Zinc Finger LIM Domains with Protein Kinase C*

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Shun’ichi Kuroda†, Chiharu Tokunaga, Yoshimoto Kiyohara, Osamu Higuchi‡, Hiroaki Konishi, Kensaku Mizuno¶, Gordon N. Gill§, and Ushio Kikkawa

From the Biosignal Research Center, Kobe University, Kobe 657, Japan, the Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-81, Japan, the Inheritance and Variation Group, PRESTO, Research Development Corporation of Japan, Kyoto 619-02, Japan, and the Department of Medicine, University of California San Diego, La Jolla, California 92039-0650

The LIM domain comprising two zinc-finger motifs is found in a variety of proteins and has been proposed to direct protein-protein interactions. During the identification of protein kinase C (PKC)-interacting proteins by a yeast two-hybrid assay, a novel protein containing three LIM domains, designated ENH, was shown to associate with PKC in an isoform-specific manner. Deletion analysis demonstrated that any single LIM domain associates with PKC in COS-7 cells and was phosphorylated by PKC in vitro. Upon treatment of the cells with phorbol ester, ENH in the membrane fraction was translocated to the cytosol fraction in vivo. Other LIM domain-containing proteins, such as Enigma and LIM-kinase 1, also interacted with PKC through their LIM domains. These results suggest that the LIM domain is one of the targets of PKC and that the LIM-PKC interaction may shed light on undefined roles of LIM domain-containing proteins.

The LIM domain is a Cys-rich domain composed of 50–60 amino acid residues with the consensus sequence (Cys-X$_2$-Cys-X$_{17-19}$-His-X$_2$-Cys$_2$)X$_2$-(Cys-X$_2$-Cys-X$_{16-20}$)Cys-X$_2$-His/Asp/Cys$_2$ (where X represents any amino acid) and is found in various proteins (1, 2); homeodomain-containing transcription factors, cytoskeletal proteins, LIM domain-only proteins, protein kinases, and proteins of undefined function. Physicochemical and structural analyses have revealed that the LIM domain is composed of two independent zinc-coordinated fingers (3, 4). Although many zinc finger motifs bind to specific DNA or RNA sequences (5), the LIM domain has been proposed to participate in protein-protein interactions (1, 2). In fact, five proteins have been reported recently as a highly specific target of each LIM domain: the LIM1 domain of zyxin binds to the LIM-only protein CRP by LIM-LIM interaction (6); the CRP forms homodimer by LIM-LIM interaction (7); the LIM domain of RBTN2 binds to the bHLH (basic-helix-loop-helix) domain of TAL1 protein (8); the LIM2 and LIM3 domains of Enigma interact with the Tyr-containing tight-turn motifs of the GDNF$^+$ receptor (GDNFR, known as a Ret tyrosine kinase) and the insulin receptor (Inr), respectively (9, 10). Although most LIM domains adopt a similar zinc-coordinated finger consisting of well-conserved amino acid sequences, no protein has been identified yet as a common target of LIM domains.

The PKC family consists of at least 11 isoforms, which play distinct roles for many cellular functions but show subtle difference of substrate specificities by in vitro phosphorylation studies (11, 12). Therefore, it is reasonable to assume that there are some mechanisms by which each PKC isoform recognizes its specific substrate proteins in vivo. Recently, several proteins associating with PKC have been emerged to govern the subcellular localization of the enzyme family (13–16).

We report here a novel PKC-binding protein containing three LIM domains, designated ENH, and show the association of PKC with LIM domains of different proteins including this novel PKC-binding protein, suggesting that protein-protein interaction with PKC is a general property of LIM domains.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Assay—The yeast two-hybrid assay (17) was conducted in the yeast strain CG-1945, a derivative of HF7c (18), by using a fusion between GAL4 DNA binding domain and the regulatory domain of rat PKC β (residues 1–340) (19) as a bait. β-Galactosidase activity in yeast cells was measured by plate assay methods. All measurements were repeated at least four times.

Expression of Epitope-tagged ENH in COS-7 Cells—We constructed two parental vectors, pTB701-FLAG and pTB701-HA, for expression of NH$_2$-terminal epitope-tagged fusion proteins in COS-7 cells, by inserting the sequences encoding FLAG (20) and HA epitope (21) under the SV40 early promoter of pTB701 (22), respectively. We constructed pTB701-FLAG-END by fusing the ENH cDNA under the FLAG epitope sequence of pTB701-FLAG. Similarly, pTB701-HA-PKC β was constructed by inserting the PKC β cDNA to pTB701-HA. HA-tagged PKC α, γ, ε, δ, α, and ε were constructed using each insert cDNA (23, 24). A kinase-negative mutant of PKC β-HA was generated by replacing Lys-371 by Met by site-directed mutagenesis, and designated as K371M PKC β-HA.

