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Lambda-Interacting Protein, a Novel Protein That Specifically Interacts with the Zinc Finger Domain of the Atypical Protein Kinase C Isotype λ/ι and Stimulates Its Kinase Activity In Vitro and In Vivo

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The members of the atypical subfamily of protein kinase C (PKC) show dramatic structural and functional differences from other PKC isotypes. Thus, in contrast to the classical or novel PKCs, they are not activated by diacylglycerol or phorbol esters. However, the atypical PKCs are the target of important lipid second messengers such as ceramide, phosphatidic acid, and 3′-phosphoinositides. The catalytic and pseudosubstrate sequences in the two atypical PKCs (λ/ιPKC and ζPKC) are identical but are significantly different from those of conventional or novel PKCs. It has been shown that microinjection of a peptide with the sequence of the pseudosubstrate of the atypical PKC isotypes but not of αPKC or εPKC dramatically inhibited maturation and NF-κB activation in *Xenopus* oocytes, as well as reinitiation of DNA synthesis in quiescent mouse fibroblasts. This indicates that either or both atypical isoforms are important in cell signalling. Besides the pseudosubstrate, the major differences in the sequence between λ/ιPKC and ζPKC are located in the regulatory domain. Therefore, any functional divergence between the two types of atypical PKCs will presumably reside in that region. We report here the molecular characterization of lambda-interacting protein (LIP), a novel protein that specifically interacts with the zinc finger of λ/ιPKC but not ζPKC. We show in this paper that this interaction is detected not only in vitro but also in vivo, that LIP activates λ/ιPKC but not ζPKC in vitro and in vivo, and that this interaction is functionally relevant. Thus, expression of LIP leads to the transactivation of a κB-dependent promoter in a manner that is dependent on λ/ιPKC. To our knowledge, this is the first report on the cloning and characterization of a protein activator of a PKC that binds to the zinc finger domain, which has so far been considered a site for binding of lipid modulators. The fact that LIP binds to λ/ιPKC but not to the highly related ζPKC isoform suggests that the specificity of the activation of the members of the different PKC subfamilies will most probably be accounted for by proteins like LIP rather than by lipid activators.

The atypical subgroup of the protein kinase C (PKC) family of isozymes is composed of two members: λ/ιPKC and ζPKC (1, 33, 38). ιPKC is the human homolog of the mouse λPKC, and both show a high degree of homology in the catalytic as well as in the regulatory domain with ζPKC from different species (32). Of great interest is the fact that both the structure and the mechanism of regulation of the two atypical PKCs by lipid activators differ from those of the other members of their family. Thus, they do not contain the Ca^{2+}-binding region, and they have only a single zinc finger-like motif, which in other PKC isoforms has been shown to be double and responsible for the activation by diacylglycerol and phorbol esters (32). Consequently, the atypical PKCs cannot be regulated by Ca^{2+}, and are insensitive to phorbol esters and diacylglycerol (1, 33, 38, 42). However, native ζPKC is activated by critical lipid second messengers like phosphatidic acid (31) and phosphatidylinositol 3,4,5-P_3 (30). Phosphatidic acid is produced as consequence of the activation of phosphatidylcholine-phospholipase D or the concerted action of phosphatidylcholine-phospholipase C and diacylglycerol kinase (12). Both phospholipases have been implicated in cell proliferation and tumor transformation (12, 20, 22, 43). Phosphatidylinositol 3,4,5-P_3 is the product of the phosphoinositide-3 kinase, which constitutes a decisive step during mitogenic signal transduction (41). On the other hand, ceramide that is generated following the activation of sphingomyelin hydrolysis by inflammatory cytokines, such as interleukin-1 or tumor necrosis factor alpha (TNF-α) (13, 18), has been shown to bind and activate ζPKC (24, 28). Taken together, all these observations pinpoint to ζPKC as a target of important lipid second messengers and suggest its role in cell signalling.

Molecular cloning of an atypical PKC isotype from *Xenopus laevis* with the regulatory domain of rat ζPKC as a probe produced a cDNA encoding a protein highly homologous to rat ζPKC, which was the only atypical isotype known at that time (10, 33). The cloned *X. laevis* atypical PKC displays an overall 72% identity at the amino acid level to ζPKC, which becomes 84% in the catalytic domain (10, 33). This led to the identification of that gene product as the *X. laevis* ζPKC (10). The more recent description of λ/ιPKC (1, 38) allowed the comparison of the *X. laevis* enzyme with all known atypical subspecies. On the basis of alignment of their sequences, the cloned *X. laevis* atypical PKC shows the highest degree of homology with λ/ιPKC, with an overall 90% identity at the amino acid level. Therefore, the cloned *X. laevis* enzyme is actually λ/ιPKC (10). Interestingly, the pseudosubstrate sequence is identical in both atypical isotypes but is significantly different from that of conventional or novel PKCs (1, 32, 38). Microinjection of a peptide with the sequence of the pseudosubstrate of the atypical PKC isotypes but not of αPKC or εPKC, dramatically inhibited maturation (10) and NF-κB (11)

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activation in *X. laevis* oocytes, as well as the reinitiation of DNA synthesis in quiescent mouse fibroblasts (3). This indicates that either or both atypical isoforms are important in mitogenic activation. Besides the pseudosubstrate, the major differences in the sequence between λ/ιPKC and ζPKC are located in the regulatory domain. Therefore, any functional divergence between the two types of atypical PKCs will presumably reside in that region. We reasoned that proteins that interact with the regulatory domain of λ/ιPKC but not with ζPKC could be potential and specific regulators of its enzymatic activity both in vitro and in vivo and that their molecular characterization would help to dissect specific pathways regulating the different atypical isotypes.

MATERIALS AND METHODS

Yeast strains and media. The genotype of the *Saccharomyces cerevisiae* reporter strain Y190, used for the two-hybrid screening, is *MATα leu2-3,112 ura3-52 trp1-901 his3-Δ200 ade2-101 gal4 Δgal80 URA3*::*GAL-lacZ LYS*::*GAL-HIS3 cyh*^{r}. The genotype of SFY526, used to test interactions between GAL4-BD and GAL4-AD fusions, is *MAT***a** *ura3-52 his 3-200 ade 2-101 lys2-801 trp1-901 leu 2-3,112 can*^{r} *gal4-542 gal80-538 URA3*::*GAL1-lacZ* (Clontech Laboratories, Inc.). Strains were grown under standard conditions in rich or synthetic medium with appropriate supplements at 30°C (39).

Two-hybrid screening and cDNA isolation. For the yeast two-hybrid screening, pYTH9λ/ιPKC^{REG} was cotransformed with the human placenta cDNA Matchmaker library in the pGAD10 vector (Clontech Laboratories, Inc.) into the Y190 yeast strain, as described previously (14, 37), and the transformants were plated to synthetic medium lacking histidine, leucine, and tryptophan but containing 20 mM 3-amino-1,2,4-triazole. The plates were incubated at 30°C for 5 days. His^{+} colonies were assayed for β-galactosidase activity by a filter assay as described below. To obtain a cDNA for the full-length lambda-interacting protein (LIP) coding sequence, a human placenta 5′-RACE-Ready cDNA (Clontech Laboratories, Inc.) was used as the template in PCR with primers RACE1 (5′-GCG AATTCTCTGAGTAGACATATCCTGG-3′) and anchor (5′-CTGGTTCGGC CCACCTCTGAAGGTTCCAGAATCGATAG-3′). The conditions of the PCR were 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, with a final extension time of 7 min at 72°C. For the second 5′-RACE reaction, primers RACE2 (5′-CTGAATTCTCATGCTGGTTAGGATAGCAG-3′) and anchor were used. Oligonucleotides were synthesized by Isogen Biosciences. DNA sequencing was done by the dideoxynucleotide chain termination method.

