarly we generated stable cell lines expressing a defective form of LIMK-2, in which a lys360 to arg point substitution was introduced into the ATP binding site to abolish kinase activity. Cell lysates were prepared from both cell lines and LIMK-2 proteins were immunoprecipitated with an anti-HA antibody followed by western blot analysis with an anti-peptide antibody made against the predicted amino acid sequence of LIMK-2. A LIMK-2 specific protein of approximately 65 kDa is detected in cell lines expressing both the wild type and kinase defective forms of LIMK-2, but is not detectable in untransfected control cells (Fig. 2A).

Because the primary sequence of LIMK-2 is predicted to encode a protein kinase, we performed an *in vitro* kinase assay with immunoprecipitated recombinant LIMK-2. Cell lysates were prepared from control 293 cells and 293 cells

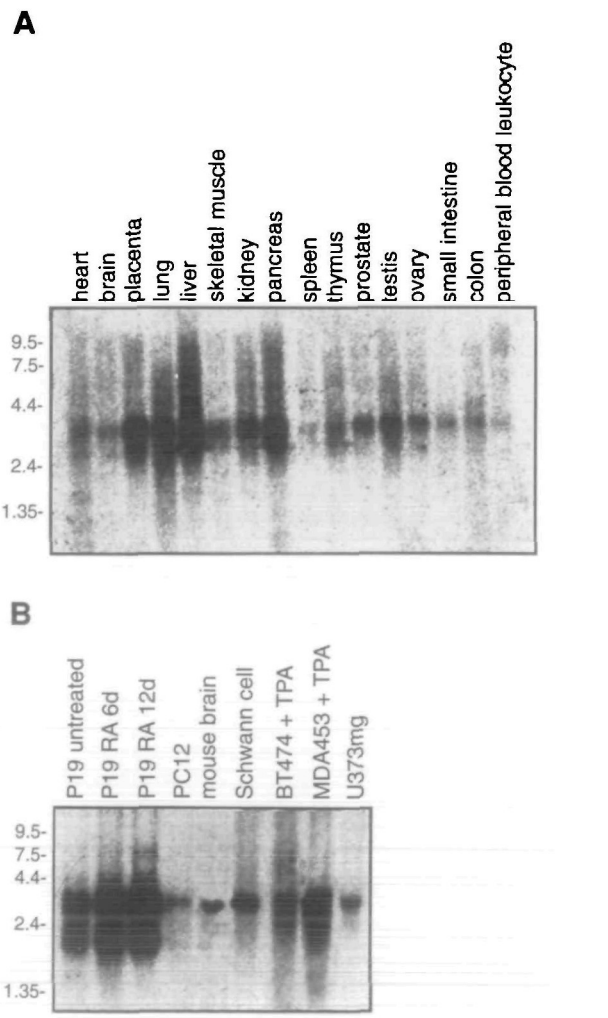

Fig. 1. **Northern blot analysis of LIMK-2 RNA expression in human tissues and mouse, rat, and human cell lines.** Northern blots were hybridized with a human LIMK-2 probe. A: Human tissue blot (Clontech) contains 2 μg poly(A)$^+$ RNA per lane. B: 2 μg poly-(A)$^+$ RNA was loaded in P19 untreated and retinoic acid (RA) treated lanes, 10 μg total RNA was loaded in all other lanes. Samples are: P19 mouse embryonal carcinoma cells, PC12 rat pheochromocytoma cells, rat Schwann cells, BT474 and MDA453 human breast carcinoma cells treated with TPA, U373mg human glioblastoma cells. Position of molecular weight markers (kbp) are indicated on the left.

