Self-association of LIM-kinase 1 mediated by the interaction between an N-terminal LIM domain and a C-terminal kinase domain

Junji Hiraoka[a], Ichiro Okano[a], Osamu Higuchi[a], Neng Yang[a], Kensaku Mizuno[a,b,*]

[a]*Department of Biology, Faculty of Science, Kyushu University, Hakozaki, Fukuoka 812-81, Japan*
[b]*PRESTO, Japan Science and Technology Corporation (JST), Seika-cho, Kyoto 619-02, Japan*

Received 1 October 1996; revised version received 1 November 1996

Abstract LIM-kinase 1 (LIMK1) and 2 (LIMK2) are members of a novel class of protein kinases containing two LIM motifs at the N-terminus. The LIM motif is thought to be involved in protein-protein interactions. We report here evidence that LIMK1 self-associates and also associates with LIMK2. In vivo and in vitro binding analyses using variously deleted mutants of LIMK1 revealed that the self-association of LIMK1 was caused by interaction between the N-terminal LIM domain and the C-terminal kinase domain. The association of LIMK1 with itself and with LIMK2 is important for understanding how activities and functions of LIMK family kinases are regulated.

Key words: Protein kinase; LIM motif; LIMK; Dimerization

1. Introduction

Protein kinases play a central role in intracellular signal-transducing pathways which regulate cell growth, differentiation and other responses. We recently identified a novel class of protein kinases, termed LIM kinases (LIMKs), composed of LIMK1 and LIMK2 [1–4]. These two kinases share characteristic structural features, consisting of the N-terminal two LIM motifs, the internal PDZ (also called DHR or GLGF)-like domain, and the C-terminal protein kinase domain. The LIM motif is a structural motif composed of two adjacent zinc fingers separated by a 2-amino acid linker [5,6]. The PDZ domain is a 90–100 amino acid motif found in a diverse set of membrane-bound proteins and enzymes [7,8]. Both of these domains were characterized as the binding surfaces of protein-protein interactions [5–8], and may be involved in regulation of the kinase activity or in the subcellular localization of LIMKs. The C-terminal kinase domains of LIMKs contain a consensus sequence of protein kinases, but are unique in that they have an unusual sequence motif (DLNSHN) in the kinase catalytic loop in subdomain VIB and a highly basic kinase insert between subdomains VII and VIII [1–4]. The unique structural features of these kinases suggest their specific roles in previously uncharacterized signaling pathways. LIMK1 is highly expressed in the brain in developing and adult mammals [1–4,9–11]. The LIMK1 gene is located on human chromosome 7q11.23 [3], and a recent report suggests that it is a gene linked to the impairment of visuospatial cognition in patients of Williams syndrome [12]. The cellular functions and mechanisms of regulation of LIMK family kinases remain unknown.

Several protein kinases form a homotypic dimer or oligomer to regulate their activity [13–16]. As the LIM and PDZ domains are thought to be involved in protein-protein interactions, and in some cases in homophilic interactions [17,18], we asked whether LIMKs would self-associate through these domains. We now provide evidence that LIMK1 self-associates and also associates with LIMK2. We also show that the self-association of LIMK1 is mediated by interactions between the N-terminal LIM domain and the C-terminal protein kinase domain.

