We have cloned and characterized a novel striated muscle-restricted protein (Cypher) that has two mRNA splice variants, designated Cypher1 and Cypher2. Both proteins contain an amino-terminal PDZ domain. Cypher1, but not Cypher2, contains three carboxyl-terminal LIM domains and an amino acid repeat sequence that exhibits homology to a repeat sequence found in the largest subunit of RNA polymerase II. cypher1 and cypher2 mRNAs exhibited identical expression patterns. Both are exclusively expressed in cardiac and striated muscle in embryonic and adult stages. By biochemical assays, we have demonstrated that Cypher1 and Cypher2 bind to α-actinin-2 via their PDZ domains. This interaction has been further confirmed by immunohistochemical studies that demonstrated co-localization of Cypher and α-actinin-2 at the Z-lines of cardiac muscle. We have also found that Cypher1 binds to protein kinase C through its PDZ domain. Phosphorylation of Cypher by protein kinase C has demonstrated the functional significance of this interaction. Together, our data suggest that Cypher1 may function as an adaptor in striated muscle to couple protein kinase C-mediated signaling, via its LIM domains, to the cytoskeleton (α-actinin-2) through its PDZ domain.

The LIM domain is a recently identified cysteine-rich motif defined by 50–60 amino acids with the consensus sequence C\(_X\)_16–23HXC\(_X\)_16–23CX\(_X\)_2(C/H/D), which contains two closely associated zinc-binding modules (1–3). The acronym “LIM” derives from three genes in which the LIM domain was first described: lin-11 from Caenorhabditis elegans (4), Isl1 from rat (5), and mec-3 from C. elegans (6). LIM domains have been identified in a variety of proteins and can be roughly classified into three groups. The first group contains both LIM domain(s) and one or more recognizable domains exclusive of homeodomains (7). Some LIM domains are nuclear proteins involved in cell lineage determination and pattern formation during development (8). Others encode proteins associated with the cytoskeleton and play a role in adhesion plaque and actin microfilament organization (9, 10). Genetic studies have demonstrated important roles of LIM domain proteins in cell lineage determination, cytoskeletal structure, and organogenesis. Disruption of several homeodomain/LIM genes has demonstrated their importance in neuronal lineage development (8). The LIM-only protein LMO2 is required for erythropoiesis at early developmental stages, as evidenced by the lack of erythropoiesis in LMO2-deficient mice, resulting in embryonic mortality (11).

Recently, it has been shown that mice lacking muscle LIM protein (MLP/CRP3) develop dilated cardiomyopathy with hypertrophy and heart failure after birth (12). MLP is a LIM-only protein, consisting of two LIM domains, that is expressed abundantly in heart and skeletal muscle (13). We hypothesized that other LIM domain-containing proteins may also play important roles in cardiac function. Characterization of these proteins will help us to better understand the function of LIM domains and may identify candidate genes involved in cardiomyopathies. Here, we report the cloning and characterization of a novel striated muscle-restricted LIM domain-containing protein that we have named Cypher owing to its homology to another LIM domain protein, Enigma.

**MATERIALS AND METHODS**

**GenBank** and EST DATABASE SEARCH—The BLAST search program (14) was used to search the GenBankTM and EST data bases. Positive clones were purchased from Genome Systems Inc.

**Library Screening**—106 bacteriophage clones from a mouse embryonic day 13 heart cDNA library (Stratagene, catalog no. 936306) were screened according to the manufacturer’s protocol. The probe for library screening and Northern blotting was synthesized by PCR with the EST clone as template and primers p1 (5’-CCAAGTGCATGGATGCGATGGAAGC-3’) and p2 (5’-TCTCTTTGATGAACTACAATAAT-3’).

**Isolation of Mouse α-Actinin-2**—A search of mouse EST clones with human α-actinin-2 cDNA revealed multiple EST clones encoding mouse α-actinin-2. One positive EST clone (AI006532) was purchased from Genome Systems Inc. Sequence analysis indicated that this clone encodes full-length mouse α-actinin-2.

