Molecular Biology of the Cell
Vol. 10, 1973–1984, June 1999

The PDZ Domain of the LIM Protein Enigma Binds to β-Tropomyosin

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Submitted December 4, 1998; Accepted March 23, 1999
Monitoring Editor: Paul T. Matsudaira

PDZ and LIM domains are modular protein interaction motifs present in proteins with diverse functions. Enigma is representative of a family of proteins composed of a series of conserved PDZ and LIM domains. The LIM domains of Enigma and its most related family member, Enigma homology protein, bind to protein kinases, whereas the PDZ domains of Enigma and family member actin-associated LIM protein bind to actin filaments. Enigma localizes to actin filaments in fibroblasts via its PDZ domain, and actin-associated LIM protein binds to and colocalizes with the actin-binding protein α-actinin-2 at Z lines in skeletal muscle. We show that Enigma is present at the Z line in skeletal muscle and that the PDZ domain of Enigma binds to a skeletal muscle target, the actin-binding protein tropomyosin (skeletal β-TM). The interaction between Enigma and skeletal β-TM was specific for the PDZ domain of Enigma, was abolished by mutations in the PDZ domain, and required the PDZ-binding consensus sequence (Thr-Ser-Leu) at the extreme carboxyl terminus of skeletal β-TM. Enigma interacted with isoforms of tropomyosin expressed in C2C12 myotubes and formed an immunoprecipitable complex with skeletal β-TM in transfected cells. The association of Enigma with skeletal β-TM suggests a role for Enigma as an adapter protein that directs LIM-binding proteins to actin filaments of muscle cells.

INTRODUCTION

Conserved protein interaction domains found in many proteins with diverse functions provide molecular recognition essential for assembling multiprotein complexes (Pawson and Scott, 1997). LIM and PDZ domains are two protein interaction motifs that are widely distributed in cells of both plants and animals (Gill, 1995; Fanning and Anderson, 1996). LIM domains, named for the three homeodomain proteins in which they were first recognized (lin-11, isl-1, and mec-3) (Jurata and Gill, 1998) are cysteine-rich modules that contain two coordinated Zn^{2+} atoms (Perez-Alverado *et al.*, 1994). Nuclear LIM domains interact with nuclear LIM interactor (Agulnick *et al.*, 1996, Jurata *et al.*, 1996) and transcription factors (Wadman *et al.*, 1994), whereas cytoplasmic LIM domains bind to protein kinases (Wu and Gill, 1994; Kuroda *et al.*, 1996; Wu *et al.*, 1996), other LIM domains (Schmeichel and Beckerle, 1994), and cytoskeletal targets (Crawford and Bekerle, 1992; Arber and Caroni, 1996; Pomies *et al.*, 1997). PDZ domains, named for the three proteins in which they were first recognized (postsynaptic density-95, discs large, and zo1 tight junction protein) (Fanning and Anderson, 1996), are ~85 amino acid β-barrel structures (Doyle *et al.*, 1996) that bind to the consensus sequence (Ser/Thr)-X-(Val/Leu/Ile) contained in targets, most commonly at the carboxyl terminus (Kim *et al.*, 1995; Kornau *et al.*, 1995; Songyang *et al.*, 1997). PDZ domains are also reported to recognize internal consensus sites (Shieh and Zhu, 1996), other PDZ domains (Brenman *et al.*, 1996), spectrin-like repeats (Xia *et al.*, 1997), LIM domains (Cuppen *et al.*, 1998), and unspecified sites (Tsunoda *et al.*, 1997). Several PDZ domain-containing proteins serve as scaffolds for assembling components of large protein complexes at cell–cell junctions and for assembling proteins involved in signal transduction. The PDZ domains of PSD-95/SAP90 display individual specific binding to the *N*-methyl-D-aspartate receptor (Kornau *et al.*, 1995), potassium channels (Kim *et al.*, 1995; Cohen *et al.*, 1996), and neuroligins (Irie *et al.*, 1997). By

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clustering these membrane-associated proteins, PDS-95 is hypothesized to mediate the formation of an asymmetric cell–cell junction maintained by the interaction of neuroligins with β-neurexins in the extracellular space (Irie *et al.*, 1997). InaD is a multi-PDZ domain protein that serves as a scaffold for assembly of signaling molecules of the *Drosophila* vision system. By binding to distinct PDZ domains of InaD, a G-protein effector molecule (phospholipase C-β), a calcium channel (TRP), and an eye PKC are assembled for efficient rhodopsin-stimulated activation of phospholipase C-β followed by PKC-mediated deactivation of the light response (Tsunoda *et al.*, 1997). Recent studies indicate that Enigma, a protein that contains both PDZ and LIM domains, is involved in the assembly of an actin filament–associated complex essential for transmission of ret/ptc2 mitogenic signaling (Durick *et al.*, 1998).

Enigma is a member of an emerging family of proteins that contain amino-terminal PDZ domains and carboxyl-terminal LIM domains (Figure 1A). There are two subclasses of the Enigma family defined by the number of LIM domains; Enigma and Enigma homology protein (ENH) (Kuroda *et al.*, 1996) have three LIM domains that are 51, 59, and 70% identical, whereas RIL (Kiess *et al.*, 1995), CLP36 (Wang *et al.*, 1995), and actin-associated LIM protein (ALP) (Xia *et al.*, 1997) each have one LIM domain with 59–67% amino acid identity. A high degree of sequence identity is apparent among the PDZ domains of family members; Enigma and ENH have 69% identity, and RIL, CLP36, and ALP have 48–69% identity. The PDZ domains of Enigma and RIL, CLP36, and ALP are 42, 47, and 44% identical (Figure 1B). Family member PDZ domains are distinguished by the amino acids Pro/Ser-Trp in place of Gly-Leu in the "Gly-Leu-Gly-Phe" signature sequence of PDZ domains (Figure 1B, boxed).

The binding of the LIM domains of Enigma and its most related family member, ENH, to protein kinases led to their discovery. The tyrosine kinases Ret and insulin receptor recognize LIM2 and LIM3 of Enigma, respectively (Wu *et al.*, 1996), and PKC β-1 binds to each of the three LIM domains of ENH (Kuroda *et al.*, 1996). RIL was discovered as a gene that underwent down-regulation in H-ras-transformed cells (Kiess *et al.*, 1995), whereas CLP36 was shown to be down-regulated in heart in response to hypoxia (Wang *et al.*, 1995). ALP was identified by reverse transcription-PCR from skeletal muscle mRNA using degenerate primers designed to amplify an amino acid sequence conserved among PDZ domains. The PDZ domain of ALP binds to the actin-binding protein α-actinin-2, and these proteins colocalize at the Z lines of skeletal muscle (Xia *et al.*, 1997).

Recent studies indicated that Enigma and ALP associate with actin filaments via their PDZ domains; the