2. Materials and methods

2.1. Plasmid construction

LIMK1 mutants used in this study are schematically shown in Fig. 1. pUC-SRα expression plasmids encoding LIMK1-HA and LIMK2-HA were constructed to contain the epitope peptide (YPYDVPDYA) of influenza virus haemagglutinin (HA) at the C-terminus of human LIMK1 (1–640) and LIMK2 (1–629), respectively. The full-length coding sequence of human LIMK1 cDNA was inserted into pBluescript (Stratagene), using a *Not*I linker (pBS-LIMK1). To generate the plasmid encoding LIMK1-HA, the cDNA fragment was amplified by polymerase chain reaction (PCR), using the upper primer 5′-GACAGCCAGTACCCA-3′ and the lower primer 5′-GCTCTAGAGGCCTCAGGCCATAGTCGGGGACGTCATAGGGGGGTAAGGCAGTCCGCTCTCGCC-3′ (containing an HA epitope, a stop codon and *Xba*I site). The PCR-amplified fragment was digested with *Sph*I and *Xba*I and ligated into the *Sph*I, *Xba*I-digested pBS-LIMK1, to replace the original *Sph*I-*Xba*I fragment of LIMK1 cDNA with the HA-tagged fragment. The resulting plasmid was cut with *Not*I, and the insert was subcloned into the pUC-SRα vector [19] to produce pUC-SRα-LIMK1-HA. To construct the expression plasmid pUC-SRα-ΔLIM-HA, pUC-SRα-LIMK1-HA was digested with *Bal*I and *Bst*EII and ligated with a *Hin*dIII linker. To construct the expression plasmid pUC-SRα-ΔInt-HA, pUC-SRα-LIMK1-HA was digested with *Bst*EII and *Pst*I and ligated. To construct the plasmid pUC-SRα-ΔK-HA, pUC-SRα-LIMK1-HA was digested with *Pst*I and *Sph*I and the *Pst*I-*Sph*I fragment within the LIMK1 sequence was removed. The plasmid coding for glutathione *S*-transferase (GST)-fused LIMK1 was constructed by inserting *Hin*cII-digested LIMK1 cDNA into the *Sma*I site of pGEX-2T (Pharmacia). The plasmid coding for GST-fused LIMK1 (4–216) (GST-N) was generated by self-ligation of the *Eco*RI-digested pGEX-LIMK1. The plasmid coding for GST-LIM was generated by digestion of pGEX-LIMK1 with *Bst*EII and *Sph*I and ligation with an *Eco*RI linker. The authenticity of the expression plasmids was confirmed by nucleotide sequence analysis.

2.2. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with the plasmid DNA using the DEAE-dextran-chloroquine method, as described [3].

2.3. Immunoprecipitation and immunoblotting

2 days after transfection, the cells were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM NaF, 1 mM

*Corresponding author. Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-81, Japan. Fax: (81) (92) 642-2645. E-mail: kmizuscb@mbox.nc.kyushu-u.ac.jp

Abbreviations: LIMK, LIM-containing protein kinase; HA, haemagglutinin; GST, glutathione *S*-transferase.

PII S0014-5793(96)01303-8

Na_3VO_4, 50 μM $ZnCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml antipain and 2 μg/ml leupeptin. After centrifugation, lysates were precleared with Protein A-Sepharose (Pharmacia) for 1 h at 4°C. The precleared supernatants were incubated with rabbit anti-LIMK1 polyclonal antibody (LK1-C) [3] and Protein A-Sepharose for 2 h at 4°C. After centrifugation, the immunoprecipitates were washed three times with washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 50 μM $ZnCl_2$, 1 mM DTT and 0.5% Nonidet P-40) and used for immunoblot analysis. Immunoblot analysis was performed as described [3], except that rabbit anti-HA polyclonal antibody (HA.11, Babco) was used as the primary antibody in some experiments.

2.4. In vitro binding assay

The plasmids coding for GST fusion proteins were transformed into *Escherichia coli* XL1-blue. Expression of GST fusion proteins was induced by 1 mM isopropyl thiogalactopyranoside (IPTG). Bacteria were collected 90 min after the addition of IPTG and suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 50 μM $ZnCl_2$, 1 mM DTT, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 2 μg/ml antipain and 2 μg/ml leupeptin. After standing on ice for 15 min, suspensions were sonicated and cleared by centrifugation at $12000 \times g$ for 20 min. The supernatants were added to glutathione-Sepharose (Pharmacia) and incubated for 2 h at 4°C. The beads were washed three times with washing buffer and used for in vitro binding assay. The lysates of COS cells expressing HA-tagged LIMK1 mutants were precleared with glutathione-Sepharose and incubated for 1 h at 4°C with GST fusion proteins immobilized on glutathione-Sepharose, prepared as above. Beads were washed three times with washing buffer and the bound proteins were solubilized in SDS sample buffer at 95°C for 10 min, run on SDS-PAGE and analyzed by western blotting with anti-HA antibody.