Immunoprecipitation and Phosphorylation Assay—COS-7 cells coexpressing FLAG-tagged ENH and HA-tagged PKC β from a 10-cm plate were suspended in 500 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 50 mM NaF, 1 mM Na$_2$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 1 tablet/50 ml complete protease inhibitor mixture tablets (Boehringer Mannheim), 1% (w/v) Triton X-100). Cleared lysates (500 μl) were incubated for 1 h on ice with 2 μg of either anti-FLAG (M2, Eastman Kodak Co.) or anti-HA (12CA5, Boehringer Mannheim) monoclonal antibody and then mixed with 20 μl of protein G-Sepharose 4 fast flow (50% slurry, Pharmacia Biotech, Uppsala, Sweden). After incubation at 4°C for 1 h with rotation, the beads were washed with lysis buffer four times. The beads were subjected to Western blotting. For phosphorylation assay, the beads were mixed with 25 μl of the reaction mixture (20 mM Tris, pH 7.5, 10 mM MgCl$_2$, 1 mM CaCl$_2$, 20 μM ATP, 8 μg/ml phosphatidyl-

The abbreviations used are: GDNF, glial-cell-line-derived neurotrophic factor; GDNFR, GDNF receptor; Inr, insulin receptor; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; kb, kilobases; TPA, 12-O-tetradecanoylphorbol-13-acetate; LIMK1, LIM-kinase 1; GST, glutathione S-transferase.

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† To whom correspondence should be addressed: Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657, Japan. Tel.: 81-78-803-1255; Fax: 81-78-803-0994; E-mail: skuroda@inherit.biosig.kobe-u.ac.jp.
serine, 0.8 mg/ml diolein). After addition of 10 μl of [γ-32P]ATP (10 mCi/ml), the reaction mixture was incubated for 15 min at 30°C. Samples were analyzed on SDS-PAGE and then autoradiographed.

In Vitro Phosphorylation Assay—Thereaction mixture (25 μl) and 50 ng of PKC βI, βII, and γ were added to 5 μg of GST-fused ENH bound to glutathione-Sepharose 4B. After addition of 1 μl of [γ-32P]ATP (10 mCi/ml), the reaction mixture was incubated for 5 min at 30°C. Samples were analyzed onto SDS-PAGE and then autoradiographed.

RESULTS AND DISCUSSION

We used a yeast two-hybrid system to identify proteins that bind to the regulatory domain of PKC βI. Six positive clones were isolated independently from a rat brain cDNA library. Sequence analysis showed that one of the positive clones encodes a novel protein containing LIM domains. A full-length cDNA clone (1,896 base pairs) was obtained by a rapid amplification of cDNA ends method from the same cDNA library (Fig. 1). The cDNA encodes a novel polypeptide sequence of 591 amino acid residues with a calculated molecular weight of 63,197. The deduced protein sequence has two Pro/Ser-rich regions (Pro/Ser-1 (residues 106–216): Pro, 18.9%; Ser, 19.8%; Pro/Ser-2 (residues 308–394): Pro, 16.1%; Ser, 23.0%).
three LIM domains (LIM1–3; residues 415–585), which resembles the molecular organization of human Enigma (455 amino acid residues) (9). Residues 120–591 of this protein show high similarity to the full-length Enigma, and approximately 37% of the amino acid residues are identical between this protein and Enigma. Therefore, we termed this protein ENH (Enigma homolog). Northern blot analysis of adult rat tissues revealed that the 1.9-kb ENH mRNA was efficiently expressed in heart and skeletal muscle, and the 4.4-kb ENH mRNA was expressed in various tissues, such as heart, brain, spleen, liver, and kidney (Fig. 2).

To investigate the in vivo interaction between ENH and PKC βI, we constructed expression vectors of FLAG-tagged ENH (ENH-FLAG) and HA-tagged PKC βI (PKC βI-HA). COS-7 cells coexpressing ENH-FLAG and PKC βI-HA were lysed, and proteins were immunoprecipitated with either anti-FLAG or anti-HA antibody. Western blotting analysis (Fig. 3A, top and bottom panels) showed that ENH-FLAG (70 kDa) associated with PKC βI-HA (80 kDa) in vivo. TPA treatment prior to the cell lysis had no effect on the association of these two proteins. Phosphorylation assay (Fig. 3A, middle panel) indicated that PKC βI-HA can phosphorylate ENH-FLAG. When a kinase-negative mutant of PKC βI-HA (K371M PKC βI-HA) was used under the same condition (Fig. 3A), although the association of ENH-FLAG with the kinase mutant was observed, the phosphorylation of ENH-FLAG was not observed. These results indicate that association of PKC βI with ENH is independent of the enzymatic activity of PKC βI.