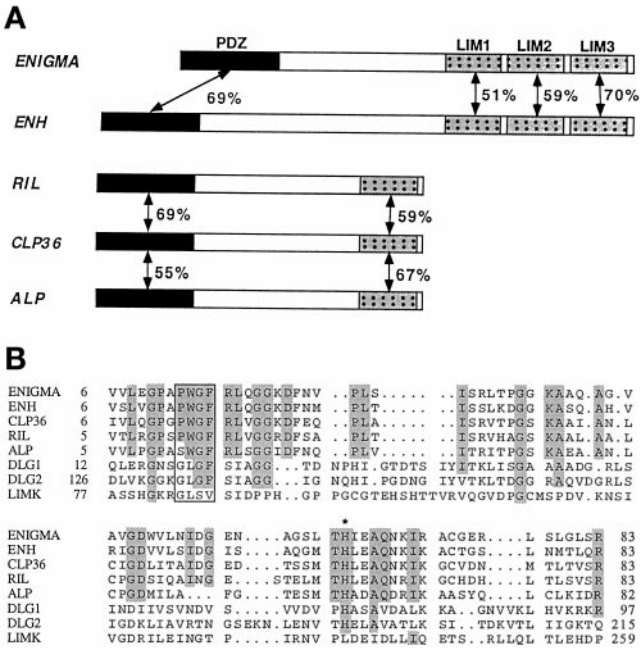

Figure 1. The Enigma family of PDZ-LIM proteins. (A) Enigma, ENH, RIL, CLP36, and ALP have conserved PDZ and LIM domains and represent a family of PDZ–LIM proteins. Percentages of amino acid identities between LIM and PDZ domains of family members are indicated. (B) Amino acid sequence alignment of PDZ domains of Enigma, ENH, RIL, CLP36, ALP, Dlg (PDZs 1 and 2), and LIM kinase. Four residues that reside in the carboxylate-binding loop of PDZ domains, the Gly-Leu-Gly-Phe sequence for which PDZ domains were originally named, are boxed. Among Enigma family members the sequence (Pro/Ser)-Trp-Gly-Phe occurs at this site. A histidine residue that is conserved in the Enigma family (His-63 in Enigma) is indicated with an asterisk. Amino acid identities among Enigma family and other PDZ domains are indicated by shading. The amino acid sequences used in this study were obtained from the following database entries: Enigma, L35240; ENH, U48247; RIL, X76454; CLP36, U23769; Dlg, M73529; LIM kinase, D26309; and ALP (Xia *et al.*, 1997).

PDZ domain of Enigma localized to actin microfilaments of fibroblasts (Durick *et al.*, 1998), and ALP localized to actin filaments that compose skeletal muscle myofibers (Xia *et al.* 1997). To investigate the linkage of Enigma to actin filaments, we sought to isolate targets of its PDZ domain. In the present study, the association of the PDZ domain of Enigma with the actin-binding protein skeletal muscle–specific β-tropomyosin (skeletal β-TM) is described. This interaction is specific for the PDZ domain of Enigma, is abolished by mutations within this PDZ domain, and requires a consensus binding site at the carboxyl terminus of skeletal β-TM. Enigma is localized at Z lines and at the boundary between the I band and Z lines of adult muscle. These findings identify skeletal β-TM as a target for the PDZ domain of Enigma and suggest that members of the Enigma family of PDZ-LIM proteins

function as adapters that localize LIM-binding proteins to actin filaments.

MATERIALS AND METHODS

Protein–Protein Interaction Screening

A λEX*lox* mouse embryonic day 16 (E16) library was screened using a ^{32}P-labeled GST fusion protein of the PDZ domain of Enigma (GST-PDZ$_{Eng}$) by the method described by Jurata *et al.* (1996). The PDZ domain (residues 2–96) of Enigma was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) using a 5′ primer that contained a *Bam*HI restriction site (5′-ATGGATCCGATTCCTTCAAAGTAGTG) and a 3′ primer that contained an *Eco*RI site (5′-ATGAATTCCTCAGGCGGAGGCCTTCTG), and the product was cloned into the *Bam*HI and *Eco*RI sites of pGEX-2TK (Pharmacia, Piscataway, NJ). GST-PDZ$_{Eng}$ was expressed from pGEX-2TK in *Escherichia coli* strain BL21, purified on glutathione-agarose (Sigma, St. Louis, MO), and eluted with glutathione. Labeling with [γ-^{32}P]ATP was performed by incubation of 2 μg of fusion protein with 1.5 μg of PKA catalytic subunit (provided by S.S. Taylor, University of California at San Diego) and 200 μCi of [γ-^{32}P]ATP in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT, for 3 h at 30°C. The probe was bound to glutathione-agarose, washed several times in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% Nonidet P-40 (Promega, Madison, WI), and eluted. Approximately 30% of the probe contained incorporated label.

Phage (1.3×10^6) were plated and incubated at 37°C until plaques were visible. Isopropyl β-D-thiogalactoside–impregnated nitrocellulose filters were placed on plates for induction at 4°C overnight. Filters were rinsed, blocked, and incubated with probe (250,000 cpm/ml) overnight at 4°C. Filters were then washed three times and exposed to X-OMat film (Eastman Kodak, Rochester, NY) for 24 h. Positive phage were plaque purified and converted to plasmid subclones.

GST Fusion Protein Interactions

GST-PDZ domains of Enigma, Dlg (Athar Chishti, St. Elizabeth's Medical Center, Boston, MA), ENH (Shun'ichi Kuroda, Biosignal Research Center, Kobe University, Kobe, Japan), and LIM Kinase (David Edwards, University of California at San Diego) were expressed in bacteria from pGEX plasmid constructs and purified on glutathione-agarose. For binding assays, 5 μg of GST fusion protein were incubated for 90 min with mixing by inversion at 4°C with solubilized in vitro translation products or cell extracts in buffer containing 20 mM HEPES, pH 7.0, 100 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitors. For C2C12 myoblasts and myotubes, one 150-mm dish was harvested per GST fusion incubation. Transfected 293 cell lysates were prepared from one 100-mm dish per anti-hemagglutinin (HA) immunoprecipitation. In all cases, beads were washed three times in the above buffer, suspended in SDS sample buffer, boiled 2 min, and separated by 10% SDS-PAGE. Dried gels were exposed for 1–4 h to BioMax film (Kodak) for in vitro translation experiments. T10-TM fusion proteins were translated from pEXlox-T10-TM, pEXlox-T10-TMc5, pEXlox-T10-TMc10, and pcDNA3-TM using the TnT quick coupled transcription/translation system (Promega).

Cell Culture

The C2C12 myogenic cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (Life Technologies, Gaithersburg, MD) containing 15% FCS (HyClone Laboratories, Logan, UT), 0.5% chick embryo extract (Life Technologies), and 2 mM L-glutamine in a humidified atmosphere of 8% CO_2 at 37°C. When cells were confluent, differentiation was induced by replacement of the medium with DMEM high glucose containing 5% horse serum (PAA Laboratories, Linz, Austria) and 2 mM L-glutamine. Myoblasts were harvested at 90% confluence, and myotubes were collected 5 d after induction of differentiation. Human embryonic kidney 293 cells were grown in DMEM high glucose with 10% cosmic calf serum (HyClone).

Cloning and Site-directed Mutagenesis

The full-length cDNA encoding the skeletal muscle isoform of TM-1 was generated by PCR using Pfu DNA polymerase (Stratagene) from a pEXlox clone isolated in the protein–protein interaction screen and cloned into pcDNA3 (Invitrogen, San Diego, CA). Site-directed mutagenesis of GST-PDZ$_{Eng}$ was accomplished using Quickchange (Stratagene) with the following primers: His63Ala, 5′-GCG GGT AGC CTC ACA GCC ATC GAA GCT CAG and 5′-CTG AGC TTC GAT GGC TGT GAG GCT ACC CGC; Gly15Ala/Phe16Ala, 5′-CTG GAG GGG CCA GCA CCT TGG GGC TTC CGG CTG CAA GGG GGC and 5′-GCC CCC TTG CAG CCG GGC GGC CCA AGG TGC TGG CCC CTC CAG. Truncations of TM were achieved by introduction of stop codons into TM-1 in pEXlox-T10-TM using Quickchange with the following primers: for the −5 mutant, 5′-GCG AGG AGC TGG ACA ACT GAG CAC TCA ATG ACA TCA CTT CC and 5′-GGA AGT GAT GTC ATT GAG TGC TCA GTT GTC CAG CTC CTC GC; and for the −10 mutant, 5′-GAA GTC TAT GCA CAG AAG TGA ATG AAG TAC AAG GCC ATC AGC and 5′-GCT GAT GGC CTT GTA CTT CAT TCA CTT CTG TGC ATA GAC TTC. Sequencing of 3′ and 5′ ends and internal sites of mutation was performed using the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, OH). Construction of pcDNA3-HA epitope–tagged Enigma and EnigmaΔPDZ (residues 275–455) were described previously (Wu *et al.*, 1996).