3. Results

3.1. Association of LIMK1 with itself and with LIMK2

To examine the self-association of LIMK1, we constructed two expression plasmids, one coding for non-tagged, full-length LIMK1 (1–647) and the other coding for C-terminally HA-tagged LIMK1 (1–640) (termed LIMK1-HA) (see Fig. 1). To avoid the cross-reaction with anti-LIMK1 antibody, the C-terminal 7 amino acid residues (corresponding to LIMK1 (641–647)) were deleted from the LIMK1-HA construct. As shown in Fig. 2, anti-LIMK1 antibody raised against the C-terminal peptide of human LIMK1 immunoprecipitated and detected non-tagged LIMK1 protein expressed in COS cells, but not HA-tagged LIMK1 (1–640). Conversely, anti-HA antibody immuno-precipitated and detected HA-tagged LIMK1 (1–640), but not non-tagged LIMK1. Thus, anti-LIMK1 and anti-HA antibodies specifically recognized the epitopes at the C-termini of intact and HA-tagged LIMK1, respectively, and cross-reactivity was not observed.

When LIMK1 and LIMK1-HA were co-expressed in COS cells and LIMK1 was immunoprecipitated with anti-LIMK1 antibody, LIMK1-HA co-precipitated with LIMK1, which was detected by immunoblot analysis with anti-HA antibody (Fig. 3A, upper panel, lane 3). Co-precipitation of LIMK2-HA with LIMK1 was also observed in a similar experiment (Fig. 3A, upper panel, lane 4). Expression of LIMK1, LIMK1-HA or LIMK2-HA in each experiment was confirmed by immunoblot analyses (Fig. 3A, middle and lower panels). Together these findings suggest that LIMK1 self-associates to form a dimer or a multimer in the cell and that it can also associate with LIMK2 to form a heterotypic complex. Based on the amounts of LIMK1-HA and LIMK2-HA in the cell lysates, as estimated by direct immunoblot analysis with anti-HA antibody, about 1.6% of LIMK1-HA and about 3.7% of LIMK2-HA expressed in COS cells co-precipitated with LIMK1.

3.2. Localization of the domains of LIMK1 required for self-association

To localize the domains of LIMK1 required for self-association, we constructed expression plasmids encoding non-tagged or HA-tagged version of ΔK, a LIMK1 mutant with a deletion of the C-terminal kinase domain (amino acid residues 297–610) (see Fig. 1). The non-tagged and HA-tagged versions of the full-size LIMK1 or ΔK were mutually co-transfected into COS cells, and the cell lysates were analyzed by immunoprecipitation with anti-LIMK1 antibody, followed

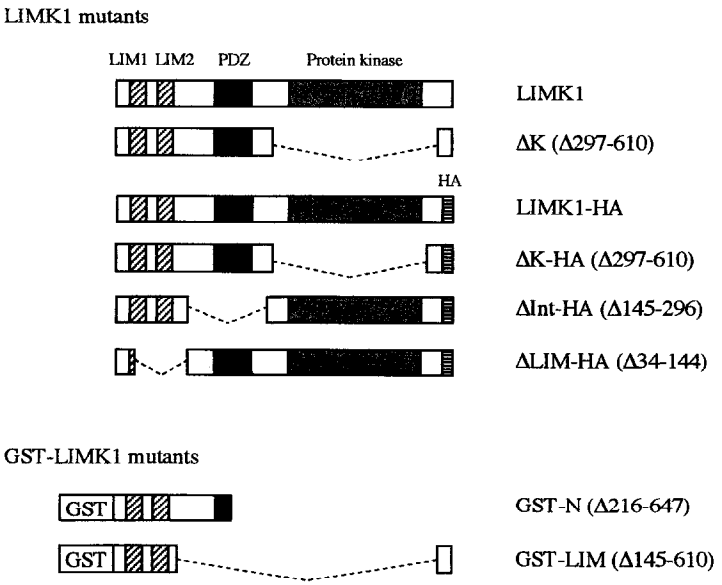

Fig. 1. Diagram showing structures of human LIMK1 and various mutants used in the present study. The hatched boxes represent LIM domains; black boxes, PDZ (DHR) domains; gray boxes, protein kinase domains; and horizontally striped boxes, HA-tag peptides. The deleted residues in each mutant are indicated in parentheses.