**Plasmid Construction**—Expression and GST fusion constructs were generated by PCR using Pfu DNA polymerase (Stratagene) followed by cloning into pcDNA3FLAG (modified from pcDNA3 (Inviogene) to include a FLAG epitope tag), pT7B01-HA (15), or pGEX-4T-1 (Amersham Pharmacia Biotech). Each plasmid was sequenced to ensure that the
open reading frame was intact and that no PCR errors had occurred in the amplified fragment.

In Situ Hybridization—A 325-bp and a 373-bp PCR product of cypher cDNA (encoding amino acids 1–84 plus 73 bp of 5′-untranslated region and amino acids 662–723 plus 190 bp of 3′-untranslated region, respectively) were subcloned into pT3/7 to generate templates for sense and antisense 35S-UTP-labeled probes. Paraffin sections at different stages of mouse development were hybridized with 10,000 cpm 35S-labeled riboprobe/ml in hybridization solution containing 50% formamide, 30 mM NaCl, 20 mM EDTA, 10 mM Na2HPO4, 10% dextran sulfate, 1× Denhardt’s solution, and 10 mM dithiothreitol. Hybridization was carried out for 14 h at 60 °C. RNase treatment and washes were performed according to Ruiz-Lozano et al. (16). Slides were dipped in Ilford K5 photographic emulsion, exposed for 3 weeks, and developed in Eastman Kodak D19 solution. Counteringstained was performed in 0.02% toluidine blue/hematoxylin, and slides were mounted in Permount solution.

GST Fusion Protein Binding Assay—Proteins were labeled with [35S]methionine using the TNT coupled reticulocyte lysate system (Promega). Each GST fusion protein (200 pmol) was bound to 20 μl of glutathione-agarose beads and washed with binding buffer (1% Triton x-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, one tablet protease inhibitor mixture/40 ml (Roche Molecular Biochemicals), 150 mM NaCl, and 0.1% bovine serum albumin in phosphate-buffered saline), and the beads were resuspended in 400 μl binding buffer. Five to ten μl of in vitro labeled protein were adjusted to 100 μl with binding buffer and added to 400 μl of GST fusion protein beads. The mixture was then incubated for 4 h at 4 °C, and beads were washed six times with 100 μl of binding buffer each time.

Cotransfection and Co-immunoprecipitation from COS-7 Cells—Plasmids (5 μg each) were cotransfected into COS-7 cells plated in 10-cm dishes at 80% confluence via Superfect (QIAGEN Inc.). Cells were harvested and immunoprecipitated essentially as described (15) using 1.5 μg of anti-FLAG (M2, Sigma) or 1 μg of anti-HA (12CA5, Roche Molecular Biochemicals) antibodies. Immunoprecipitated complexes or 5% of the volume of cell extract used for immunoprecipitation was immunoblotted with anti-FLAG or anti-HA antibodies.

Phosphorylation Assay—The phosphorylation assay was performed essentially as described by Kuroda et al. (15). Briefly, the immunoprecipitated beads were mixed with 25 μl of reaction buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM ATP, 8 μg/ml phosphatidylserine, 20 μg/ml phosphatidylserine, 20 μg/ml phosphatidylserine, 20 μg/ml phosphatidylserine, 20 μg/ml phosphatidylserine), and incubated for 60 min at 30 °C. The phosphorylated proteins were resolved on an SDS-PAGE gel transferred to a nitrocellulose membrane and subjected to immunoblotting with anti-FLAG or anti-HA antibodies.
and 0.8 mg/ml diolein). Following addition of 1 μl of [γ-32P]ATP (10 mCi/ml), the mixture was incubated at 30 °C for 15 min. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

**SDS-Polyacrylamide Gel Electrophoresis and Western Immunoblotting—**SDS-polyacrylamide gel electrophoresis was performed as described in the protocols of the TNT coupled reticulocyte lysate system. Western immunoblot analyses were performed following the procedures provided by Millipore Corp. Detection was performed via chemiluminescent techniques (ECL, Amersham Pharmacia Biotech).

**Antibodies and Immunohistochemistry—**Rabbit anti-mouse Cypher polyclonal antibodies were raised against keyhole limpet hemocyanin-conjugated polypeptide SGGPSESASRPPWVTDDSFS (amino acids 474–493 of Cypher1) by Research Genetics.