Transfection and Coimmunoprecipitation

HA-tagged Enigma and TM-1 were cloned into pcDNA3, and plasmids were transfected into 293 cells (one 100-mm dish per sample) using Superfect 1 (Qiagen, Hilden, Germany). After 60 h, cell extracts were prepared by solublization on ice with 20 mM HEPES-HCl, pH 7.0, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, followed by centrifugation at 12,000 rpm for 10 min at 4°C. Incubation with anti-HA mouse mAb 12CA5 (BabCO, Emeryville, CA) and protein A-Sepharose (Sigma) was for 90 min at 4°C. Anti-tropomyosin mouse mAb T-2780 (Sigma) was used at a dilution of 1:1000 for Western blotting. For detection, HRP-coupled anti-mouse (Amersham, Arlington Heights, IL) antibodies were used with enhanced chemiluminescence development (Amersham). Stripping of Western blots was achieved by incubation for 30 min at 70°C in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.8% β-mercaptoethanol, followed by extensive washing and blocking in 1% BSA.

In Situ Hybridization

In situ hybridization was performed as described by Angerer *et al.*, (1987). The RNA probe was generated using the Riboprobe System (Promega) with a *Not*I fragment of the mouse Enigma cDNA encoding the first 152 amino acids and 87 bp of 5′ untranslated sequence. Briefly, 14-μm frozen sections of paraformaldehyde (PF)-fixed tissue were pretreated with 10 μg/ml proteinase K and by acetylation in 0.1 M triethanolamine and 0.25% acetic anhydride. Tissue was hybridized overnight at 60°C in hybridization buffer (4× SSC, 50% formamide, 1× Denhardt's solution, 500 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, 10% dextran sulfate, 1 mM DTT, 1 mM EDTA, and 10 mM Tris-HCl) containing 1×10^7 cpm/ml ^{35}S-labeled probe. After hybridization, tissue was rinsed in 4× SSC, treated with 20 μg/ml RNase, and then desalted by a series of washes in SSC and 0.1 mM DTT. Tissue was then incubated at 65°C for 30 min in 0.1× SSC before dehydration in ethanol series containing 0.5× SSC and 1 mM DTT. For autoradiography, tissue

sections were exposed to Biomax-MR film (Kodak) overnight before dipping in NTB2 liquid emulsion to estimate the intensity of signal. Emulsion-dipped tissue sections were developed in D19 developer (Kodak) and fixative before dehydration and mounting in DePek (BDH Laboratory Supply, Poole, England). Photographic slides (35 mm) were taken using dark-field microscopy on a Nikon (Tokyo, Japan) Optiphot and scanned in a Nikon LS-1000 35-mm film scanner. Scanned images were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) for figure presentation.

Histology and Immunolocalization

For immunofluorescence or immunogold labeling of cryosections, mouse calf muscle, stretched on a stick, was fixed in 4% PF and 0.1 M phosphate buffer, pH 7.5 for 20 min, followed by 8% PF and 0.1 M phosphate buffer for 2 h at room temperature. Muscle was cut into 0.5-mm cubes, cryoprotected by infusing with 20% polyvinylpyrrolidone in 1.84 M sucrose for 4 h at room temperature, and frozen in liquid nitrogen. All the following incubations were done at room temperature for 1 h. Semithin 1-μm cryosections were prepared and incubated with anti-Enigma (rabbit polyclonal, 3941), anti-tropomyosin (mouse monoclonal, Sigma T-3651), or phalloidin-FITC followed by incubation in cross-absorbed FITC-conjugated donkey anti-mouse or TRITC-conjugated donkey anti-rabbit F(ab')2. Anti-Enigma antibody was generated by immunization with the amino-terminal 152 residues. Anti-tropomyosin is directed to an epitope in the amino terminus shared among tropomyosins. Ultrathin cryosections were prepared and incubated with anti-Enigma and then with anti-tropomyosin. Antibodies were diluted in 10% FCS and PBS followed by 10-nm or 5-nm gold-conjugated goat anti-rabbit or anti-mouse immunoglobulin G. Grids were stained in 2% neutral uranyl acetate for 20 min, absorption stained with 0.2% uranyl acetate, 0.2% methyl cellulose, and 3.2% polyvinyl alcohol, and observed in a JEOL (Peabody, MA) 1200 EX-11 microscope.

Because optical filter sets are not in exact registry, the alignment of digital images captured with different filters must be corrected for proper comparison. The optical filter sets used to capture digital immuofluorescent images of Enigma and tropomyosin were aligned using a standard provided by Dr. Velia Fowler (Scripps Research Institute, La Jolla, CA) and with the technical assistance of Ryan Littlefield (Scripps Research Institute, La Jolla, CA) as follows. The green and red optical filters were used to capture digital images of tropomodulin and phalloidin distribution in chick myofibrils. The image of tropomodulin captured using the green filter was shifted a certain amount of pixels relative to the red to correctly overlay the images from the two filter sets. Once the appropriate pixel shift was determined using the localization patterns of topomodulin and phalloidin in myofibrils as a standard, the same pixel shift was applied to images of Enigma and tropomyosin.

The distribution of Enigma was independently scored by three observers using eight separate electron microscopic sections. Relative distributions are averages per unit area assigned to the I band, Z line, including the boundary between the I band and Z line, and A band zone. Control experiments for nonspecific staining included incubating sections with secondary antibody alone and alternating the order in which primary antibodies were added in double-labeling experiments.

RESULTS

The PDZ Domain of Enigma Binds to Skeletal β-TM

To identify protein targets of the PDZ domain of Enigma, a mouse E16 phage expression library (1.3×10^6 phage) was screened using ^{32}P-labeled GST-PDZ_{Eng} as a probe. The majority of the positive clones were identified as skeletal β-TM. Tropomyosins are well-characterized components of actin filaments that bind directly to F-actin and have distinct functions in muscle, where they are involved in the calcium-dependent regulation of muscle contraction, and in fibroblasts, where they are constituents of actin filament structures with undefined functions (Lees-Miller and Helfman, 1991). Several isoforms of tropomyosin, some specific for skeletal muscle, heart, and brain, are products of alternative promoter usage and alternative mRNA splicing of at least two genes (α and β) in humans (Helfman *et al.*, 1986; Wieczorek *et al.*, 1988; Lees-Miller *et al.*, 1990). The β gene encodes a minimum of three tropomyosin isoforms that result from the use of alternate exons: β-tropomyosin, also known as fibroblast TM-1, skeletal muscle–specific β-TM, and β-TM-2. Sequencing of the 3′ ends of tropomyosin clones isolated in this screen revealed that only skeletal β-TM is recognized by the PDZ domain of Enigma. Skeletal muscle and fibroblast tropomyosins differ in amino acid sequences that may correspond to structural alterations in functional domains (MacLeod, 1982; Lewis *et al.*, 1983). One such difference is observed in the carboxyl termini of these isoforms such that skeletal β-TM possesses an amino acid sequence (Thr-Ser-Leu) that has the consensus characteristics (Ser/Thr-X-Val/Leu/Ile) of proteins targeted by PDZ domains (Songyang *et al.*, 1997), whereas fibroblast and smooth muscle tropomyosin terminates with Asn-Asn-Leu, a sequence not predicted to be a PDZ domain interaction site. Thus, skeletal β-TM was identified as a target of the PDZ domain of Enigma and contains a carboxyl-terminal PDZ domain target sequence.

Specificity of PDZ Domain Binding to Skeletal β-TM

As a test for specificity of Enigma binding to skeletal β-TM, the GST-PDZ domains of Enigma, Dlg, ENH, and LIM kinase were examined for their ability to bind skeletal β-TM fusion proteins. The PDZ domain of ENH is 69% identical to that of Enigma and thus provided a stringent test of specificity. GST-PDZ domains were expressed in bacteria, immobilized on glutathione-agarose, and incubated with ^{35}S-skeletal β-TM generated by in vitro translation. A Coomassie blue stain of the various GST-PDZ domains is shown in Figure 2A, lower panel. A 60-kDa fusion protein (T10-TM) resulted from translation of a full-length skeletal β-TM clone fused at its 5′ end to T7 gene 10 encoded in the pEXlox vector. The GST-PDZ domain of Enigma bound to the T10-skeletal β-TM protein, whereas the PDZ domains of ENH, Dlg, and LIM-kinase did not interact with T10-skeletal β-TM, indicating specificity in the binding of the PDZ domain of Enigma to skeletal β-TM (Figure 2A).