β-Galactosidase filter assays. Yeast strains were patched to synthetic medium lacking leucine and tryptophan, incubated for 3 days at 30°C, and transferred to a nitrocellulose filter (5). The filter was placed on aluminum foil atop a sea of liquid nitrogen for 20 s and then immersed in liquid nitrogen for 1 to 2 s. The filter was allowed to come to room temperature and then placed on top of Whatman no. 1 paper that had been prewetted in Z buffer containing 0.75 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml (25). The filters were incubated for 3 h at 30°C. Blue coloration is indicative of β-galactosidase activity.

Plasmids. pYTH9λ/ιPKC was constructed from full-length *X. laevis* ζPKC derived by PCR (oligonucleotides 5′-GCGAATTCCATGCCGACACAGAGG G-3′ and 5′-CGAGGTCGACGGTATCG-3′) and the template pBluescriptζ PKC (3) at the *Eco*RI site of pYTH9. pYTH9λ/ιPKC^{REG} was constructed from the regulatory domain of *X. laevis* ζPKC (amino acids 1 to 250) and was made in the same way as pYTH9λ/ιPKC but with the oligonucleotides 5′-GCGAATTC CATGCCGACACAGAGGG-3′ and 5′-TTGGAATTCTCAGCTAGATGGGC TCTTTCCAC-3′. pYTH9λ/ιPKC$^{REG\text{-}C167S}$ was obtained by replacing Cys-167 with a serine residue by PCR-directed mutagenesis. Deletions of the regulatory domain were used to construct pYTH9λ/ιPKC^{126} (oligonucleotides 5′-GCGAA TTCCATGCCGACACAGAGGG-3′ and 5′-GCCGAATTCTCATCCATTTG CACAATAC-3′ at the *Eco*RI site of pYTH9) and pYTH9λ/ιPKC^{ZF} (oligonucleotides 5′-CTGCTCGAGGCTGTATTGTGCAA-3′ and 5′-GCCGAAT TCTCATCCATTTGCACAATAC-3′, subcloned into the *Sal*I-*Eco*RI sites of pYTH9). pYTH9λ/ιPKC^{CAT} contains the catalytic domain (oligonucleotides 5′-GCGAATTCCTGGGACTCCAAGACTT-3′ and 5′-CGAGGTCGACGGT ATCG-3′) at the *Eco*RI site of pYTH9. pYTH9ζPKC was constructed from full-length rat brain ζPKC derived by PCR (oligonucleotides 5′-GGAGAATTC CATGCCCAGCAGGACC-3′ and 5′-ATTCCCGGGTCACACGGACTCCT CA-3′) at the *Eco*RI-*Sma*I sites of pYTH9.pYTH9ζPKC^{REG} was constructed from the regulatory domain (amino acids 1 to 250) made the same way as pYTH9ζPKC but with oligonucleotides 5′-GGAGAATTCCATGCCCAGCA GGACC-3′ and 5′-AGGAATTCTCACTGAGAGATTTTGATCCCATC-3′ and with subcloning into the *Eco*RI site of the same vector. Deletions of the regulatory domain yielded pYTH9ζPKC^{126} (oligonucleotides 5′-GGAGAATTC CATGCCCAGCAGGACC-3′ and 5′-TTGGAATTCTCAGCCGTTGGCTCG GTAT-3′) and pYTH9ζPKC^{ZF} (oligonucleotides 5′-ATTGAATTCGCTATAC CGAGCCAACGGC-3′ and 5′-GCCGAATTCTCAATCTACTGGAGGCTC TTG-3′) at the *Eco*RI site of pYTH9. pYTH9αPKC^{REG} was constructed by subcloning a *Nco*I-*Bam*HI fragment, encompassing the regulatory domain of bovine αPKC, into the *Nco*I-*Bgl*II sites of pYTH9. pYTH9εPKC^{REG} was obtained by subcloning a *Nco*I-*Pvu*II fragment, containing the regulatory domain of mouse εPKC, into the *Nco*I-*Sma*I-digested pYTH9. This vector is an influenza virus hemagglutinin (HA) epitope-tagged pYTH6 (Trp1 marker) derivative vector. pGBT9Raf^{REG}, containing the regulatory domain of c-Raf-1, was obtained by PCR with primers 5′-GCAGAATTCGAGCACATACAGGGA-3′ and 5′-TCCGGATCCTACTCATGTAGCCAA-3′ and with p627-Raf-1 as the template, and inserted at *Eco*RI-*Sal*I sites. pGBT9Raf, pGBT9Raf^{CAT}, and pGBT9 Mos have been described previously (29). pGAD10-LIP contains the full-length cDNA LIP and was amplified by PCR with oligonucleotides 5′-GCGAATTCAT GCTGCATGAACTGGACGGT-3′ and 5′-CGGAATTCCGAGAATCTGAAT GCAT-3′ and subcloned into the *Eco*RI site of pGAD10.

Plasmids GST-λ/ιPKC^{REG}, GST-λ/ιPKC^{126}, and GST-λ/ιPKC^{ZF} were generated by PCR with pBluescript-ζPKC as the template (3) and the primers 5′-AATACGACTCACTATAG-3′ and 5′-TTGGAATTCTCAGCTAGATGGGC TCTTTCCAC-3′ for GST-λ/ιPKC^{REG}, 5′-ATTGGATCCACACAGAGGGA CAACAC-3′ and 5′-GCCGAATTCTCATCCATTTGCACAATAC-3′ for GST-λ/ιPKC^{126}, and 5′-ATTGGATCCCTGTATTGTGCAAATGG-3′ and 5′-GCC GAATTCTCATCCATTTGCACAATAC-3′ for GST-λ/ιPKC^{ZF}. The PCR products were digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI-*Eco*RI-digested pGEX-2TK (Pharmacia), except for λ/ιPKC^{REG}, which was digested with *Xmn*I and *Eco*RI and subcloned into *Sma*I-*Eco*RI sites. Plasmids GST-ζPKC^{REG}, GST-ζPKC^{126}, and GST-ζPKC^{ZF} were generated by PCR with MBP-ζPKC as the template (8) and the primers 5′-ATTGGATCCAGCAGGACCG ACCCCAA-3′ and 5′-AGGAATTCTCACTGAGAGATTTTGATCCCATC-3′ for GST-ζPKC^{REG}, 5′-ATTGGATCCAGCAGGACCGACCCCAA-3′ and 5′-TTGGAATTCTCAGCCGTTGGCTCGGTAT-3′ for GST-ζPKC^{126}, and 5′-AT TGGATCCCTATACCGAGCCAACGGC-3′ and 5′-GCCGAATTCTCAATC TACTGGAGGCTCTTG-3′ for GST-ζPKC^{ZF}. The PCR products were digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI-*Eco*RI-digested pGEX-2TK. pGEX-2TK constructs were transformed into *Escherichia coli* JM101, and expression of glutathione-*S*-transferase (GST) fusion proteins and their purification on glutathione-Sepharose were carried out as specified by the manufacturer. MBP-LIP(R4) contains the partial cDNA clone of LIP inserted in the *Eco*RI site of pMALc2 (New England Biolabs). The MBP-LIP(R4) fusion protein was expressed in *E. coli* and purified by binding to an amylose resin as specified by the manufacturer. Expression and purification of hnRNPA1 have been described previously (29).