expressing either the epitope-tagged wild type or kinase defective forms of LIMK-2, and LIMK-2 protein was immunoprecipitated using an anti-HA antibody. The *in vitro* kinase assay was performed both with and without myelin basic protein (MBP) as a substrate. Auto-phosphorylation of LIMK-2 was observed in immunoprecipitates from cells expressing wild type LIMK-2, both with and without MBP, although less in the presence of MBP (Fig. 2B). No phosphorylation of the kinase defective form of LIMK-2 was detected either with or without MBP included in the reaction (Fig. 2B). Aliquots of each immunoprecipitate were analyzed by HA immunoblot, with equivalent levels of protein detected in each sample (data not shown). In addition to autophosphorylation, immunoprecipitates of wild type LIMK-2 but not the kinase defective LIMK-2 were capable of phosphorylating MBP. An *in vitro* kinase assay with both wild type and kinase defective LIMK-2 was also performed without ^{32}P and then analyzed by immunoblot with anti-phosphotyrosine antibody. No tyrosine phosphorylation of either LIMK-2 itself or the MBP substrate was detected (data not shown).

Identification of Endogenous LIMK-2 and an Associated Phosphoprotein—We analyzed the expression of endogenous LIMK-2 protein in cell lines of mouse, rat, and human origin, chosen for analysis based on RNA expression data. Extracts of each cell type were immunoprecipitated with a LIMK-2 specific antibody and the immunoprecipitates were subjected to immunoblot analysis with the same antibody. A native LIMK-2 protein of 65 kDa, similar in size to the epitope tagged recombinant protein, was specifically detected in every cell line examined, including neuronal, glial, and breast carcinoma lines (Fig. 3A). The smaller 50 kDa protein detected in some samples is due to cross reactivity with the antibody heavy chain. In some cell lines LIMK-2 migrates as a doublet (*e.g.* PC12) and in the case of P19, the pattern (single band to doublet) changes upon retinoic acid induced neuroectodermal differentiation (Fig. 3A).

As a first approach to characterizing the endogenous LIMK-2 protein, we determined if it is phosphorylated on tyrosine when isolated from cell extracts. The LIMK-2 antibody was stripped from the western blot in Fig. 3A and the blot was reprobed with an anti-phosphotyrosine antibody. While LIMK-2 itself is not recognized by the anti-phosphotyrosine antibody, it appears to co-precipitate with a tyrosine phosphoprotein of approximately 140 kDa in every cell line examined except COS cells transfected with a LIMK-2 expression vector and MDA453. However in the latter breast carcinoma cell line LIMK-2 did associate with a larger 200 kDa tyrosine phosphoprotein (Fig. 3B). The identity of the LIMK-2-associated tyrosine phosphoprotein is unknown, although LIMK-2 did not appear to

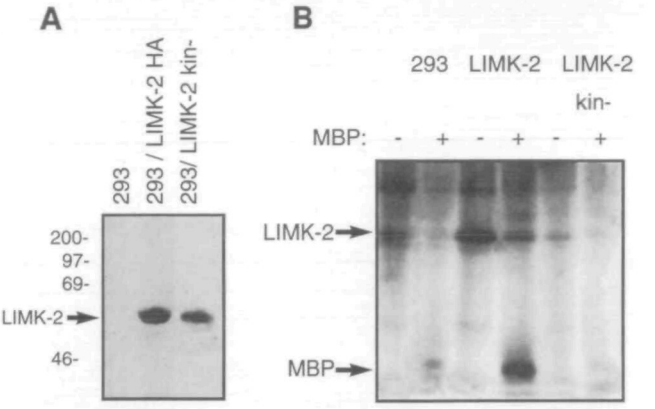

Fig. 2. **LIMK-2 overexpression in 293 cells and *in vitro* kinase activity.** A: HA-tagged LIMK-2 proteins were immunoprecipitated from either control 293 cells (293), 293 cells overexpressing wild type LIMK-2/HA (293/LIMK-2 HA), or 293 cells overexpressing kinase defective LIMK-2/HA (293/LIMK-2 kin-) with an anti-HA antibody and immunoblotted with an anti-LIMK-2 antibody. B: LIMK-2/HA proteins were immunoprecipitated from the indicated cell lines with an anti-HA antibody and subjected to an *in vitro* kinase reaction in the presence or absence of MBP. The positions of phosphorylated MBP and LIMK-2 are indicated.