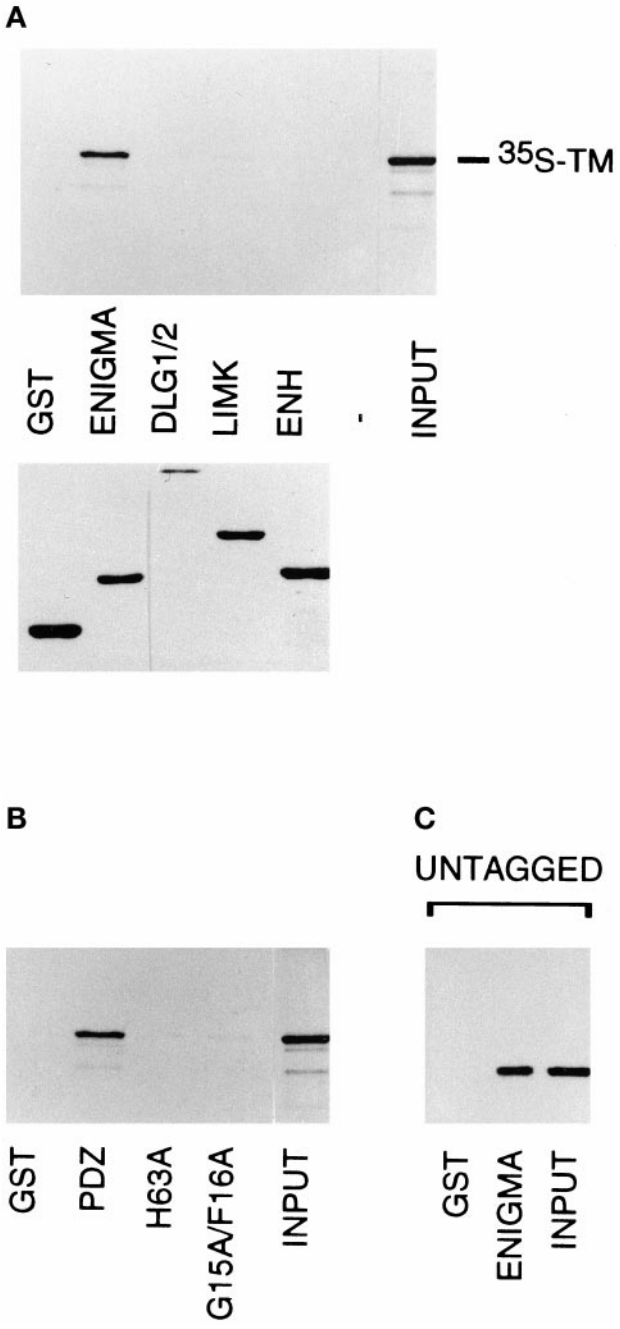

Figure 2. Specificity of the interaction of the PDZ domain of Enigma with TM-1. (A) Detergent extracts of skeletal β-TM fusion protein (^{35}S-T10-TM) generated by in vitro translation were probed with glutathione-agarose–coupled GST or GST-PDZ domains of Enigma, Dlg (PDZs 1 and 2), LIM kinase, and ENH. T10-TM binds selectively to the PDZ domain of Enigma. A Coomassie blue–stained gel of the various GST-PDZ domains is shown in the lower panel. Input represents 2% of the T10-TM extract incubated with GST-PDZ domains. (B) Demonstration of the inability of GST-PDZ_{Eng} mutants His63Ala and Gly15Ala/Phe16Ala to interact with T10-TM. Input represents 1.5% of the T10-TM extract incubated with GST-PDZ domains. (C) Untagged TM-1 binds to the PDZ domain of Enigma to the same extent as the corresponding fusion protein. Input represents 2% of the TM-1 extract incubated with GST-PDZ domains.

Mutation of the PDZ Domain Results in Loss of Interaction with Skeletal β-TM

To confirm the requirement for structural integrity of the PDZ domain for interaction with skeletal β-TM, mutations were introduced into the PDZ domain of Enigma. Mutants in the context of the GST-PDZ fusion protein were tested for their ability to bind to in vitro–translated T10-skeletal β-TM. Sites of mutation were selected based on the crystal structure of PDZ-3 of PSD-95 (Doyle *et al.*, 1996) and on sequence conservation among Enigma family members. Hydrogen bonding contacts between the PDZ domain of PSD-95 and target peptide side chains shown by the crystal structure indicate that His-372 of the PDZ domain plays an important role in forming a hydrogen bond with the hydroxyl group of a threonine residue at the −2 position of the target peptide. Mutation of the homologous His-63 of Enigma, which is conserved among Enigma family members (Figure 1B, asterisk), to Ala (H63A) was hypothesized to destabilize the PDZ domain interaction with the threonine residue at position −2 in skeletal β-TM. When this substitution was made the GST-H63A-PDZ domain no longer bound ^{35}S-labeled T10-skeletal β-TM (Figure 2B). Within the carboxylate-binding loop of the PDZ domain, hydrogen bonding to the carboxyl terminus of the peptide target is achieved in part through peptide interaction with amide nitrogens on the protein backbone of the residues Gly-Leu-Gly-Phe of PSD-95. The homologous sequence in Enigma, Pro-Trp-Gly-Phe, was mutated to Pro-Trp-Ala-Ala to destabilize and disrupt target binding. GST-G15A/F16A-PDZ did not bind to T10-skeletal β-TM (Figure 2B). To be certain that the T7 gene 10 fusion moiety did not influence PDZ binding, full-length skeletal β-TM was translated in vitro and incubated with GST-PDZ_{Eng} (Figure 2C). Skeletal β-TM bound to the same extent as the corresponding fusion protein. Thus, mutations in the PDZ domain of Enigma that are predicted to disrupt target interaction abolished the binding of GST-PDZ_{Eng} to T10-skeletal β-TM.

The Carboxyl-terminal Sequences of Skeletal β-TM Are Required for PDZ Domain Binding

The majority of PDZ domains bind to the carboxyl termini of target proteins (Songyang *et al.*, 1997), although other modes of interaction, such as PDZ–PDZ (Brenman *et al.*, 1996) and PDZ–spectrin-like repeats (Xia *et al.*, 1997), have been reported. Because skeletal β-TM terminates with an amino acid sequence consistent with a PDZ domain target, we tested the requirement of the extreme carboxyl terminal residues of skeletal β-TM for PDZ domain interaction using truncation mutants. The carboxyl terminal 5 or 10 amino acids of skeletal β-TM were deleted via insertion of stop codons. Mutated proteins were translated in vitro

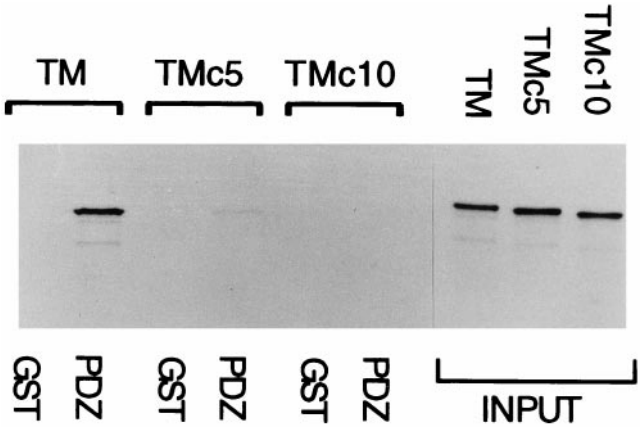

Figure 3. Interaction of the PDZ domain of Enigma requires the carboxyl terminus of skeletal β-TM. Truncation mutants having deletions of 5 or 10 amino acids from the carboxyl terminus of skeletal β-TM (TMc5 and TMc10, respectively) were translated in vitro as T10-TM fusion proteins and incubated with GST-PDZ_{Eng} or GST alone. Mutant skeletal β-TM proteins did not bind to GST-PDZ_{Eng}. Input represents 2% of translation reactions of skeletal β-TM, TMc5, and TMc10 incubated with GST beads.