To make the HA-tagged plasmids, pCDNA3-HA was constructed as follows. The following pair of complementary oligonucleotides was synthesized, annealed, and ligated into pCDNA3 (Invitrogen) digested with *Hin*dIII and *Eco*RI, to create pCDNA3-HA vector: 5′-AGCTTGCCGCCACCATGTATGATGTTCCTGAT TATGCTAGCCTCCCGGGG-3′ and 5′-AATTCCCCGGGAGGCTAGCAT AATCAGGAACATCATACATGGTGGCGGCA-3′. To construct pCDNA3-HA-ζPKC and pCDNA3-HA-ζPKC^{MUT}, a fragment corresponding to the full-length wild-type or kinase-inactive mutant of rat brain ζPKC was obtained by PCR with pBluescriptζPKC or pSelectζPKC^{MUT}, respectively, as template and with the primers 5′-GGAGAATTCCATGCCCAGCAGGACC-3′ and 5′-ATGT CTAGACACGGACTCCTCAGCAGA-3′, digested with *Eco*RI and *Xba*I, and subcloned into the *Eco*RI-*Xba*I sites of pCDNA3-HA. pSelectζPKC^{MUT} was derived by directed mutagenesis from pSelectζPKC and the mutagenic oligonucleotide 5′-GATTTACGCCATGTGGGTGGTGAAGAAGGAGC-3′. pSelectζ PKC was obtained by subcloning the *Eco*RI fragment containing full-length ζPKC from pBluescriptζPKC into pSelect (Promega). pCDNA3-HAλ/ι-PKC and pCDNA3-HAλ/ι-PKC^{MUT} were generated by PCR with pBluescriptλ/ιPKC or pSelectλ/ιPKC^{MUT} (3) as the template, respectively, and the primers 5′-GCG AATTCCATGCCGACACAGAGGG-3′ and 5′-CGAGGTCGACGGTATCG-3′. The PCR products were cut with *Eco*RI and ligated to pCDNA3HA previously cut with the same enzyme. To obtain the HA-tagged kinase-inactive mutants of λ/ιPKC and ζPKC, CMV-LIP(R4) was generated by PCR with primers 5′-TCGAAGCTTATGGGTCTTGAACATCCTAT-3′ and 5′-CGAGAATCTG AATGCAT-3′ and with pGAD10-LIP(R4), the original clone obtained from the yeast two-hybrid screen, as the template and subcloned into *Hin*dIII and *Xba*I sites of pRcCMV (Invitrogen). All constructs were confirmed by nucleotide sequence analysis, and the expression of the fusion proteins was confirmed by immunoblotting.

Northern (RNA) blots. Samples (10 μg) of total RNA from different human tissues (Clontech Laboratories, Inc.) were run on formaldehyde-agarose gels, blotted onto Hybond-N^{+} filters, and hybridized overnight at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.02% bovine serum albumin)–10 mM Tris-Cl (pH 7.5)–0.5% sodium dodecyl sulfate (SDS) to 50% formamide–200 μg of denaturated salmon sperm DNA per ml–50 μg of tRNA per ml with a ^{32}P-labelled cDNA probe of LIP (clone R4). The filters were washed for 15 min at room temperature in 2× SSC, three times (1 h each time) at 56°C with 0.1× SSC–0.5% SDS, and 5 min at room temperature with 2× SSC.

In vitro binding studies. GST, GST-λ/ιPKC, and GST-ζPKC were incubated with the resin-bound MBP or MBP-LIP(R4) at the protein concentrations specified in the figure legends. The binding-reaction mixtures contained 85 mM NaCl, 10% glycerol, 0.6 mM guanine nucleotide, and 6 mM free magnesium.

After 60 min at 4°C, the resin was sedimented and washed with 0.5 ml (10 bed volumes) of 10 mM Tris-HCl (pH 7.6)–10% glycerol–5 mM $MgCl_2$–1 mM dithiothreitol–0.1% Triton X-100–0.1 mM phenylmethylsulfonyl fluoride. The bound proteins were boiled in sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred, and immunoblotted with an anti-GST antibody (Santa Cruz Biotechnology).

Antibody production and immunoblot analysis. Antibodies against LIP were prepared by immunizing rabbits with the MBP-LIP(R4) fusion protein. For immunoblot analysis, 30 μg of cellular protein was resolved in SDS–10% polyacrylamide gels. The protein was then electrophoretically transferred onto a nitrocellulose filter (Promega) and incubated with preimmune serum or anti-LIP antibody. Bands were visualized with the ProtoBlot AP System (Promega).

Transfections. Subconfluent cultures of Cos cells in 100-mm plates were transfected by the calcium phosphate method (Gibco, BRL) with 20 μg of either pCDNA3-HA, pCDNA3-HA-λ/ιPKC, or pCDNA3-HA-ζPKC. Plasmid DNA was removed 4 h later, and the cells were incubated for 16 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Then, medium was replaced with medium containing 0.5% fetal calf serum and the incubation was continued for 16 h, followed by an additional 8 h in serum-free medium. Cultures were then extracted with lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), protease inhibitors] and immunoprecipitated with 2 μg of anti-HA antibody (12CA5; Boehringer, Mannheim, Germany) per mg of protein extract. Immunoprecipitates were washed seven times with lysis buffer containing 0.5 M NaCl. For the in vitro kinase assay, immunocomplexes were incubated with 1 μg of recombinant bacterially produced heterogeneous nuclear ribonucleoprotein A1 (29) in the presence or absence of 1 μg of MBP, MBP-LIP(R4), or phosphatidylserine as sonicated vesicles (50 μg/ml) and 5 to 10 μCi of [γ-^{32}P]ATP (100 μM) in kinase buffer (35 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, 1 mM phenylphosphate) for 30 min at 30°C in a final volume of 20 μl. Reactions were stopped by the addition of concentrated sample buffer. Samples were boiled for 3 min and separated by SDS-PAGE followed by autoradiography.

Subconfluent cultures of Cos cells in 100-mm-diameter plates were transfected with 5 μg of either pCDNA3-HA, pCDNA3-HA-λ/ιPKC, or pCDNA3-HA-ζPKC together with 20 μg of CMV or CMV-LIP(R4). Plasmid DNA was removed 4 h later, and cells were incubated for 16 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Then the medium was replaced with medium containing 0.5% fetal calf serum and the incubation was continued for 16 h, followed by an additional 8 h in serum-free medium. Cultures were then either untreated or stimulated with 10% fetal calf serum for 5 min. Immunoprecipitates and kinase assays were performed as above. In another set of experiments, subconfluent cultures of Cos cells were transfected with different amounts of plasmid CMV-LIP(R4) together with 500 ng of 3EConALuc reporter plasmid. In some experiments, these plasmids were cotransfected with 5 μg of the different λ/ιPKC expression vectors. After 4 h, the DNA-containing medium was removed and cells were made quiescent by serum starvation for 36 h. Then the cells were either untreated or stimulated with human TNF-α (500 U/ml) for 6 h as a control, extracts were prepared, and luciferase activity was determined as described previously (2). Data are expressed in terms of relative luciferase activity units, calculated as (light emission from experimental sample-light emission of lysis buffer alone) per microgram of cellular protein in the sample. In another set of experiments, Cos cells were transfected with the κB reporter plasmid with different concentrations of either empty vector or expression plasmids for wild-type and kinase-inactive mutants of λ/ιPKC and ζPKC, and the experiments were processed as above.

In vivo association. Quiescent NIH 3T3 fibroblasts, either untreated or stimulated according to the experiments, or proliferating HeLa cells were extracted with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 25 μg of leupeptin per ml, 25 μg of aprotinin per ml, 25 μg of pepstatin per ml, and 1 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with an anti-λ/ιPKC-ζPKC antibody (Gibco, BRL) either in the absence or in the presence of competing peptide as described previously (3). The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-LIP antibody.