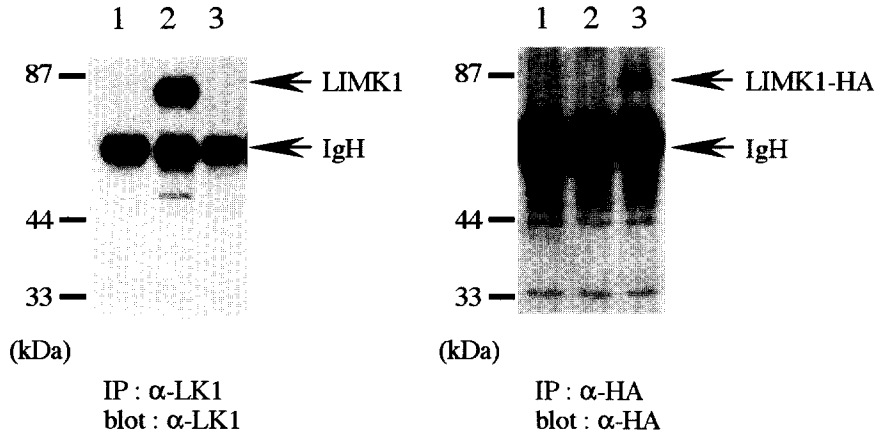

Fig. 2. Specificity of anti-LIMK1 and anti-HA antibodies. COS-7 cells were transfected with the pUC-SRα expression vector (lane 1) or the vector encoding either LIMK1 (lane 2) or LIMK1-HA (lane 3). Cell lysates were immunoprecipitated with anti-LIMK1 (α-LK1) or anti-HA epitope (α-HA) antibody, run on SDS-PAGE and immunoblotted with the same antibodies, as indicated. Arrows indicate the predicted positions of LIMK1, LIMK1-HA and immunoglobulin heavy chain (IgH). The positions of molecular weight marker proteins are indicated on the left. IP, immunoprecipitation.

by immunoblotting with anti-HA antibody. As shown in Fig. 3B (top panel), LIMK1-HA co-precipitated with LIMK1 and ΔK, and ΔK-HA co-precipitated with LIMK1. On the other hand, co-precipitation of ΔK-HA with ΔK was never evident. These observations suggest that the N-terminal half of LIMK1 is involved in the self-association of LIMK1, but this association is not mediated by homotypic interactions between the N-terminal halves of LIMK1 molecules, rather