We next examined whether ENH is a substrate for PKC by incubating bacterially expressed GST-ENH hybrid with purified rat cPKC. The GST-ENH fusion protein was phosphorylated by PKC in vitro, whereas GST alone was not (Fig. 3B, top and bottom panels). H1 histone, known as a good substrate for PKC (26), was more efficiently phosphorylated than GST-ENH by PKC (Fig. 3B, middle panel). H1 histone was known to be rapidly phosphorylated by PKC, and after prolonged reaction approximately 1.8 mol of phosphate was incorporated into every mole of H1 histone (26). Under similar conditions, the reaction with GST-ENH was relatively slow. After prolonged reaction, approximately 0.9 mol of phosphate was incorporated per mol of GST-ENH. These data suggest that ENH is a good substrate of PKC at least under the conditions employed.

Western blotting of subcellular fractions of COS-7 cells coexpressing ENH-FLAG and PKC βI-HA showed that ENH-FLAG is equally localized in both cytosol and membrane fractions (Fig. 3C). After TPA treatment, ENH-FLAG in the membrane fraction disappeared, and the amount of ENH-FLAG in the cytosol fraction increased. When a PKC inhibitor, such as calphostin C (100 nM) (27), was added for 15 min prior to TPA treatment (Fig. 3C), ENH-FLAG remained in the membrane fraction. Staurosporine (100 nM), a general inhibitor of Ser/Thr protein kinases (28), also blocked the TPA-induced translocation of ENH. When we used ENH-HA instead of ENH-FLAG, the same results were obtained. Thus, ENH interacts with PKC βI in vivo, and activation of PKC results in translocation of ENH from membrane to cytosol.

We next delineated the region of ENH that interacts with the regulatory domain of PKC βI by the yeast two-hybrid system. A series of deletion mutants of ENH as GAL4 DNA binding domain hybrids were examined for the interaction with the regulatory domain of PKC βI (Fig. 4A). The NH2-terminal region of ENH (residues 1–414; 8LIM) is not required for PKC binding, whereas each LIM domain (LIM1, LIM2, LIM3) can bind to PKC βI. In addition, the COOH-terminal half of the LIM1 domain (1/2 LIM1) was unable to interact with PKC βI. These results indicate that the region of ENH essential for the PKC βI binding is a single intact LIM domain. Using the regulatory domain of various PKCs as a bait, it was shown that residues 415–591 of ENH (ENH LIM1–3 domain) were able to interact with PKC βI, γ, and ε but not with PKC α, δ, and ζ. To further delineate the region interacting with the ENH LIM domains, we assayed deletion mutants of the regulatory domain of PKC βI and ε (Fig. 4B). Mutants harboring the NH2-terminal V1 region of either PKC βI or ε showed an intact ability to bind to the ENH LIM1–3 domain. The V1 region of PKC is thus critical for the ENH-PKC interaction. Since the V1 regions of PKCs show high diversity (29), it is likely that the LIM domains of ENH may identify PKC isoforms by a specific sequence of the V1 region rather than the conformation of PKC molecule.

Because human Enigma (9) has three highly related LIM domains at its COOH-terminal (Enigma LIM1–3 domain), we investigated the interaction of Enigma LIM domains with PKC by a pull-down assay. Cell lysates from the COS-7 cells expressing one of the HA-tagged PKCs (α, βI, γ, δ, ε, and ζ) were mixed with GST or GST-Enigma LIM1–3, and the proteins bound to glutathione-Sepharose 4B were analyzed with anti-HA antibody. The Enigma LIM1–3 domain was found to interact with PKC α, βI, and ζ, but not with PKC γ, δ, and ε (Fig. 5A, top panel), indicating that LIM domains of Enigma and ENH have individual specificities for PKC isoforms. Previous studies showed that the LIM2 and LIM3 domains of Enigma bind to GDNF-R and InsR, respectively (9, 10). Each LIM domain of Enigma was found to associate with PKC, as in the case of ENH. These results suggest the possibility that one LIM domain interacts with more than one molecule. Enigma has been
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postulated to be either a scaffold or an anchoring protein that coordinates the subcellular localization and activity of GDNFLR and InsR (9, 10) and also associates with PKC as described above. Since ENH shows the same molecular organization as Enigma, ENH might have a role similar to Enigma and its subcellular localization is controlled by PKC.

By the pairwise alignment analysis of LIM domains, David et al. (1) demonstrated that LIM domains are classified into five discrete groups (groups A to E). All LIM domains of ENH and Enigma belong to group D. We tested LIM domains from other groups using human LIM-kinase 1 (LIMK1) (30, 31), which efficiently bound to LIMK1, were lysed, and proteins were immunoprecipitated with either anti-LIMK1 (30, 31) or anti-HA antibody. Western blotting (Fig. 5C, third and fourth panels) indicated that the LIM2 domain of LIMK1 (group B) is critical for the binding between LIMK1 and PKC γ. Further analysis of other groups of LIM domains should yield insight into the general role of the LIM-PKC interaction.

In this study, we demonstrated that the LIM domains selectively bind to PKC isoforms in vitro and in vivo. The LIM-PKC interaction may be an important clue for the understanding of PKC isoform-specific functions in vivo and the roles of LIM domain-containing proteins.

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