For immunofluorescent staining, mouse hearts were fixed by perfusion and frozen in liquid nitrogen-cooled isobutane. Cryostat sections (16 μm) were collected onto Superfrost Plus-coated slides (Fisher). All steps were conducted at room temperature. Sections were washed in 0.1 M phosphate-buffered saline for 3-10 min. Blocking was performed for 30 min in phosphate-buffered saline containing 3% normal donkey serum, 1% bovine serum albumin, 1% cold water fish gelatin, 1 mM glycine, and 0.1% Triton X-100 (blocking buffer). Primary antibodies were applied as a mixture for 1 h. The anti-Cypher antibody was diluted 1:500, and the anti-actinin antibody was diluted 1:100. After several washes in blocking buffer diluted 1:3, sections were incubated in a mixture of donkey anti-rabbit fluorescein isothiocyanate and donkey anti-mouse RedX antibodies (Jackson ImmunoResearch Laboratories, Inc.), with a final dilution of 1:100. Sections were washed for 30 min in phosphate-buffered saline and coverslipped using Gelvatol antifade medium. Imaging was performed using a Bio-Rad MRC 1024 confocal microscope with a krypton-argon laser. Alignment between the red and green channels was calibrated using multicolor fluorescent beads (Molecular Probes, Inc.). The plate was prepared using Adobe Photoshop Version 6.0.

**RESULTS**

**Cloning cypher—**To clone novel LIM domain-containing gene(s) that might be important for cardiac development and/or function, the amino acid sequence of the LIM-only protein MLP (13) was used to search the mouse EST data base via the BLAST search program (14). We found an EST clone (W09036) that encoded a novel LIM domain-containing protein. To determine the expression pattern of this gene, Northern blot analyses were performed using the protocols of the TNT coupled reticulocyte lysate system. Western immunoblot analyses were performed following the procedures provided by Millipore Corp. Detection was performed via chemiluminescent techniques (ECL, Amersham Pharmacia Biotech).
60 positive clones were selected, subcloned, and partially sequenced. Two out of the 20 clones potentially containing full-length cDNAs were fully sequenced, and the sequences was deposited in GenBank™.

Sequencing revealed that the cDNA encodes a novel polypeptide of 723 amino acid residues with a predicted Mr of 77,000. A data base search with the deduced amino acid sequence indicated that the putative protein contains a PDZ domain at its amino terminus and three LIM domains at its carboxyl terminus. The proteins with highest homology to this novel protein are Enigma (17) and the Enigma homologue (ENH) (15). Owing to this homology to Enigma, we have named this protein Cypher.

Fig. 3. In situ hybridization analysis of cypher mRNA expression during early mouse development. A, Cypher1 was restricted to the myocardium (mc) at day 9 postcoitus. No expression in the endocardium (ec) or other embryonic structures was detected at these stages. B, at days 11.5–12 postcoitus, cypher1 mRNA expression remained myocardial in both atrial (a) and ventricular (v) chambers. Non-cardiac expression of Cypher1 was undetectable. The data shown here were obtained with a 373-bp probe that uniquely recognizes cypher1 mRNA. An identical pattern of expression was observed with a 325-bp probe for the N-terminal region common to cypher1 and cypher2 mRNAs (data not shown).

Fig. 4. In situ hybridization analysis of Cypher at embryonic day 14.5. Dorsal (A), ventral (B), and diaphragm (C) areas of parasagittal paraffin sections are shown. Every striated muscle analyzed expressed cypher1 at high levels, including skeletal muscle (sm), intercostal muscles (icm), atria (a), ventricle (v), pulmonary trunk (pt), and diaphragm (dph). No expression in the liver (lv) and cartilage primordium (cp). The expression signal was also detected in the pulmonary trunk. Sections shown here were hybridized with a 373-bp probe for the unique C terminus of Cypher1. An identical pattern of expression was observed with a 325-bp probe for the N-terminal region that is common to cypher1 and cypher2 mRNAs.
that there might be two alternatively spliced mRNAs (data not shown). To determine the full-length cDNA sequence of each isoform, an additional EST clone (W98628, Genome Systems Inc.) was fully sequenced, and the full-length cDNA sequence was deposited in GenBank™. As shown in Fig. 2B, the deduced polypeptide contains 288 amino acid residues with a predicted Mr of 31,430. We have designated this shorter splice variant cypher2 and the longer variant cypher1. Cypher2 does not contain either the C-terminal LIM domains or the Y/S/T/P/S/T/P amino acid repeats. Alignment of Cypher1 and Cypher2 indicated that they share two large identical regions: a 107-amino acid stretch beginning with the N-terminal PDZ domain and another 68-amino acid stretch just downstream of the PDZ domain (Fig. 2A).