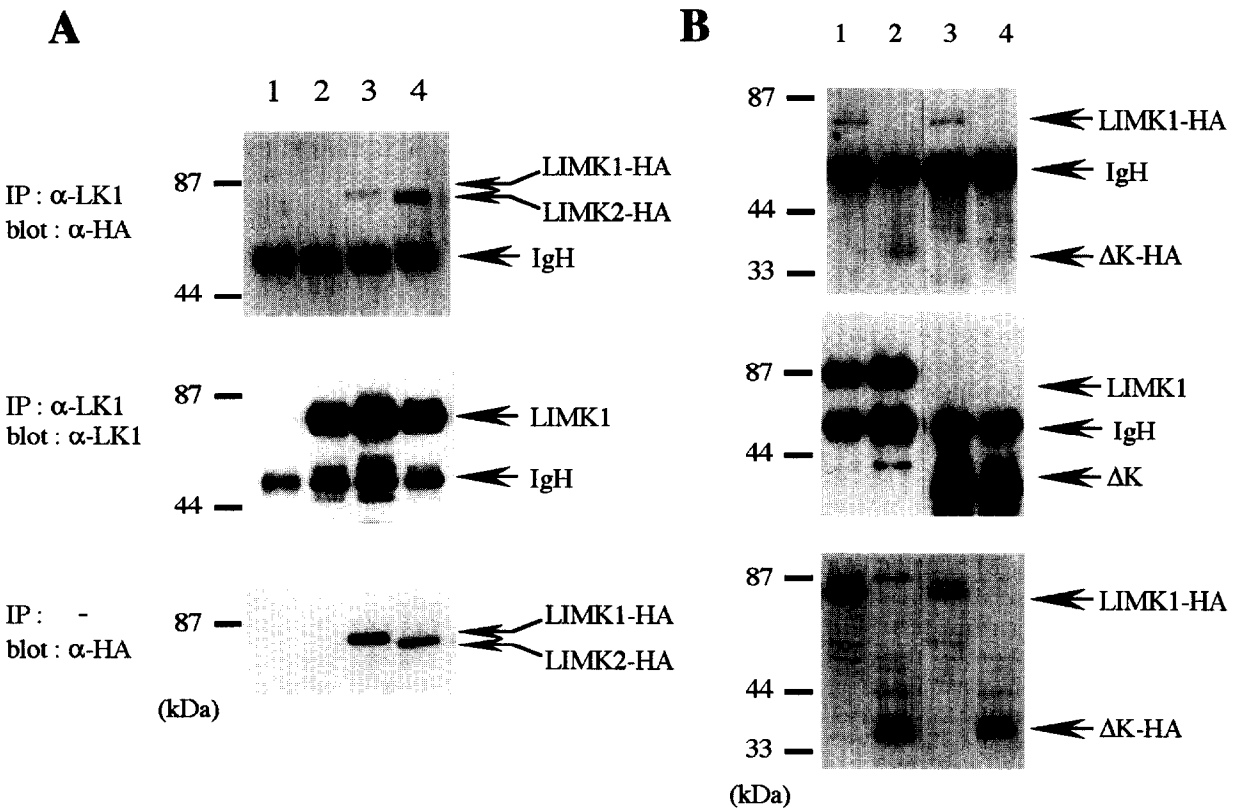

Fig. 3. Self-association of LIMK1 and association between LIMK1 and LIMK2 in COS cells. (A) Co-immunoprecipitation of LIMK1-HA and LIMK2-HA with LIMK1. COS-7 cells were transfected with SRα vector alone (lane 1) or co-transfected with LIMK1+SRα vector (lane 2), LIMK1+LIMK1-HA (lane 3), or LIMK1+LIMK2-HA (lane 4). Cell lysates (0.3 ml each) were immunoprecipitated with anti-LIMK1 antibody, run on SDS-PAGE and blotted with anti-HA antibody (upper panel) or anti-LIMK1 antibody (middle panel). Initial cell lysates (10 μl) were also directly run on SDS-PAGE and immunoblotted with anti-HA antibody (lower panel). IgH indicates the position of immunoglobulin heavy chain. (B) Co-precipitation analyses of LIMK1 and ΔK. COS-7 cells were co-transfected with LIMK1+LIMK1-HA (lane 1), LIMK1+ΔK-HA (lane 2), ΔK+LIMK1-HA (lane 3), or ΔK+ΔK-HA (lane 4). The cell lysates were immunoprecipitated and immunoblotted, as in (A). The positions of molecular weight marker proteins are indicated on the left.