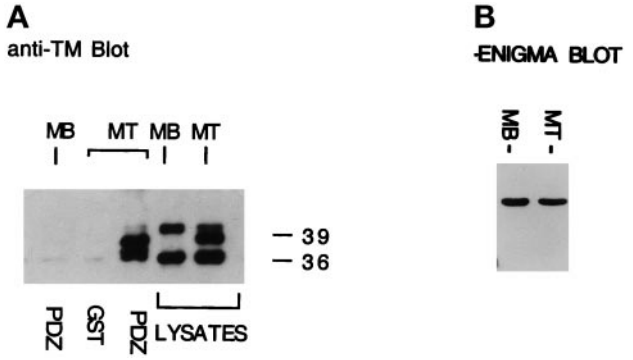

Figure 4. Association of the PDZ domain of Enigma with TM isoforms in C2C12 myotubes. (A) Detergent extracts of C2C12 myoblasts (MB) or myotubes (MT) were incubated with GST or GST-PDZ_{Eng} immobilized on glutathione-agarose. Two predominant forms of TM were detected in C2C12 lysates by Western blotting with anti-TM antibodies: 40- and 36-kDa forms were present in myoblasts, whereas myotubes expressed 39- and 36.5-kDa form. GST-PDZ_{Eng} selectively bound to the myotube-specific 39- and 36.5-kDa TM isoforms. Lysates represent 5% of the input cell extract before incubation with GST beads. (B) Anti-Enigma Western blot showing similar levels of Enigma expression in myoblasts (MB) and myotubes (MT).

and incubated with GST-PDZ_{Eng}. The removal of 5 or 10 carboxyl terminal amino acids of tropomyosin (TMc5 and TMc10) abolished binding to the PDZ domain of Enigma (Figure 3). Thus, the PDZ domain of Enigma bound to the carboxyl terminal sequence of skeletal β-TM predicted to be a PDZ binding site.

The PDZ Domain of Enigma Binds to Tropomyosin Isoforms Expressed in C2C12 Myotubes

To verify that the PDZ domain of Enigma binds to endogenous skeletal β-TM, differentiated C2C12 myogenic cells were used as a source of skeletal TM isoforms. As shown in Figure 4, anti-tropomyosin antibodies directed against a common exon recognize two predominant forms of tropomyosin with apparent molecular masses of 40 and 36 kDa in myoblasts. Two similar isoforms were present in mouse fibroblasts and presumably correspond to fibroblast TM-1 and TM-2 (Lees-Miller and Helfman, 1991). Upon differentiation into myotubes, a 39-kDa form appears, which migrates at an apparent molecular mass consistent with that of the skeletal muscle–specific β isoform (Lees-Miller and Helfman, 1991). A tropomyosin at the ~36.5-kDa position was also observed. Detergent extracts of myoblasts and myotubes were probed with agarose-coupled GST or GST-PDZ_{Eng}. GST-PDZ_{Eng} bound to the myotube-specific ~39-kDa tropomyosin isoform and also bound to the ~36.5-kDa form. GST-PDZ_{Eng} did not bind to either isoform expressed in myoblasts. Thus, tropomyosin isoforms that are upregulated in differentiated C2C12 myotubes are recognized by the PDZ domain of Enigma. The 36.5-kDa form in myotubes presumably represents skeletal α-tropomyosin, which has a carboxyl-terminal Thr-Ser-Ilu sequence.

To determine whether Enigma expression is modulated during myogenesis, protein levels in myoblasts and myotubes were compared by Western blotting. As shown in Figure 4B, Enigma protein levels are similar before and after differentiation of C2C12 cells, indicating that Enigma expression is not differentially regulated in C2C12 myoblasts and myotubes.

Coimmunoprecipitation of Enigma and TM

To determine whether Enigma and skeletal β TM form a complex in intact cells, HA-tagged Enigma and skeletal β-TM were expressed in 293 cells and immunoprecipitated using anti-HA antibodies. The endogenous tropomyosin isoforms recognized by the anti-tropomyosin antibody are expressed at low, nearly undetectable levels in 293 cells in comparison with tropomyosin levels observed in fibroblasts, CV-1, A431, and other cell lines examined (our unpublished results). In addition, 293 cells are unlikely to contain skeletal muscle tropomyosin isoforms, because muscle-specific tropomyosin exons are suppressed in nonmuscle cells (Helfman *et al*., 1986). Therefore, 293 cells offered an exceptionally low background for skeletal β-TM expression. Approximately 90% of the expressed skeletal β-TM was soluble when cells were extracted in buffer containing 1% Triton X-100.

HA-Enigma and skeletal β-TM formed a complex that was immunoprecipitated by anti-HA antibodies,

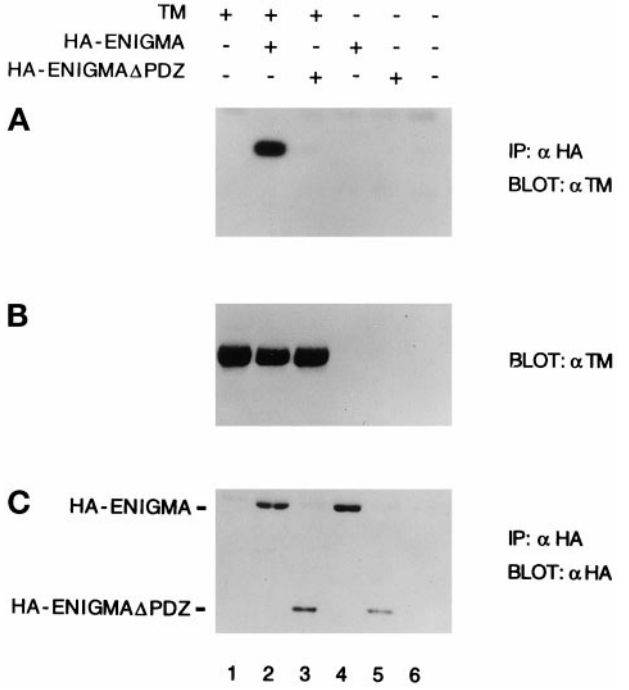

Figure 5. Coimmunoprecipitation of HA-Enigma and skeletal β-TM in transfected 293 cells. Proteins were expressed via transient transfection of 293 cells using the indicated pcDNA3 constructs: lane 1, skeletal β-TM; lane 2, skeletal β-TM and HA-tagged full-length Enigma; lane 3, skeletal β-TM and HA-tagged Enigma LIM domains (HA-EnigmaΔPDZ); lane 4, HA-tagged full-length Enigma; lane 5, HA-EnigmaΔPDZ; and lane 6, empty vector. Antibodies against the HA epitope were used to coprecipitate HA-Enigma with associated proteins. (A) Anti-TM Western blot indicates that skeletal β-TM associates with HA-Enigma (lane 2) but not with HA-EnigmaΔPDZ (lane 3). (B) Anti-TM Western blot of transfected cell lysates before immunoprecipitation (2% of input). (C) The blot in A was stripped and reprobed with anti-HA antibodies. Bands at ~56 and ~25 kDa indicate that both HA-Enigma (lanes 2 and 4) and HA-EnigmaΔPDZ (lanes 3 and 5) were immunoprecipitated by the anti-HA antibodies.

whereas HA-Enigma lacking the PDZ domain (HA-EnigmaΔPDZ) did not interact with skeletal β-TM (Figure 5A). An anti-TM blot of cell lysates before HA immunoprecipitation indicated high levels of tropomyosin expression in transfected cells (Figure 5B). To confirm the expression of HA-Enigma in transfected cells, the blot shown in Figure 5A was stripped and reprobed with anti-HA antibodies. Both HA-Enigma at ~56 kDa and HA-EnigmaΔPDZ at ~25 kDa were present in anti-HA-immunoprecipitates (Figure 5C). Thus, a PDZ domain-dependent complex of Enigma and skeletal β-TM formed in cells.