Nucleotide sequence accession number. The GenBank accession number of the LIP sequence is U32581.

RESULTS

Functional role of λ/ιPKC and ζPKC. Since transfection of a dominant negative mutant of λ/ιPKC (identified as ζPKC in references 3, 4, 7, and 9) inhibited a number of cell functions including the activation of κB-dependent promoters (4, 7), it was of interest to determine whether a dominant negative mutant of ζPKC similarly affected this parameter. Therefore, either Cos cells (Fig. 1) or NIH 3T3 cells (not shown) were transfected with different concentrations of expression vectors for HA-tagged ζPKC or λ/ιPKC, which were either wild type or dominant negative mutants along with a κB-driven reporter plasmid. Interestingly, cotransfection of the wild-type λ/ιPKC significantly stimulated the κB-dependent promoter activity, with a maximum around 15 μg (Fig. 1A). Transfection of ζPKC, although significantly less potent at lower concentrations, displayed a similar degree of activation of the reporter at high expression levels (Fig. 1A). Transfection of dominant negative λ/ιPKC severely reduced the activation of this reporter activity, with complete inhibition at around 20 μg (Fig. 1B), whereas a similar level of reduction was obtained with 30 μg of the ζPKC expression vector. Figure 1C shows a representative immunoblot in which the expression levels of the four transfected constructs were very similar. Taken together, these results indicate that although the stimulation of the κB-dependent promoter activity appears to be more sensitive to the transfection of λ/ιPKC, no obvious differences were observed between the atypical isotypes at higher expression levels. This was as expected, because of the near identity of the catalytic domains of λ/ιPKC and ζPKC. The different sensitivities of the κB-dependent promoter activity to low concentrations of λ/ιPKC and ζPKC could be due to the sequence divergences existing in their regulatory domain. The identification of proteins that selectively interact with the regulatory region of λ/ιPKC but not with ζPKC would help us to understand the potential functional and/or regulatory differences between the atypical PKCs.

Yeast two-hybrid screen. To identify potential specific activators of λ/ιPKC, the regulatory domain of that kinase (amino acids 1 to 250) was fused with the DNA-binding domain of the yeast GAL4 protein (pYTH9λ/ιPKC^{REG}) to serve as the "bait" to screen a human placenta Matchmaker cDNA library. Colonies that grew on yeast dropout media lacking Leu, Trp, and His but containing 20 mM 3-amino-1,2,4-triazole and that were blue when assayed by X-Gal colony filter assay were selected. Twelve positive colonies that stained intensely blue within 20 min were obtained from 2.5×10^6 screened. The expression of His-3 and LacZ in these colonies was shown to depend on the GAL4 fusion protein by retransforming the recovered plasmids into yeasts containing the bait construct (Fig. 2A, a and b; Table 1). The sequences of the 12 clones revealed that they were identical and partial (clone R4; 1,568 bp). To obtain the missing 5′ region, a human placenta 5′-RACE Ready cDNA (Clontech Laboratories, Inc.) was used. This procedure yielded an additional 5′ fragment of 453 bp. With that sequence, the appropriate oligonucleotide was used in a second 5′-RACE reaction, which produced another 1.3-kb fragment, including the initiation codon and some of the 5′ untranslated region. The resulting full-length cDNA clone encodes for a novel protein of 713 amino acids, with a predicted molecular mass of 79.7 kDa, and with no exact matches in the cDNA and protein data banks searched (Fig. 3). From now on, this protein will be termed LIP (lambda-interacting protein). Northern blot analysis was done with RNAs derived from different human tissues. LIP appears to be expressed in the placenta, brain, lung, and spleen (Fig. 4).

Specificity of the interaction of LIP with the λ/ιPKC regulatory domain. The specificity of the interaction between λ/ιPKC^{REG} and LIP was tested with an unrelated molecule, pYTH9-lamin, which fails to transactivate the reporter constructs (Fig. 2A, c; Table 1). Of note, the catalytic domain of λ/ιPKC did not interact with LIP whereas the full-length protein did show interaction, albeit less strongly than the regulatory region (Fig. 2A, d and e; Table 1). This indicates that the interaction of LIP with λ/ιPKC takes place through the regulatory domain. To determine whether LIP could also bind to the regulatory domain of ζPKC, this region (amino acids 1 to

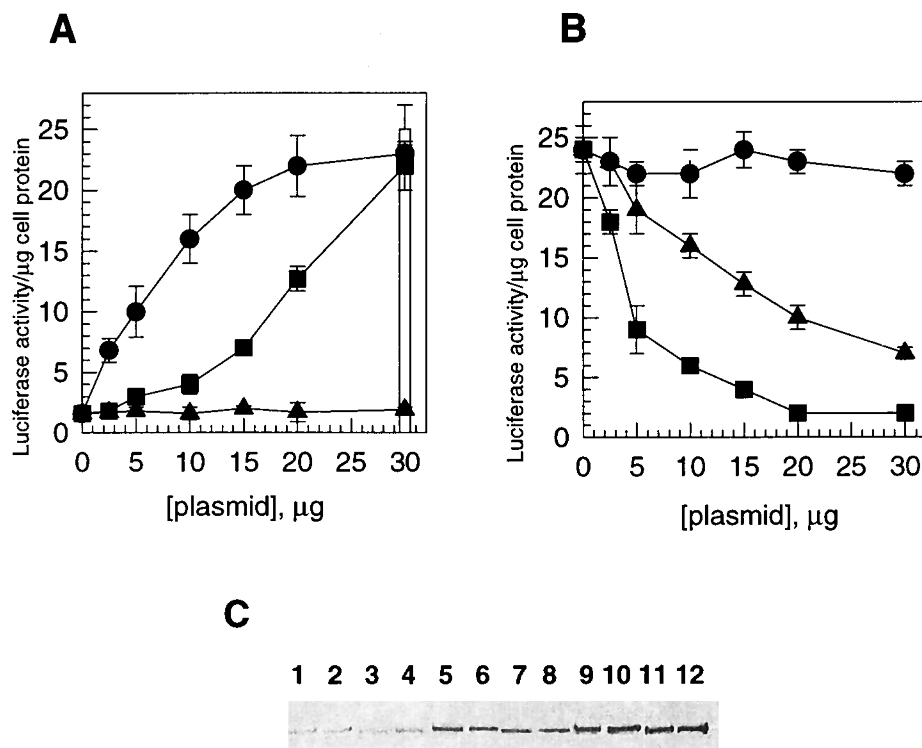

FIG. 1. Role of λ/ιPKC and ζPKC in the stimulation of κB-dependent promoter activity. (A) Cos cells were transfected with different concentrations of either empty plasmid (▲) or expression vectors for HA-tagged wild-type λ/ιPKC (●) and ζPKC (■), along with 500 ng of the κB enhancer-directed luciferase reporter plasmid (3EconALuc). After 4 h, the DNA-containing medium was removed and cells were made quiescent by serum starvation for 24 h. Then extracts were prepared, and the luciferase activity was determined as described in Materials and Methods. As a control, cells transfected with the maximal concentration of empty plasmid were stimulated with TNF-α (500 U/ml) for 4 h (empty bar). Results are the mean ± standard deviation (SD) of three independent experiments with incubations in duplicate. (B) Cos cells were transfected with different concentrations of either empty plasmid (●) or expression vectors for HA-tagged kinase-inactive mutants of λ/ιPKC (■) and ζPKC (▲), along with 500 ng of the κB enhancer-directed luciferase reporter plasmid (3EconALuc). After 4 h, the DNA-containing medium was removed and cells were made quiescent by serum starvation for 24 h. Then cells were stimulated with TNF-α (500 U/ml) for 4 h. Extracts were prepared, and the luciferase activity was determined as described in Materials and Methods. Results are the mean ± SD of three independent experiments with incubations in duplicate. (C) Representative immunoblot with anti-HA antibody in which the expression levels of the four transfected constructs were determined. Cells were transfected with 2.5 μg (lane 1), 10 μg (lane 5), or 30 μg (lane 9) of wild-type ζPKC, with 2.5 μg (lane 2), 10 μg (lane 6) or 30 μg (lane 10) of kinase-inactive ζPKC; with 2.5 μg (lane 3), 10 μg (lane 7) or 30 μg (lane 11) of wild-type λ/ιPKC; or with 2.5 μg (lane 4), 10 μg (lane 8), or 30 μg (lane 12) of kinase-inactive λ/ιPKC.