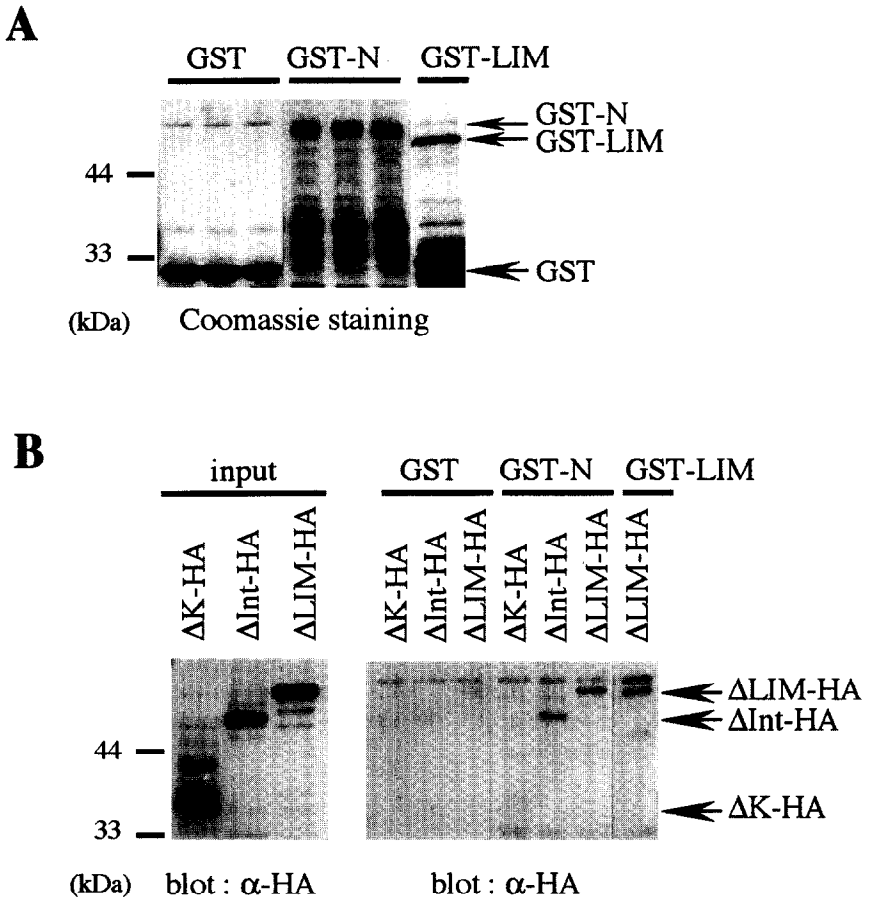

Fig. 4. In vitro binding analyses to localize the domains required for LIMK1 self-association. (A) Expression of GST fusion proteins. GST, GST-N and GST-LIM were expressed in *E. coli*, purified by glutathione-Sepharose and run on SDS-PAGE. Proteins were visualized with Coomassie blue staining. (B) In vitro binding assay. Cell lysates of COS cells transfected with the indicated plasmids were run on SDS-PAGE and immunoblotted with anti-HA antibody (input). The HA-tagged LIMK1 mutants expressed in COS cells were incubated with GST, GST-N or GST-LIM bound to glutathione-Sepharose. The bound materials were run on SDS-PAGE and analyzed by immunoblotting with anti-HA antibody (GST, GST-N and GST-LIM). The positions of molecular weight marker proteins are indicated on the left.

interactions occur between the N-terminal and C-terminal halves of LIMK1.

To further localize regions required for LIMK1 self-association, a series of HA-tagged LIMK1 deletion mutants was expressed in COS cells and the potential to interact with the GST fusion protein of the N-terminal half (residues 4–215) of LIMK1 (GST-N) was examined. The HA-tagged LIMK1 mutants used are shown in Fig. 1; ΔLIM-HA, ΔInt-HA and ΔK-HA represent the HA-tagged LIMK1 mutants lacking the LIM domain (amino acid residues 34–144), the internal region containing PDZ-like domain (residues 145–296), and the kinase domain (residues 297–610), respectively. GST-N and GST were immobilized on glutathione-Sepharose and incubated with the HA-tagged LIMK1 mutants, then the pellets were analyzed by immunoblotting with anti-HA antibody. As shown in Fig. 4B, GST-N bound the mutants lacking the LIM domain (ΔLIM-HA) and the internal PDZ-like domain (ΔInt-HA), but did not bind ΔK-HA. There was no binding of any of the HA-tagged LIMK1 mutants to the control GST. We also detected binding of ΔLIM-HA with GST-LIM, a GST fusion protein of LIMK1 with deletion of residues 145–610 (Figs. 1 and 4B). Taken together, these results indicate that the N-terminal LIM domain interacts with the C-terminal kinase domain and the self-association of LIMK1 can probably be explained by this interaction.