Enigma is Expressed in Muscle Cell Types during Embryogenesis

To determine the pattern of expression for Enigma, in situ hybridization studies were performed using embryonic and adult tissue. Enigma mRNA was predominantly detected in skeletal muscle tissues of E13.5 mouse embryos. Figure 6, A and B, shows dark-field images of sagittal sections through the E13.5 mouse embryo that were hybridized with a ^{35}S-labeled mouse Enigma probe and counterstained with hematoxylin. Enigma mRNA is detected in skeletal muscles, including the intrinsic muscle mass of the tongue (Figure 6A) and latissimus (Figure 6B). These high levels of expression in skeletal muscle contrast with undetectable expression throughout most of the nervous system, liver, and developing skeleton. In addition to skeletal muscle expression, Enigma transcripts were also present in cells associated with the equatorial zone of proliferation within the developing lens (Figure 6, B and C). Enigma expression in hippocampal cells of the adult brain was also observed (Figure 6D). Both the equatorial region of the embryonic lens and the adult hippocampus are regions marked by cells that are either proliferating or leaving the cell cycle. At early embryonic stages, Enigma was expressed throughout the embryo in subsets of mesenchymal, neural, and ectodermal tissue. Although the pattern of Enigma expression in the early embryo did not distinguish muscle precursors within the somite from other mesodermal derivatives, at E13.5 the presence of Engima transcripts in mesodermal derivatives was predominantly confined to muscle tissue (Figure 6, A and B).

Enigma and Skeletal β-TM Colocalize Near the Z Line in Adult Muscle

Immunofluorescence and immunoelectron microscopy were used to compare the subcellular distribution of Enigma, tropomyosin, and actin in adult muscle tissue. Phalloidin staining of myofibrils marks the location of the actin-rich I band and Z line (Figure 7B). Dual staining on the same tissue section revealed that Enigma was present at the Z line that lies along the midline of the I band (Figure 7, A and C). In addition, Enigma was detected along a subset of transverse filaments extending along the muscle fiber (Figure 7D). Tropomyosin was present in a doublet pattern near the Z line (Figure 7E). Engima and tropomyosin codistribution near the Z line was apparent by dual immunofluorescent staining of the same tissue section (Figure 7, D and E).

To more precisely detect the distribution of Enigma and tropomyosin within the sarcomere, immunoelectron microscopy was performed on ultrathin sections of adult skeletal muscle tissue. Enigma was predominantly detected along the Z line of the sarcomere and at the boundary of the Z line and I band, as indicated by distribution of 10-nm gold particles (Figure 8, A–C). Tropomyosin was detected throughout the I band, including the boundary between the I band and Z line (Figure 8, A and C), as indicated by the distribution of 5-nm gold particles (Figure 8, A–C). Quantitation of

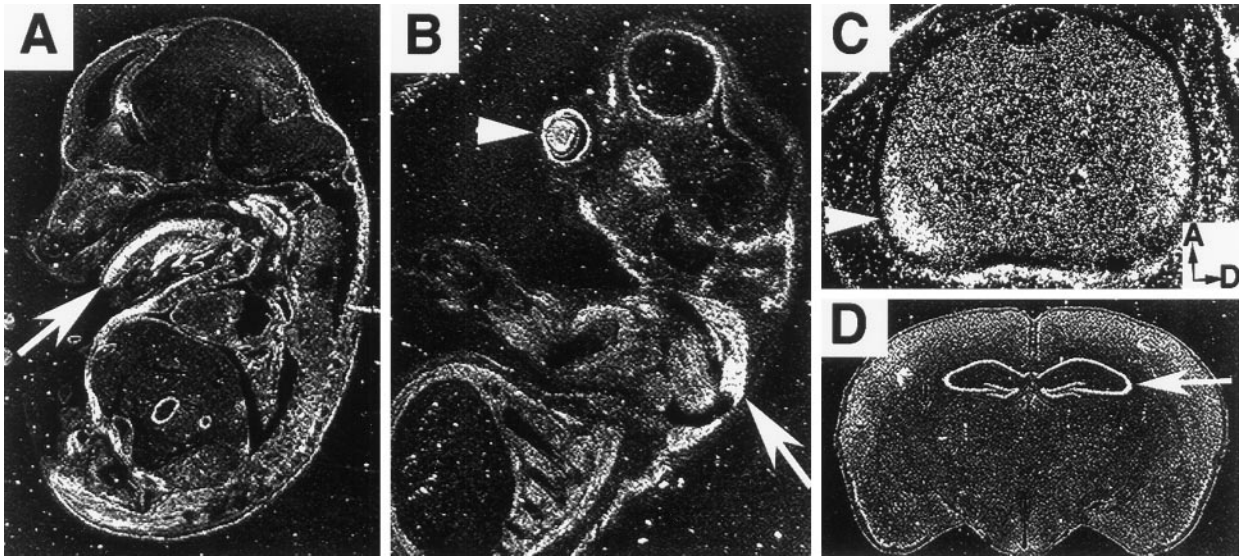

Figure 6. Expression of Enigma in skeletal muscle, brain, and other tissues. (A) In situ hybridization of an E13.5 mouse embryo sagittal section shows Enigma mRNA expression in skeletal muscle. The arrow indicates Enigma expression within the intrinsic and extrinsic muscle mass of the tongue. (B) Expression is observed in epaxial, intercostal, and other skeletal muscles at the brachial level, including the latissimus dorsi muscle, indicated by the large arrow. Lens tissue is indicated by an arrowhead. (C) Higher magnification of lens tissue shown in B. The arrow marks the location of Enigma-expressing lens cells adjacent to the equatorial zone of proliferation. For orientation, anterior (A) and dorsal (D) axes are indicated in the inset. (D) Enigma mRNA is evident within the adult hippocampus, as indicated by the arrow.

10-nm gold particles confirmed preferential localization of enigma at the Z line (Table 1). Therefore, Enigma and tropomyosin are both found at the boundary between the I band and Z line within adult muscle tissue.

DISCUSSION

The present study demonstrates that the actin-binding protein skeletal-specific tropomyosin specifically associates with the PDZ domain of Enigma. The abundant expression of Enigma in skeletal muscle is consistent with an in vivo interaction with a skeletal muscle-specific target. ALP, a muscle-specific Enigma family member, also associates with an abundant skeletal muscle actin-binding protein, α-actinin-2 (Xia *et al.*, 1997). Several PDZ domain proteins, including neurabin (Nakanishi *et al.*, 1997) and afadin (Mandai *et al.*, 1997), interact directly with actin via F-actin–binding domains. Enigma and ALP represent a class of PDZ proteins that associate with actin filaments indirectly through binding to F-actin–binding proteins. Although both family members are likely to have roles in skeletal muscle, the observation that Enigma is localized via its PDZ domain to the actin micofilament network of nonmuscle cells (Durick *et al.*, 1998) suggests that Enigma function extends to other tissues. The observation that Enigma mRNA is expressed in nonmuscle tissues also suggests that Enigma function is not confined to skeletal muscle and that other interactors in addition to skeletal β-TM exist. Enigma is therefore likely to associate with actin filaments in nonmuscle cells via another actin-associated protein that has not been identified. There is precedence for the recognition of multiple targets by PDZ domain proteins. Alternative complexes of the PDZ domains of PSD-95/SAP90 occur in tissue-specific contexts: in neuronal populations, PSD-95 interacts with neuronal nitric oxide synthase via PDZ–PDZ interactions, whereas in skeletal muscle, localization of neuronal nitric oxide synthase is mediated through PDZ–PDZ interactions between PSD-95 and α-syntrophin (Brenman *et al.*, 1996).