250) from rat brain ζPKC was fused with the DNA-binding domain of the yeast GAL4 protein (pYTH9ζPKC^{REG}) and its ability to interact with LIP in the two-hybrid system was determined. Interestingly, LIP does not interact with the regulatory domain of rat ζPKC or with the regulatory domains of αPKC or εPKC (Fig. 2A, f; Table 1), indicating that its binding to λ/ιPKC is highly specific for this atypical isotype. On the other hand, Raf-1 is a serine/threonine kinase with an overall structure similar to that of the atypical PKCs (33). Both type of kinases are critical components downstream of Ras in mito-

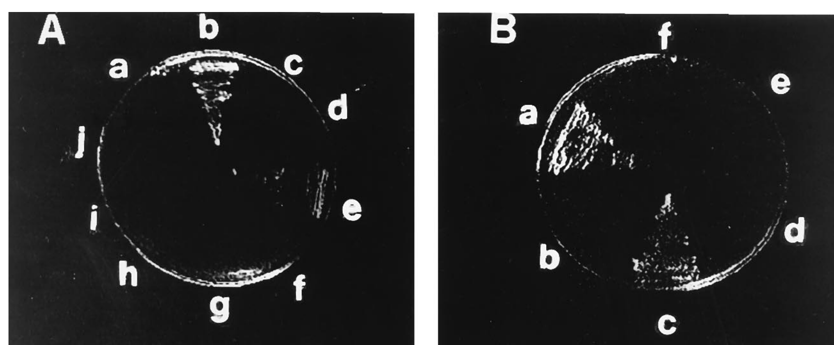

FIG. 2. Specificity of the interaction of LIP with λ/ιPKC^{REG}. The *S. cerevisiae* SFY526 reporter host strain was transformed with pGAD10-LIP and the following plasmids: (A) pYTH9 (a), pYTH9λ/ιPKC^{REG} (b), pGBT9Lamin (c), pYTH9λ/ιPKC^{CAT} (d), pYTH9λ/ιPKC (e), pYTH9ζPKC^{REG} (f), pGBT9Raf^{CAT} (g), pGBT9Raf (h), pGBT9Raf^{REG} (i), and pGBT9Mos (j); (B) pYTH9λ/ιPKC^{REG} (a), pYTH9λ/ιPKC^{126} (b), pYTH9λ/ιPKC^{ZF} (c), pYTH9ζPKC^{REG} (d), pYTH9ζPKC^{126} (e), and pYTH9ζPKC^{ZF} (f). Individual Leu^{+} Trp^{+} transformants were streaked to synthetic medium plates lacking tryptophan, leucine, and histidine. The plates were incubated at 30°C for 3 days. Essentially identical results were obtained in another two experiments.

TABLE 1. β-Galactosidase activity induced by interaction between the regulatory domain of λ/ιPKC and LIP

GAL4-BD fusion	β-Galactosidase activity for GAL4-AD fusion[a]:	
	pGAD10	pGAD10-LIP
pYTH9	−	−
pYTH9λ/ιPKC^{REG}	−	+
pYTH9λ/ιPKC	−	+
pYTH9λ/ιPKC^{126}	−	−
pYTH9λ/ιPKC^{ZF}	−	+
λ/ιPKC$^{REG\text{-}C167S}$	−	−
pYTH9λ/ιPKC^{CAT}	−	−
pYTH9ζPKC^{REG}	−	−
pYTH9ζPKC	−	−
pYTH9ζPKC^{126}	−	−
pYTH9ζPKC^{ZF}	−	−
pYTH9αPKC^{REG}	−	−
pYTH9εPKC^{REG}	−	−
pGBT9Raf^{REG}	−	−
pGBT9Raf	−	−
pGBT9Mos	−	−
pGBT9Lamin	−	−

[a] β-Galactosidase activity was determined by a filter assay for the yeast strains containing the indicated plasmids as described in Materials and Methods. Plus signs represent a positive indication of β-galactosidase activity (blue color) in filter assays. Essentially identical results were obtained in three other independent experiments.

genic cascades (3, 6, 9, 10, 15–17, 19). Therefore, it was of great interest to determine whether Raf-1 would interact with LIP. The catalytic domain (Fig. 2A, g; Table 1), the full-length Raf-1 (Fig. 2A, h; Table 1), and the regulatory region (Fig. 2A, i; Table 1) of Raf-1 all failed to interact with LIP. The product of c-*mos* that is another serine/threonine kinase critically involved, like Raf-1 and the atypical PKCs, in mitogenic signal transduction in *X. laevis* oocytes and mammalian cells (36) did not interact with LIP (Fig. 2A, j; Table 1). Therefore, these data collectively indicate that LIP specifically binds to the regulatory domain of λ/ιPKC but not to other structural and functional related kinases.

LIP binds specifically to the zinc finger of λ/ιPKC. To map the region in the regulatory domain of λ/ιPKC where LIP binds, cDNA fragments corresponding to amino acids 1 to 126 and amino acids 127 to 194 (corresponding to the zinc finger region) of λ/ιPKC and ζPKC, respectively, were fused with the DNA-binding domain of the yeast GAL4 protein and their ability to interact with LIP in the two-hybrid system was determined. Interestingly, only the zinc finger and the whole regulatory domain of λ/ιPKC displayed LIP-binding activity (Fig. 2B; Table 1). No binding was detected with the different ζPKC fragments or with the region encompassing amino acids 1 to 126 of λ/ιPKC (Fig. 2B; Table 1). As an important control, disruption of the cysteine finger by mutation of cysteine 167 to serine in the λ/ιPKC regulatory domain completely abolished the binding of LIP (Table 1). To our knowledge, this is the first report of a protein that interacts with a PKC through the putative zinc finger lipid-binding domain.

LIP interacts with λ/ιPKC in vitro and in vivo. To further investigate the interaction between LIP and λ/ιPKC, the cloned partial LIP cDNA (clone R4) was expressed as an MBP fusion protein and tested to see if it could interact in vitro with either the region encompassing amino acids 1 to 126, the zinc finger, or the whole regulatory domain of both λ/ιPKC and ζPKC, which have been expressed as GST fusion proteins (Fig. 5A). Two different concentrations (200 or 400 nM) of MBP or MBP-LIP(R4) were incubated with 800 nM GST fusion proteins for 1 h at 4°C, after which MBP and MBP-LIP(R4) were immobilized on amylose resin. Following extensive washing with binding buffer plus 0.1% Triton X-100, bound MBP fusion proteins and any associated proteins were boiled in sample buffer, fractionated by SDS-PAGE, and immunoblotted with anti-GST antibody to detect binding of the different GST fusion proteins to LIP. Of note, GST-λ/ιPKC^{ZF} and GST-λ/ιPKC^{REG} but not GST, GST-λ/ιPKC^{126}, or any GST-ζPKC fusion proteins bound to MBP-LIP(R4) (Fig. 5B; data not shown for ζPKC). Also, no binding of GST-λ/ιPKC^{ZF} or GST-λ/ιPKC^{REG} to MBP alone was detected (Fig. 5B). Taken together, these data indicate that LIP specifically binds in vitro to the zinc finger domain of λ/ιPKC. Staining of a parallel gel confirmed that all the reactions contained equal molar amounts of GST (Fig. 5C) and MBP (not shown) fusion proteins.