4. Discussion

LIM domains are found in a variety of proteins, including transcription factors, cytoskeletal proteins, protein kinases and other signaling proteins [5,6]. Other studies provided evidence that the LIM domains are involved in protein-protein

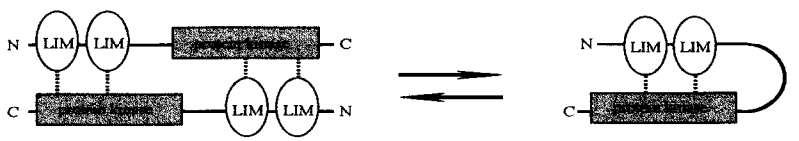

Fig. 5. A putative model for the self-association of LIMK1. LIMK1 may exist in a monomer and in a dimer form, in which the N-terminal LIM domain associates with the C-terminal kinase domain intramolecularly and intermolecularly, respectively.

interactions. For example, cysteine-rich protein (CRP) associates with itself and with zyxin, through LIM-LIM interactions [17,20]. The LIM domains of Enigma recognize the tyrosine-containing short sequence motifs in the insulin receptor and Ret receptor tyrosine kinase [21,22]. The LIM domains of LIM only protein-1 and -2 (LMO-1 and LMO-2) interact with the basic helix-loop-helix domains of TAL1, TAL2 and LYL1 [23]. In the present study, we showed that the LIM domain of LIMK1 probably interacts with the protein kinase domain. Thus, the LIM domains act as conserved protein-binding modules in various signaling systems. However, no apparent sequence similarity has been found within the targets for the LIM domains, suggesting that the individual LIM domains likely have diverse binding specificities. This is in contrast to the SH2 and SH3 domains, which also function as protein binding modules in various signaling pathways but do have narrow target specificities to short sequence motifs, the phosphotyrosine-containing and proline-rich motifs, respectively [24]. The mechanisms by which the LIM domains recognize such diverse targets will be elucidated by ongoing studies on refinement of target sequences and three-dimensional structure analyses of LIM-target complexes.

As seen in Raf, Akt, double-strand RNA-dependent protein kinase (PKR) and receptor tyrosine kinases, dimerization or oligomerization of protein kinases leads to promotion of their kinase activity [13–16]. However, it is not clear at present whether this is also the case for LIMK1, since the kinase activity of the monomer form and the multimer form of LIMK1 must be separated and measured separately.

The binding potential of the N-terminal LIM domain to the C-terminal kinase domain suggests that this interaction may also function intramolecularly, as illustrated in Fig. 5. In the cases of LIM-homeodomain proteins such as Mec-3, Isl-1 and Xlim-1, it was proposed that the LIM domain interacts with the homeodomain within the molecule and that this interaction inhibits the DNA binding activity of the homeodomain [5,25–27]. This inhibitory effect is thought to be released by association of the LIM domain with other activator proteins [5]. In a similar manner, the intra- and intermolecular interactions between the LIM domain and the protein kinase domain of LIMK1 may negatively regulate its catalytic function, and LIMK1 may be activated by intermolecular interactions of the LIM domain with other activator proteins. In order to clarify mechanisms of the kinase activity of LIMK1, the kinase activity of LIMK1 mutants destroyed in the LIM domain will have to be determined and the LIM-binding proteins will need to be identified.

Acknowledgements: We thank Drs. K. Toyoshima and Y. Fujiki for encouragement, and M. Ohara for critical comments. This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan and the Japan Science and Technology Corporation.