Despite the existence of additional targets, interaction and colocalization of Enigma and skeletal β-TM are likely to be significant features of muscle. Because Enigma is targeted to F-actin via its PDZ domain in fibroblasts (Durick *et al.*, 1998), it is likely that the subcellular distribution of Enigma in muscle is also regulated by PDZ domain interactions. We show that Enigma forms a complex with skeletal tropomyosin in C2C12 myotubes. The observed PDZ domain interactions may be responsible for anchoring Enigma to the margin of the Z line and I band, where skeletal β-TM is distributed. Once anchored by its PDZ domain, the LIM domains of Enigma could recruit kinases to the cytoskeleton, as in fibroblasts (Durick *et al.*, 1998).

Alternatively, the Enigma–skeletal β-TM interaction may be relevant to cytoskeletal assembly in muscle, a process that requires tropomyosin (Kagawa *et al.*,

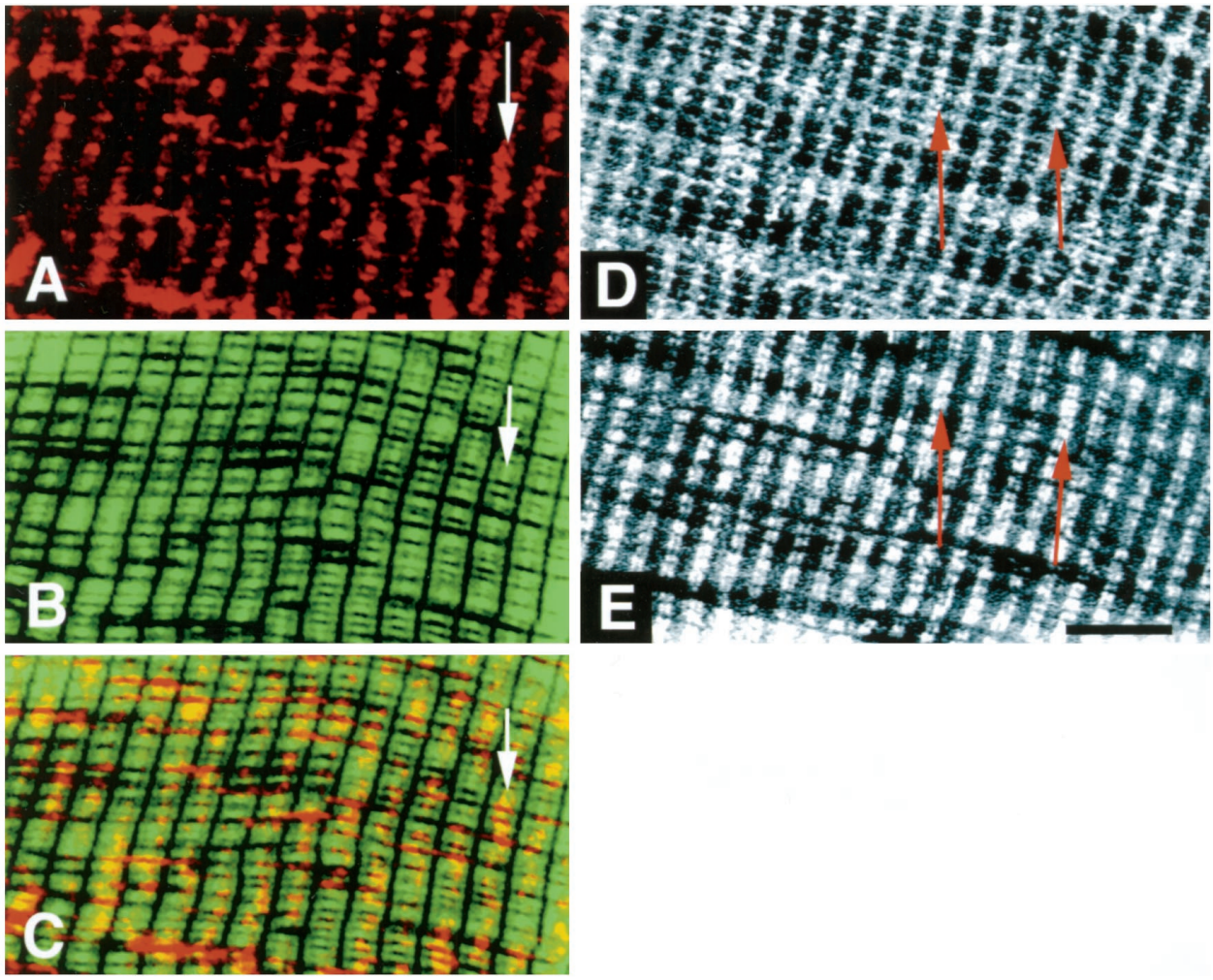

Figure 7. Localization of Enigma in skeletal muscle. (A) Immunofluorescence of semithin cryostat sections shows that Enigma is present at the Z line and some transverse filaments in adult muscle sarcomeres. Enigma is present along the Z line as indicated by the arrow. (B) Phalloidin-FITC staining of the same tissue section as in A shows the distribution of actin at the I band and at the Z line, which lies at the midline of the I band. The arrow marks the actin-rich Z line. (C) Digitally merging of the images of A and B shows codistribution of Enigma (red) and Phalloidin-stained actin (green). Regions where Enigma and actin overlap appear yellow, as indicated by the arrow. Arrows in A–C mark the same location in the tissue section. (D) Enigma is detected at the Z line, as indicated by the arrow, and along a subset of transverse filaments. (E) Skeletal β-TM is also distributed near the Z line (arrowhead) in a doublet pattern on the same tissue section as in D. Bar, ~3 μM.

1997). Codistribution of tropomyosin with Enigma at the boundary between the I band and the Z line supports the biological relevance of the Enigma–skeletal β-TM interaction in cytoskeletal assembly. Tropomyosin isoforms bind the actin thin filaments and are assembled into homodimers or heterodimers in a head-to-tail mechanism as a result of an interaction between the amino terminus and carboxyl terminus (Gimona *et al.*, 1995; Warren *et al.*, 1995). The carboxyl-terminal residues of α-tropomyosin also provide a functional domain that determines actin affinity (Hammell and Hitchcock-DeGregori, 1996), and the PDZ domain of Enigma appears to also recognize skeletal α-TM. If Enigma–skeletal β-TM and skeletal β-TM–skeletal–β-TM interactions are mutually exclusive, distribution of Enigma along thin filaments may be directed by a target protein other than skeletal β-TM, because the skeletal β-TM carboxyl termini would not be available for Enigma binding. However, at the barbed end of the actin thin filament, the skeletal β-TM carboxyl termini does not interact with another skeletal β-TM protein and is available for binding of Engima. Codistribution of Enigma and skeletal β-TM along the boundary between the Z line and I band suggests that the PDZ domain of Enigma binds the exposed carboxyl terminus of skeletal β-TM located at the barbed end of some actin thin filaments. The association between Enigma and the carboxyl ter-