To confirm the existence of two transcripts, Northern blot analysis was repeated using a probe corresponding to the N-terminal PDZ domain found in both Cypher1 and Cypher2. As shown in Fig. 1B, two hybridizing bands were observed. One was ~5 kilobases, corresponding to the size of the transcript previously observed by Northern blot analysis utilizing a probe corresponding to the C-terminal LIM domain sequence of Cypher1. The second was ~2 kilobases, which corresponds to the expected size for cypher2 mRNA. The results of this Northern blot analysis indicated that both cypher1 and cypher2 are expressed in adult heart and skeletal muscle and also, to a lesser extent, in lung. The cypher1/cypher2 transcript ratio is 1:4. Expression in lung is likely to reflect invasion of the pulmonary vein by the myocardium (24, 25). This explanation is in agreement with results seen for expression of cypher mRNA during embryogenesis (Fig. 4).

mRNA Expression Pattern of cypher during Embryonic Development—Two different probes were used to compare the expression patterns of cypher1 and cypher2. A 325-bp probe for the N-terminal region that is common to cypher1 and cypher2 mRNAs and another 373-bp probe for the unique C terminus of cypher1 mRNA. The results with each of the probes indicated that cypher1 and cypher2 mRNAs exhibited an identical pattern of expression. The results shown here were obtained with the 373-bp probe for the unique C terminus of cypher1 mRNA.

In situ hybridization performed on mouse embryonic sections from day 8.5–14.5 postcoitus embryos indicated that cypher mRNA was first expressed in a myocardium-specific manner at days 8.5–9 postcoitus and remained cardiac-restricted until day 12 (Fig. 3). cypher mRNA was strongly expressed throughout the heart in all stages examined.

Beginning at day 12.5 postcoitus, non-cardiac striated muscles started to express cypher at very low levels (data not shown). At embryonic day 14.5, cypher was expressed at high levels in both cardiac and skeletal muscle. Strong expression of cypher at this stage was also detected in striated muscles of the tongue, thoracic and abdominal muscles, leg, and diaphragm. Within the heart, cypher mRNA appeared to be restricted to the myocardium in both atrial and ventricular chambers. No hybridization signal was detected in the endocardium. An expression signal was also detected in the pulmonary trunk (Fig. 4). This expression pattern of cypher at embryonic days 14–15 is in agreement with results obtained by Northern blot analysis using adult tissues.

Both Cypher1 and Cypher2 Bind to α-Actinin-2 via Their PDZ Domains—Recently, it has been shown that the PDZ domain of ALP (actinin-associated LIM protein) can bind to α-actinin-2 (22). As the PDZ domain of Cypher shares high homology with that of ALP (57% identity and 76% positive; data not shown), we hypothesized that Cypher might also interact with α-actinin-2. To test this hypothesis, we cotransfected FLAG-tagged Cypher1 or Cypher2 with HA-tagged...
mouse α-actinin-2 expression vectors into COS-7 cells. The transfected cells were lysed, and proteins were immunoprecipitated with either anti-FLAG or anti-HA antibody. Western blot analyses showed that both Cypher1 and Cypher2 can interact with α-actinin-2 in vivo (Fig. 5, B and C).

To test whether the region of Cypher that interacts with α-actinin-2 is the PDZ domain, we made a series of deletion mutants of Cypher fused to GST. Assays in which GST fusion proteins were incubated with [35S]methionine-labeled α-actinin-2 showed that the 84-amino acid N-terminal PDZ domain bound to α-actinin-2. Other regions of Cypher did not bind to α-actinin-2 (Fig. 5D). Similar results were obtained in the immunoprecipitation assay with proteins isolated from cotransfected COS-7 cells (Fig. 5B).