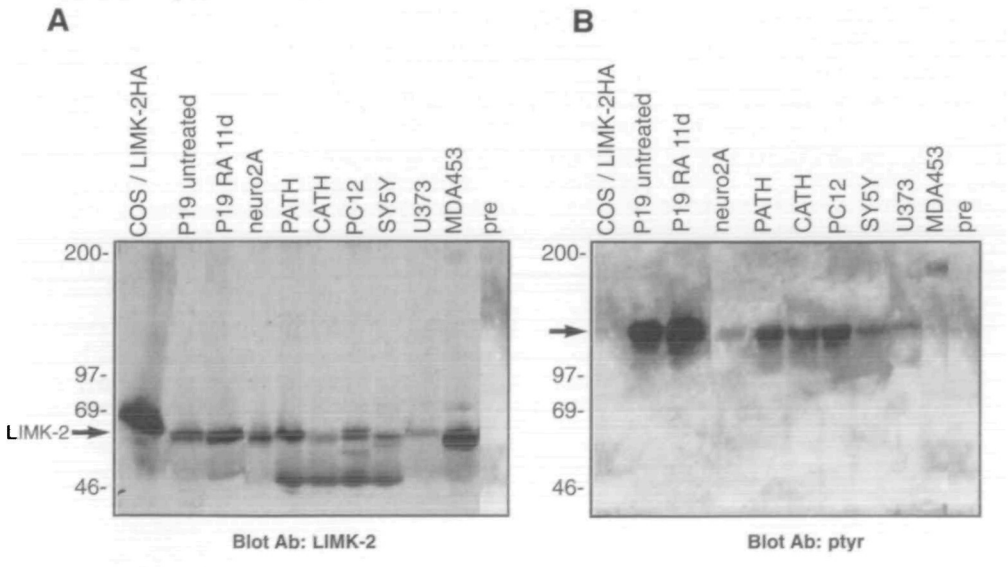

Fig. 3. **Endogenous LIMK-2 protein expression and association of tyrosine phosphoprotein.** A: Cell extracts were immunoprecipitated and immunoblotted with an anti-LIMK-2 antibody. The position of LIMK-2 is indicated on the left. The samples are: COS/LIMK-2 HA: COS cells transiently transfected with LIMK-2/HA; P19, untreated and retinoic acid (RA) treated for 11 days; Neuro2A mouse neuroblastoma cell line; PATH and CATH: rat adrenal (Path2) and brain (Cath.a) dopaminergic cells lines (*33*); PC12 rat pheochromocytoma cell line; SY5Y human neuroblastoma cell line; U373 human glioblastoma cell line; MDA453 human breast carcinoma cell line; pre, immunoprecipitate of P19 cell extract using LIMK-2 pre-immune serum. B: The blot from (A) was stripped and reprobed with an anti-phosphotyrosine antibody. The indicated band is aproximately 140 kDa. Molecular weight markers (kDa) are indicated on the left.

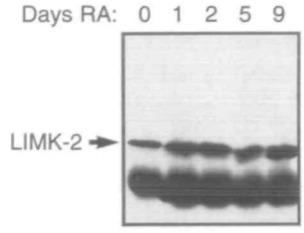

Fig. 4. **Endogenous LIMK-2 protein expression during P19 RA differentiation.** P19 cells were induced to differentiate with RA for the days indicated. Cell lysates were immunoprecipitated and subjected to immunoblotting with an anti-LIMK-2 antibody.