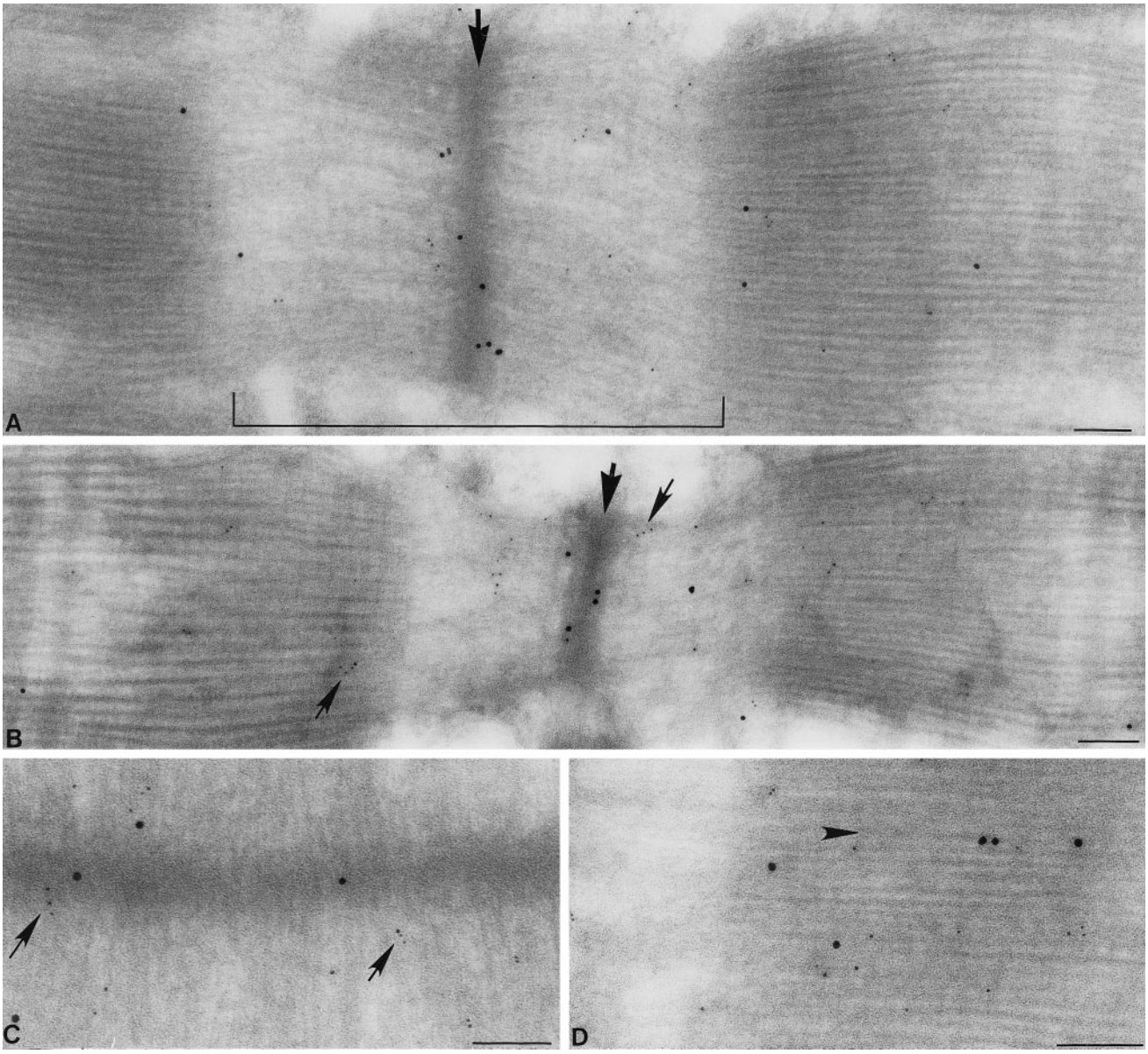

Figure 8. Immunoelectron microscopic analysis of the distribution of Enigma in skeletal muscle. Immunoelectron micrographs show the presence of Enigma (10-nm gold) and skeletal TM (5-nm gold) in ultrathin sections of adult muscle. (A–C) Enigma (large black dots) is predominantly detected at the boundary of the I band and Z line, whereas skeletal TM (small dots) is detected at the boundary of the I band and Z line and throughout the I band. The arrow in A indicates the Z line, and the bracket spans the I band. The large arrow in B marks the Z line, and small arrows mark skeletal TM detected in the I band and along thin filaments. Arrows in C indicate skeletal TM located at the boundary between the I band and Z line. The arrow in D marks Enigma distribution along a transverse filament. Bar, 0.1 μm.

minus of skeletal β-TM may therefore connect the actomyosin-free carboxyl ends of skeletal β-TM to the Z line and possibly prevent actin binding and homodimerization of skeletal β-TM at the Z line. Interaction between the PDZ domain of Enigma and the carboxyl terminus of skeletal β-TM is therefore of considerable interest.

Characterization of the recognition sequence required for the interaction between Enigma and skeletal β-TM indicated that the canonical PDZ target sequence (Thr-X-Leu-COO-) of skeletal β-TM was the primary site of PDZ binding. Although α-actinin contains a carboxyl-terminal PDZ consensus binding site (Ser-Asp-Leu-COO-), ALP interaction was mapped to internal spectrin-like repeats (Xia *et al.*, 1997). Thus, Enigma and ALP recognize targets of distinct natures, with the PDZ domain of Enigma binding to a carboxyl-terminal target and the PDZ domain of ALP bind-

Table 1. Enigma sarcomeric distribution

Region	anti-Enigma immunogold particles/mm^2
A band	5.7
I band	5.8
Z line	44.6

Eight immunoelectron micrographs were scored for number of anti-Enigma immunogold particles located in the area of three sarcomeric regions, A band, I band, and Z line. Values are the average number of particles per unit area.

ing to an internal sequence in its target. With additional data, a trend among family members to recognize a particular target type, internal or carboxyl-terminal, may become evident. Other Enigma family members may also have muscle-specific PDZ domain targets, because expression of ENH, ALP, CLP36, and RIL mRNAs is observed in skeletal muscle and heart (Kiess *et al.*, 1995; Wang *et al.*, 1995; Kuroda *et al.*, 1996; Xia *et al.*, 1997).

Enigma, ALP, and many other extranuclear LIM domain-containing proteins are localized to the cytoskeleton (Xia *et al.*, 1997; Durick *et al.*, 1998; Jurata and Gill, 1998). Zyxin and CRP are localized along actin filament bundles and at adhesion plaques (Sadler *et al.*, 1992). Zyxin, which contains three carboxyl-terminal LIM domains, binds to α-actinin via its proline-rich amino terminus (Schmeichel and Beckerle, 1994), whereas CRP1 binds to α-actinin via its first LIM domain (Pomies *et al.*, 1997). The overexpression of the CRP family member muscle LIM protein (MLP), which localizes to actin filaments, promotes differentiation of C2C12 myoblasts to myotubes (Arber and Caroni, 1996), whereas antisense MLP inhibits C2C12 differentiation (Arber *et al.*, 1994). Deletion of the MLP gene results in dilated cardiomyopathy in which cardiomyocyte architecture is disrupted (Arber *et al.*, 1997). Although the mechanisms through which the various LIM domain-containing proteins affect muscle and nonmuscle cytoskeleton function are unknown, proper LIM target localization may prove to be essential.

The biological function of Enigma is likely to be that of an adapter that, via its PDZ domain, localizes LIM-binding proteins to actin filaments of both skeletal muscle and nonmuscle tissues. The conservation of PDZ and LIM domains among Enigma family members, the recognition of protein kinases by the LIM domains of Enigma and ENH, and the identification of the actin-binding proteins β-tropomyosin and α-actinin as PDZ targets of Enigma and ALP suggest that targeting of protein kinases to actin filaments may emerge as a general function of members of the Enigma family.

ACKNOWLEDGMENTS

We are grateful to Tammie McQuist for expert assistance with immunolocalization studies, Marilyn Farquhar for advice and discussion, and Michele Yeo, Ryan Littlefield, Angels Almenar-Queralt, and Dr. Velia Fowler for helpful discussion and for technical assistance. We thank S. Kuroda for the ENH cDNA, A. Chishti for pGEX Dlg1/2, D. Edwards for pGEX-LIM Kinase PDZ, S.S. Taylor for the protein kinase A catalytic subunit, K.L. Carraway, III, for the C2C12 cell line, L. Jurata for helpful screening protocols, A. Nesterov for comments on the manuscript, M. Wilson for isolation of mouse Enigma cDNA, and Marie Truong for technical assistance. These studies were supported by National Institutes of Health grant 5PO 1 CA58689. P.M.G. was supported by National Institutes of Health National Research Service Award DK-09320. D.A.K. was supported by National Institutes of Health training grant T32HL-07770.

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