References

[1] Mizuno, K., Okano, I., Ohashi, K., Nunoue, K., Kuma, K., Miyata, T. and Nakamura, T. (1994) Oncogene 9, 1605–1612.
[2] Ohashi, K., Toshima, J., Tajinda, K., Nakamura, T. and Mizuno, K. (1994) J. Biochem. 111, 636–642.
[3] Okano, I., Hiraoka, J., Otera, H., Nunoue, K., Ohashi, K., Iwashita, S., Hirai, M. and Mizuno, K. (1995) J. Biol. Chem. 270, 31321–31330.
[4] Nunoue, K., Ohashi, K., Okano, I. and Mizuno, K. (1995) Oncogene 11, 701–710.
[5] Sanchez-Garcia, I. and Rabbitts, T.H. (1994) Trends Genet. 10, 315–320.
[6] Dawid, I.B., Toyama, R. and Taira, M. (1995) C.R. Acad. Sci. Paris 318, 295–306.
[7] Ponting, C.P. and Phillips, C. (1995) Trends Biochem. Sci. 20, 102–103.
[8] Gomperts, S.N. (1996) Cell 84, 659–662.
[9] Bernard, O., Ganiatsas, S., Kannourakis, G. and Dringen, R. (1994) Cell Growth Diff. 5, 1159–1171.
[10] Cheng, A.K. and Robertson, E.J. (1995) Mech. Dev. 52, 187–197.
[11] Pröschel, C., Blouin, M.-J., Gutowski, N.J., Ludwig, R. and Noble, M. (1995) Oncogene 11, 1271–1281.
[12] Frangiskakis, J.M., Ewart, A.K., Morris, C.A., Mervis, C.B., Bertrand, J., Robinson, B.F., Klein, B.P., Ensing, G.J., Everett, L.A., Green, E.D., Prochel, C,. Gutowski, N.J., Noble, M., Atkinson, D.L., Odelberg, S.J. and Keating, M.T. (1996) Cell 86, 59–69.
[13] Langland, J.O. and Jacobs, B.L. (1992) J. Biol. Chem. 267, 10729–10736.
[14] Datta, K., Franke, T.F., Chan, T.O., Makris, A., Yang, S.-I., Kaplan, D.R., Morrison, D.K., Golemis, E.A. and Tsichlis, P.N. (1995) Mol. Cell. Biol. 15, 2304–2310.
[15] Farrar, M.A., Alberola-Ila, J. and Perlmutter, R.M. (1996) Nature 383, 178–181.
[16] Heldin, C.H. (1995) Cell 80, 213–223.
[17] Feuerstein, R., Wang, X., Song, D., Cooke, N.E. and Liebhaber, S.A. (1994) Proc. Natl. Acad. Sci. USA 91, 10655–10659.
[18] Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C. and Bredt, D.S. (1996) Cell 84, 757–767.
[19] Takebe, Y., Seiki, M,. Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472.
[20] Schmeichel, K.L. and Beckerle, M.C. (1994) Cell 79, 211–219.
[21] Wu, R.-Y. and Gill, G.N. (1994) J. Biol. Chem. 269, 25085–25090.
[22] Wu, R.-Y., Durick, K., Songyang, Z., Cantley, L.C., Taylor, S. and Gill, G.N. (1996) J. Biol. Chem. 271, 15934–15941.
[23] Wadman, I., Li, J., Bash, R.O., Forster, A., Osada, H., Rabbitts, T.H. and Baer, R. (1994) EMBO J. 13, 4831 4839.
[24] Pawson, T. (1995) Nature 373, 573–580.
[25] Xue, D., Tu, Y. and Chalfie, M. (1993) Science 261, 1324–1328.
[26] Sanchez-Garcia, I., Osada, H., Forster, A. and Rabbitts, T.H. (1993) EMBO J. 12, 4243–4250.
[27] Taira, M., Otani, H., Saint-Jeannet, J.-P. and Dawid, I.B. (1994) Nature 372, 677–679.