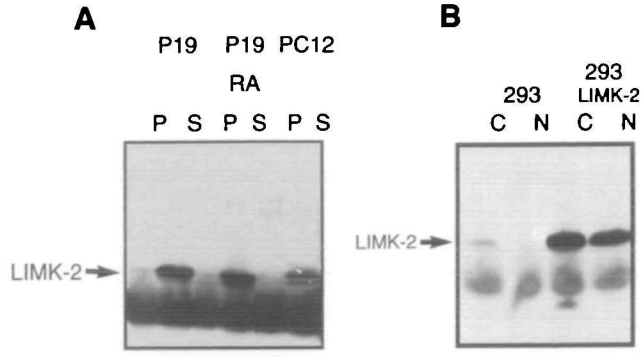

Fig. 5. **Subcellular localization of endogenous LIMK-2 protein.** A: Triton X-100 insoluble pellet (P) and soluble (S) cell fractions were prepared from untreated P19 cells (P19), P19 cells treated with RA for 10 days (P19 RA), and PC12 cells. Lysates were immunoprecipitated and immunoblotted with an anti-LIMK-2 antibody. B: Cytoplasmic (C) and nuclear (N) fractions were prepared from control and LIMK-2 transfected 293 cells. Cell lysates were immunoprecipitated and immunoblotted with an anti-LIMK-2 antibody.

co-precipitate with p120, β-catenin, or tyro3, tyrosine phosphoproteins of similar size (data not shown) (*25-28*).

LIMK-2 protein was detected as a single band in undifferentiated P19 cells but was identified as a doublet after 11 days of retinoic treatment (Fig. 3A). To determine more precisely the time of appearance of the LIMK-2 doublet during neuroectodermal differentiation, cell lysates were prepared from a time course of P19 cells induced to differentiate with retinoic acid for 0-9 days. These extracts were immunoprecipitated with a LIMK-2 specific antibody and subjected to Western immunoblot analysis with the same antibody. A single band is detected in undifferentiated P19 cells as well as cells treated for 1 or 2 days with retinoic acid (Fig. 4). By 5 days of retinoic acid treatment, however, LIMK-2 is detected as a doublet, which persists through 9 days of differentiation. Note that the 140 kDa associated tyrosine phosphoprotein in present in extracts from undifferentiated as well as retinoic acid treated cells (Fig. 3B).

LIMK-2 Protein Resides in Soluble and Nuclear Fractions—The sub-cellular localization of LIMK-2 protein was determined by examining Triton soluble and insoluble cell extracts from cells in which LIMK-2 had been detected as a doublet (see Fig. 3A). The extracts were prepared from P19 cells, either untreated or induced to differentiate with retinoic acid, as well as from PC12 cells. LIMK-2 protein was detected by specific immunoprecipitation followed by immunoblotting. LIMK-2 protein was localized entirely to a Triton X-100 soluble fraction in all the cell extracts (Fig. 5A), suggesting that it is not associated with insoluble cytoskeletal elements. In addition, both species of the LIMK-2 protein were localized to the same fraction, and there was no detectable change in localization during P19 retinoic acid induced differentiation.

Because the kinase domain of LIMK-2 contains a short, highly basic insert region which has been suggested to be a nuclear localization signal (*15*), and because other LIM domain containing proteins are nuclear proteins, we sought to determine whether LIMK-2 was in the nucleus. Nuclear and cytoplasmic fractions were prepared from untransfected 293 cells and from 293 cells expressing wild type LIMK-2. These extracts were examined for LIMK-2 protein by immunoprecipitation followed by immunoblot analysis. LIMK-2 protein was detected in both cytoplasmic and nuclear fractions of both cell lines (Fig. 5B).

DISCUSSION

In the present study we have used a PCR based cloning strategy to isolate a novel protein kinase containing LIM domains which we designate as LIMK-2. The human sequence and the rat LIMK-2 orthologue have recently been reported (*18, 21*). The LIMK-2 cDNA was originally cloned from primary astrocytes, however Northern blot and immunoblot analyses show that it is expressed in a wide variety of tissues and cell types, including but not limited to those of neuronal origin. The related rat LIMK-1 and mouse Limk1/Kiz-1 genes are expressed predominantly in neural tissue (*15-17, 19*) while rat LIMK-2 is ubiquitously expressed (*18*), similar to the pattern we and others describe for human LIMK-2 (*21*). The distinct patterns of expression observed for the two LIM kinase family members suggests they may regulate discrete cellular processes.