To determine whether LIP and λ/ιPKC can also interact in vivo, we next used the MBP-LIP(R4) fusion protein to immunize rabbits and generate an antibody against LIP. This antiserum specifically recognizes a band of 80 kDa in immunoblots of extracts from HeLa cells that is not seen with the preimmune serum. The molecular mass of that band is in good agreement with that predicted from the protein sequence (Fig. 3). Actively proliferating HeLa cells were immunoprecipitated, either in the absence or in the presence of the competing peptide, with an antibody that recognizes an epitope present in both λ/ιPKC and ζPKC. Immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-LIP antibody. Interestingly, results from Fig. 6A demonstrate that LIP is specifically detected in the immunoprecipitates performed in the absence of competing peptide, indicating the in vivo association of LIP with λ/ιPKC and/or ζPKC in proliferating cells. Since the antibody used during the immunoprecipitation does not discriminate between λ/ιPKC and ζPKC, the interaction between LIP and ζPKC in vivo cannot be completely ruled out, although the experiments described above and in the next section make this possibility highly unlikely. We next determined whether the association of LIP with λ/ιPKC in vivo can be triggered by serum. Quiescent NIH 3T3 cells were stimulated with serum for different times, after which cell lysates were immunoprecipitated with the anti-λ/ιPKC-ζPKC antibody and the immunoprecipitates were analyzed by immunoblotting with anti-LIP antibody, following their fractionation by SDS-PAGE. Of note, quiescent cells displayed little or no binding of LIP to λ/ιPKC but their association was dramatically increased by stimulation with serum (Fig. 6B). This increased activation was maximal by 10 min (Fig. 6B). All the above data suggest that LIP may be an important regulator of λ/ιPKC activity in response to mitogenic activation.

LIP is a potent activator of λ/ιPKC but not of ζPKC in vitro and in vivo. Because LIP binds specifically to the zinc finger of λ/ιPKC and that region in classical PKC isoforms has been shown to mediate the binding of stimulants (32, 34), we sought to determine if the interaction of LIP with λ/ιPKC can regulate its enzymatic activity both in vitro and in vivo. Therefore, serum-starved Cos cells were transfected with HA protein epitope-tagged *X. laevis* λ/ιPKC or rat brain ζPKC and the expressed proteins were immunopurified with the anti-HA monoclonal antibody 12CA5. The addition of phosphatidylserine (a classical activator of all PKCs) to the immunoprecipitates significantly activated both λ/ιPKC and ζPKC (Fig. 7). Interestingly, the addition of 1 μg of MBP-LIP(R4) but not of MBP alone produced a dramatic activation of HA-λ/ιPKC but not of ζPKC, comparable to that produced by phosphatidylserine (Fig. 7). Therefore, LIP not only binds specifically to

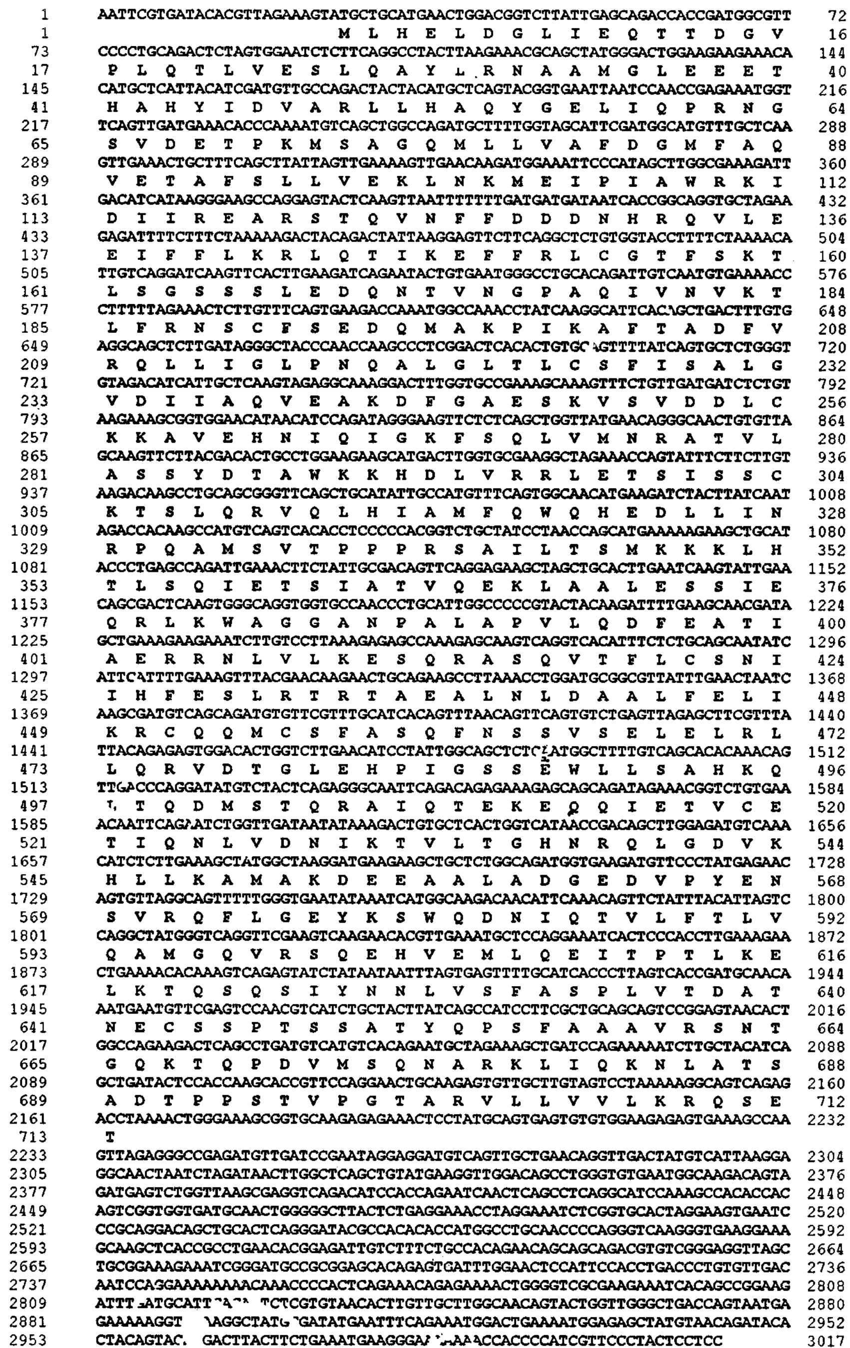

FIG. 3. DNA and deduced amino acid sequences of LIP. Nucleotides and amino acids are numbered on the left and right. Amino acid residue 1 is the putative initiator methionine. The single-letter amino acid code is used. Both strands of the cDNA were sequenced.