**Cypher Co-localizes with α-Actinin-2 in Vivo at the Z-Lines in Cardiac Muscle**—To determine whether Cypher co-localizes with α-actinin-2 in vivo in cardiac tissue, we performed double-staining immunohistochemistry on cryosections of adult mouse heart utilizing antibodies that specifically recognize either staining immunohistochemistry on cryosections of adult mouse with antibodies against α-actinin and Cypher1, double immunofluorescence staining of a cryostat section from adult mouse heart with antibodies against α-actinin (red) and Cypher1 (green). Superimposition of the two images demonstrated the overlap in staining for both proteins (orange). Bar = 20 μm.

**Fig. 6. Cypher co-localizes with α-actinin-2 at Z-lines in cardiac muscle.** A, Western blot analysis of Cypher. Protein extracts from adult mouse kidney (K), liver (L), and heart (H) were run on SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and then probed with polyclonal antibodies raised against a keyhole limpet hemocyanin-conjugated polypeptide with the sequence SGGPSESASRPWVT-DDSFS (amino acids 474–493 of Cypher1). The size of the detected protein is in agreement with the predicted molecular weight of Cypher1. B, proteins were incubated with [35S]methionine-labeled ENH and bound to nin-2 showed that the 84-amino acid N-terminal PDZ domain of ENH can selectively bind to PKC α, but not PKCβ1, ε, or γ. The LIM domains of Enigma can selectively bind to PKCα, β1, and γ, but not to PKCα, δ, or ζ. The LIM domains of Enigma can selectively bind to PKCα, β1, and γ, but not to PKCγ, δ, or ε (15). To test whether or not Cypher can bind to PKC isoforms, we performed cotransfection assays with FLAG-tagged Cypher1 or Cypher2 with HA-tagged isoforms of PKC. As shown in Fig. 7, Cypher1 (but not Cypher2) can bind to all six tested PKC isoforms (α, β1, γ, δ, ε). Since Cypher1 (but not Cypher2) contains three LIM domains, we next tested whether the region of Cypher1 that interacts with PKC is the LIM domains. The results are shown in Fig. 7. As with full-length Cypher1, the Cypher1 LIM domains can interact with all six tested PKC isoforms.

To examine the functional significance of the association of Cypher1 and different isoforms of PKC and to determine whether Cypher1 can be phosphorylated by PKC, we performed immunoprecipitation and phosphorylation assays. The results, as shown in Fig. 7C, demonstrated that Cypher1 is phosphorylated by PKC.

**DISCUSSION**

We have identified a novel PDZ and LIM domain-containing protein, Cypher. *cypher* mRNA has two splice variants that we have termed *cypher*1 and *cypher*2. *cypher*1 and *cypher*2 have an identical expression pattern during development and in adult tissues, both being exclusively restricted to cardiac and other striated muscle. This expression profile suggests that Cypher plays an important role in the development and/or function of striated muscle.

PDZ domains interact with at least four distinct protein sequences. These include (S/T)XV (26–28); other PDZ domains, as hetero- or homo-oligomers (29, 30); LIM domains (31); and spectrin-like repeats in α-actinin-2 (22). Each PDZ domain may be capable of specifically binding to more than one protein. For example, the PDZ domain of PICK1 binds both to itself and to the C terminus of PKCa. Mutations of specific amino acids in the PDZ domain can selectively abolish binding to PKCa, but do not affect homo-oligomerization (30).

Utilizing both *in vitro* GST fusion binding assays and cell
Cypher Binds to α-Actinin-2 and Protein Kinase C

It has been shown that the LIM2 and LIM3 domains of the Cypher family member Enigma interact with the Tyr-containing tight-turn motifs of the Ret tyrosine kinase (glial cell line-derived neurotrophic factor receptor) and the insulin receptor, respectively (17, 32). Future studies will determine whether the LIM domains of Cypher act as adaptors for signaling mediated by these receptors.

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