The LIMK-2 sequence has motifs predictive of both tyrosine kinases and serine/threonine kinaes; however, attempts to demonstrate tyrosine phosphorylation of LIMK-2, isolated from cells grown under many growth factor and agonist stimuli, have been unsuccessful. In this report we have demonstrated autophosphorylation of LIMK-2, and also confirm other reports of its phosphorylation of the exogenous substrate MBP. Phosphotyrosine immunoblotting of the *in vitro* kinase reactions, including phosphorylated MBP, detected no tyrosine phosphoproteins. In other studies, phosphoamino acid analysis of autophosphorylated mouse LIMK-1, from an *in vitro* kinase reaction, demonstrated phosphorylation predominantly on serine, although one group also detected phosphotyrosine (*17*), while another group detected phosphothreonine (*19*). In addition, phosphoamino acid analysis of MBP, used as a substrate by rat LIMK-1 and hLIMK-1 and -2, demonstrates phosphorylation mainly on serine, with less on threonine, but no phosphotyrosine was detected (*18, 21*). Taken together these data suggest that the predominant kinase activity of LIM kinases, both auto- and substrate phosphorylation, is directed toward serine residues.

Although we have not detected tyrosine phosphorylation of LIMK-2 isolated from cells under a variety of conditions, suggesting LIMK-2 does not itself have tryosine kinase

activity nor is it a substrate for a tyrosine kinase, we have identified a co-precipitating tyrosine phosphoprotein of approximately 140 kDa in nearly every cell line in which LIMK-2 is endogenously expressed. Interestingly a 140 kDa phosphoprotein has also been found associated with hLIMK-1 (*21*). Based on the molecular weight of the associated phosphoprotein, we checked two proteins associated with an E-cadherin mediated adhesion complex, β-catenin and p120, both known to be tyrosine phosphorylated (*26, 27, 29*). Neither of these proteins correspond to the 140 kDa protein associated with LIMK-2. LIM domains are known to mediate protein-protein interactions, in some cases through homophilic interactions *via* the LIM domain. A single LIM domain in the chicken cytoskeletal protein zyxin has been shown to be necessary and sufficient to associate with a LIM domain in the chicken CRP protein (*30*). At the present time there are no known LIM domain proteins in the size range of the tyrsoine phosphoprotein associated with LIMK-2. It is possible that LIMK-2 associates either with other novel LIM domain proteins or other non-LIM proteins, thus participating in signal transduction processes as part of a multi-protein complex. The spacer region of LIMK-2, which shows similarity to the C-terminal half of the PDZ or DRG/DHR motif, is another potential region for protein-protein interactions involving LIMK-2. A similar partial PDZ motif has been described in *Caenorhabditis elegans* lin-2A, *Drosophila* cmg, and rat CASK (*24, 31*).

Since there is no detectable change in the phosphorylation state or kinase activity of LIMK-2 following a variety of signal transduction stimuli attempted (growth factors, calcium ionophore, phorbol ester treatment), it is difficult to link LIMK-2 to any known signal transduction pathways. Therefore we sought to gain some insight into the function of LIMK-2 by determining its subcellular localization. LIMK-2 was found exclusively in a Triton soluble fraction of cell extracts, making it unlikely that it associates with cytoskeletal elements similar to other LIM proteins such as zyxin and paxillin (*11, 13*). The detection of LIMK-2 in a nuclear fraction of cell extracts supports the identification of a nuclear localization signal in the kinase insert region. A nuclear localization signal has also been noted for human LIMK (*15*). Kiz-1 has also been shown by immunofluoresence to be in the nucleus of neurons (*16*). Other non-kinase LIM proteins are also nuclear, although many are transcription factors which possess a DNA binding domain distinct from their LIM domain (*5-7*). Although the LIM domain is a zinc binding domain with a predicted finger-like structure, there is to date no evidence that the LIM domain itself can bind DNA (*32*).

The accumulating evidence suggests that LIM domain proteins, including the novel member of the kinase family described here, contain a newly characterized sequence motif that facilitates their involvement in signal transduction processes. The determination of specific protein-protein interactions involving LIM proteins will allow the identification of either existing or novel pathways through which LIM proteins function to regulate cellular processes.

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