λ/ιPKC but also is a potent activator of this kinase in vitro. The addition of LIP plus phosphatidylserine together did not produce any additive or synergistic effect (not shown). To determine whether this is also true in vivo, quiescent Cos cells were transfected with either HA-λ/ιPKC or HA-ζPKC and were simultaneously cotransfected with either an empty plasmid or an expression plasmid for LIP, after which they were stimulated with serum for 5 min, and both transfected PKCs were recovered with anti-HA antibody. Interestingly, expression of LIP dramatically activated λ/ιPKC but not ζPKC in vivo to an extent comparable to that produced by serum (Fig. 8). The same stimulation was observed when λ/ιPKC and LIP were transfected into NIH 3T3 cells (results not shown). Taken together, these results indicate that LIP is a novel protein capable of specifically and directly binding to the atypical λ/ιPKC isoform both in vitro and in vivo, which leads to its specific activation.

Transactivation of a κB-dependent promoter by LIP. Since the activation by overexpression of λ/ιPKC leads to the transactivation of κB-dependent promoters (7) (Fig. 1), transfection of a LIP expression vector should conceivably be able to activate that reporter plasmid. Results in Fig. 9 demonstrate that

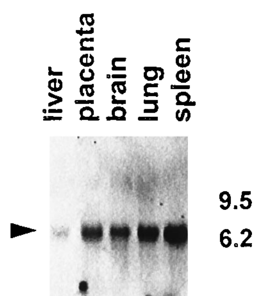

FIG. 4. LIP tissue distribution. Total RNA (10 μg) from the indicated human tissues was run in formaldehyde-agarose gels, blotted onto a nylon filter, and hybridized to a cDNA probe for LIP, as described in Materials and Methods. Each RNA sample was normalized by ethidium bromide staining. The sizes (in kilobases) of RNAs are given to the right of the gel. Essentially identical results were obtained in another two experiments.

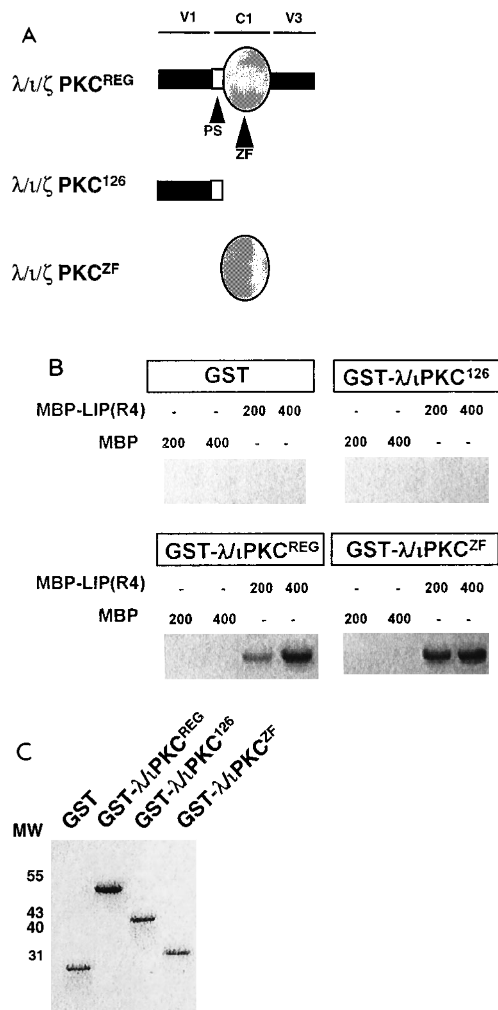

FIG. 5. Interaction of LIP and λ/ιPKC in vitro. (A) Purified GST or GST fusion proteins encoding amino acids 1 to 126 (GST-λ/ιPKC^{126}), the zinc finger (GST-λ/ιPKC^{ZF}), or the whole regulatory domain (GST-λ/ιPKC^{REG}) of λ/ιPKC at 800 nM were incubated with either 200 or 400 nM MBP or MBP-LIP(R4) for 1 h at 4°C, and the MBP fusion proteins were then immobilized on amylose resin. (B) After extensive washing, bound MBP fusion proteins and any associated proteins were boiled in sample buffer, fractionated by SDS-PAGE, and immunoblotted with anti-GST antibody to detect binding of the different GST fusion proteins. (C) Staining of a parallel gel confirming that all the reaction mixtures contained equal molar amounts of GST fusion proteins. Essentially identical results were obtained in another two experiments. PS, pseudosubstrate; ZF, zinc finger; MW, molecular weight (in thousands).

the transfection of a LIP expression vector promotes a dramatic activation of a κB-dependent promoter to an extent similar to that produced by TNF-α (7). Interestingly, cotransfection of a λ/ιPKC expression plasmid significantly synergizes with LIP to transactivate the κB-reporter plasmid (Fig. 10). Conversely, transfection of a kinase-inactive dominant negative mutant of λ/ιPKC severely impairs the activation of the κB promoter by LIP (Fig. 10). However, transfection of the kinase-inactive dominant negative mutant of ζPKC does not produce any effect on the stimulation of that promoter by LIP (not shown). All the above data indicate that the activation by LIP of λ/ιPKC in vivo is physiologically relevant.

DISCUSSION

PKCs form a large family of lipid-activable kinases that play pivotal roles in cell signalling. The so-called classical or conventional PKCs are critically involved in cell processes associated with the generation of increased cytosolic Ca^{2+} levels and diacylglycerol (32), such as those produced by the activation of the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-P_2 (32). More recently, increased interest has been focused on the understanding of the functional role, as well as the mechanisms that regulate the novel and atypical PKC isoforms. Both kinds of PKC subtypes have been implicated in the control of key cellular events such as cell proliferation. Thus, overexpression of the novel εPKC leads to increased cell growth whereas overexpression of δPKC provokes the opposite effect (26, 32). Regarding the atypical PKC isotypes, it has been recently demonstrated that the microinjection of a peptide with the sequence of the pseudosubstrate of these isoforms dramatically inhibits oocyte maturation (10), cell proliferation (3), and NF-κB activation (11). The pseudosubstrate region of ζPKC is identical to that of λ/ιPKC but is significantly different from that of conventional and novel PKCs (32), indicating that either or both atypical isoforms are important in cell signalling. The recent report of the sequence of λ/ιPKC as a distinct atypical isotype (1, 38) has allowed the identification of the previously cloned *X. laevis* ζPKC isoform as actually λ/ιPKC. Inhibition of λ/ιPKC by antisense oligonucleotides (10) or by transfection or microinjection of a kinase-inactive dominant negative mutant (3, 7, 9) produced a severe reduction of important cell signalling parameters such as the stimulation of the κB-dependent promoter activity (4, 7). We show here that transfection of kinase-inactive mutants of either λ/ιPKC or ζPKC severely impaired the activation of a κB-dependent promoter. Also, the overexpression of the wild-type genes of either atypical isotypes activated this reporter construct. This is a reliable and sensitive biological readout of the mitogenic and inflammatory responses. Although both isotypes similarly regulate the κB-promoter activity, λ/ιPKC constructs were significantly more potent than ζPKC constructs were (Fig. 1). The fact that the two kinases displayed comparable behavior at high concentrations (Fig. 1) was to some extent predictable from the identity of their catalytic domains. Although the major differences between the atypical isoforms are located in their regulatory regions, both isotypes are activated by ceramide with similar kinetics and both bind Ras in vitro with identical affinity (23). Therefore, we reasoned that if functional differences underlay the sequence divergences existing in the regulatory domain of the two atypical isotypes, this could more probably be unveiled by the molecular characterization of hy-

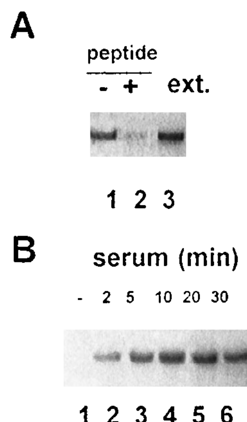

FIG. 6. Interaction of LIP and λ/ιPKC in vivo. (A) A 100-μg portion of protein from actively proliferating HeLa cells was immunoprecipitated with an antibody that recognizes an epitope of λ/ιPKC and ζPKC either in the absence (lane 1) or in the presence (lane 2) of competing peptide. Immunoprecipitates along with 20 μg of protein of whole-cell extract (lane 3) were separated by SDS-PAGE and immunoblotted with an anti-LIP antibody. Essentially identical results were obtained in another three experiments. (B) Extracts from quiescent NIH 3T3 cells that were either untreated (lane 1) or stimulated with 10% serum (lanes 2 to 6) were immunoprecipitated with anti-λ/ιPKC-ζPKC antibody and immunoblotted with anti-LIP antibody as described for panel A. Essentially identical results were obtained in another three experiments.

pothetical novel regulatory proteins that could potentially interact with either λ/ιPKC or ζPKC and specifically regulate either activities. This could account for the different sensitivities of the κB-dependent promoter activity to low concentrations of λ/ιPKC versus ζPKC. We report here the molecular cloning and characterization of a novel protein, termed LIP, that specifically interacts with the zinc finger domain of λ/ιPKC but not ζPKC. This interaction is detected by three different experimental approaches: (i) the yeast two-hybrid system used to clone it; (ii) in vitro assays with recombinant bacterially expressed LIP and different λ/ιPKC- and ζPKC-expressed fragments; and (iii) coimmunoprecipitation experiments. What is really interesting about LIP is that it specifically activates λ/ιPKC but not ζPKC in vitro as well as in vivo and that its association with this PKC is dramatically activated following the mitogenic stimulation of quiescent cells. Therefore, although the two atypical PKC subspecies display similar functions, we demonstrate here the existence of at least one regulatory protein that is capable of specifically activating one of them but not the other in vitro and in vivo. The fact that expression of LIP leads to the activation of a κB-dependent promoter in a λ/ιPKC-dependent manner underscores the likely physiological role of this novel PKC activator.

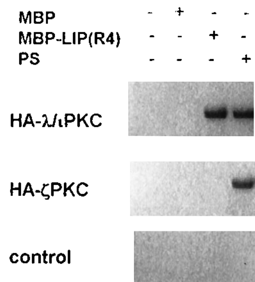

FIG. 7. Activation of λ/ιPKC but not ζPKC by LIP in vitro. Cos cells were transfected with either control plasmid or expression vectors for HA-tagged ζPKC or λ/ιPKC, after which they were made quiescent by serum starvation, and the expressed proteins were immunoprecipitated with an anti-HA antibody, as described in Materials and Methods. The effect of 1 μg of MBP, 1 μg of MBP-LIP(R4), or phosphatidylserine (PS) (50 μg/ml) on regulation of the enzymatic activity of HA-ζPKC or HAλ/ιPKC was determined. The two kinases were expressed at identical levels (not shown). Essentially identical results were obtained in another two experiments.

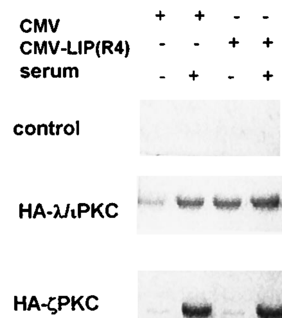

FIG. 8. Activation of λ/ιPKC but not ζPKC by LIP in vivo. Cos cells were transfected with either control plasmid or expression vectors for HA-ζPKC or HA-λ/ιPKC along with either control plasmid (CMV) or a LIP(R4) expression vector, after which they were made quiescent by serum starvation and were either untreated or stimulated with 10% serum for 10 min. Then the HA-tagged PKCs were immunoprecipitated and their activity was determined as described in Materials and Methods. The two kinases were expressed at identical levels (not shown). Essentially identical results were obtained in another two experiments.

A number of PKC-interacting proteins have recently been identified and cloned. Thus, PICK1, a perinuclear protein that binds to the catalytic domain of PKCα and is phosphorylated in vitro by this PKC isotype, has been described (40). Also, the characterization of another class of PKC-binding proteins, collectively termed RACKs, has been reported (27, 35). Interestingly, they appear to be important for the subcellular localization of activated classical PKC subspecies, a process that seems critical for their functionality in vivo (27, 35). One of the most remarkable aspects of LIP is that it activates λ/ιPKC most probably by directly binding to the kinase zinc finger-like domain, a region thought to be stimulated only by lipids and their pharmacological analogs (32, 34). From the data presented here, the zinc finger domain of the PKCs should be considered a site for activation of these enzymes, not only by lipids but also

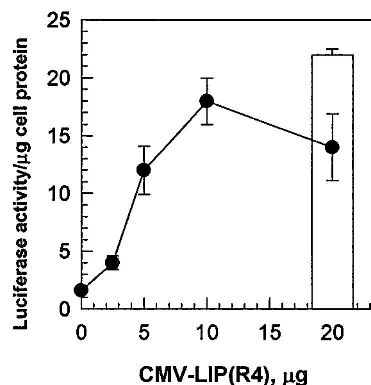

FIG. 9. Transfection of a LIP expression vector is sufficient to transactivate a κB-dependent promoter. Cos cells were transfected with different concentrations of plasmid CMV-LIP(R4) along with 500 ng of the κB enhancer-directed luciferase reporter plasmid (3EconALuc). After 4 h, the DNA-containing medium was removed and cells were made quiescent by serum starvation for 24 h. Then extracts were prepared and the luciferase activity was determined as described in Materials and Methods. As a control, cells transfected with empty plasmid were stimulated with TNF-α (500 U/ml) for 4 h (open bar). Results are the mean ± SD of three independent experiments with incubations in duplicate.

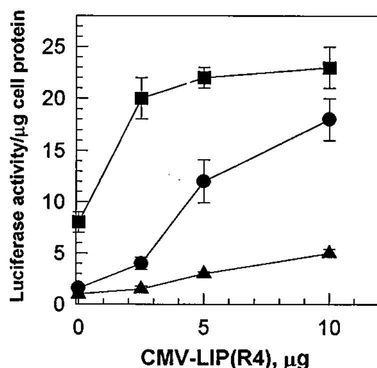

FIG. 10. Dependence on λ/ιPKC for the transactivation of a κB promoter by LIP. Cos cells were transfected with different concentrations of plasmid CMV-LIP(R4) along with 500 ng of the κB enhancer-directed luciferase reporter plasmid (3EconALuc) and either a control plasmid (●) or expression vectors for wild-type λ/ιPKC (■) or a kinase-inactive mutant of λ/ιPKC (▲). After 4 h, the DNA-containing medium was removed and cells were made quiescent by serum starvation. Then extracts were prepared and the luciferase activity was determined as described in Materials and Methods. Results are the mean ± SD of three independent experiments with incubations in duplicate.

by functionally relevant regulatory proteins. Actually, a very recent report shows that the zinc finger domain of εPKC serves to localize this isoform to the Golgi apparatus (21). Whether this has a functional repercussion in the activity of εPKC deserves further investigation. Since LIP binds to λ/ιPKC but not ζPKC, one can speculate on the existence of distinct LIP-like proteins specific for the different PKC isotypes. If further work confirms this model, it will mean that the specificity for the activation of the different PKC isoforms cannot be totally accounted for by lipid mediators but that specific PKC zinc finger-binding proteins are most probably also involved. This implies a change in the way we have so far envisioned the mechanism of PKC regulation.

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