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Identification of a Novel *Drosophila* Protein Kinase Highly Homologous to Protein Kinase N (PKN)

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We identified a novel *Drosophila* gene, Dpkn (*Drosophila* protein kinase related to PKN), encoding a putative protein serine/threonine kinase. Although the cDNA obtained was incomplete at its 5′-terminal region, the deduced amino acid sequence of its kinase domain exhibits a high degree of similarity to protein kinase N (PKN), which has a kinase domain related to protein kinase C (PKC) and leucine zipper-like sequences in the amino terminal region. Expression of Dpkn was observed throughout *Drosophila* development, although its expression level decreased at later stages of embryogenesis. The expression of Dpkn is first detected in the newly formed mesodermal cell layer and is then restricted to the developing somatic musculature, indicating a possible role of Dpkn in the development of somatic muscles in *Drosophila*.

The protein kinase C (PKC) family of serine-threonine protein kinases are activated by calcium, diacylglycerol, and phorbol esters, and play important roles in regulating a variety of cellular functions, including developmental processes (1, 2, 3, 4). Members of the PKC family have diverse expression profiles in vertebrates; some are widely expressed in different tissues, while others have more restricted expression patterns (3, 4, 5, 6). Thus far, three PKC genes in the fruit fly *Drosophila melanogaster* have been identified (7, 8, 9). They include DPKC53E, a homologue of the mammalian PKCα (8), DPKC98E, a homologue of the mammalian PKCδ (9), and eye-PKC, a mammalian PKCα homologue expressed exclusively in photoreceptor cells of the visual system (9).

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Abbreviations used: PKC, protein kinase C; PKN, protein kinase N; Dpkn, *Drosophila* protein kinase related to PKN.

A gene encoding a novel serine/threonine protein kinase, PKN, whose kinase domain is related to those of members of the PKC family, has recently been cloned from *Xenopus*, rat and human (10, 11). Interestingly,

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   1 GGTCGCGGCCACTTTGGCAAGGTGATTCTGTCCCAATTGCGAAGCAACAACCAGTACTAC  60
   1  G  R  G  H  F  G  K  V  I  L  S  Q  L  R  S  N  N  Q  Y  Y  20

  61 GCTATTAAGGCACTGAAGAAGGGAGACATCATTGCCCGCGACGAAGTGGAGTCCCTGCTT 120
  21  A  I  K  A  L  K  K  G  D  I  I  A  R  D  E  V  E  S  L  L  40

 121 AGCGAAAAGCGTATCTTCGAGGTGGCCAACGCCATGCGCCATCCGTTCTTAGTTAACTTG 180
  41  S  E  K  R  I  F  E  V  A  N  A  M  R  H  P  F  L  V  N  L  60

 181 TATTCGTGCTTCCAGACTGAGCAACACGTATGCTTTGTGATGGAATACGCTGCTGGCGGA 240
  61  Y  S  C  F  Q  T  E  Q  H  V  C  F  V  M  E  Y  A  A  G  G  80

 241 GATTTGATGATGCACATCCACACGGACGTGTTCCTAGAGCCGAGAGCCGTTTTCTACGCC 300
  81  D  L  M  M  H  I  H  T  D  V  F  L  E  P  R  A  V  F  Y  A 100

 301 GCTTGTGTGGTTCTGGGCCTGCAGTACCTGCACGAGAACAAGATCATCTACCGGGACCTG 360
 101  A  C  V  V  L  G  L  Q  Y  L  H  E  N  K  I  I  Y  R  D  L 120

 361 AAGCTGGACAATTTGCTTTTGGACACGGAAGGATATGTGAAAATTGCGGACTTTGGTTTG 420
 121  K  L  D  N  L  L  L  D  T  E  G  Y  V  K  I  A  D  F  G  L 140

 421 TGCAAGGAGGGCATGGGCTTTGGTGATCGCACGGGCACTTTCTGTGGTACGCCCGAGTTT 480
 141  C  K  E  G  M  G  F  G  D  R  T  G  T  F  C  G  T  P  E  F 160

 481 CTGGCACCGGAAGTGCTCACGGAAACTTCGTACACACGAGCTGTGGATTGGTGGGGCTTG 540
 161  L  A  P  E  V  L  T  E  T  S  Y  T  R  A  V  D  W  W  G  L 180

 541 GGTGTGTTGATCTTTGAGATGTTGGTTGGTGAGTCCCCATTCCCTGGTGACGATGAGGAG 600
 181  G  V  L  I  F  E  M  L  V  G  E  S  P  F  P  G  D  D  E  E 200

 601 GAAGTATTCGATTCAATTGTCAACGATGAGGTGCGCTATCCGCGCTTCCTGTCGCTGGAG 660
 201  E  V  F  D  S  I  V  N  D  E  V  R  Y  P  R  F  L  S  L  E 220

 661 GCCATAGCCGTGATGCGTAGGCTTTTGCGCAAGAATCCAGAGAGACGTCTGGGATCTTCG 720
 221  A  I  A  V  M  R  R  L  L  R  K  N  P  E  R  R  L  G  S  S 240

 721 GAACGGGATGCGGAGGATGTTAAGAAACAGGCATTCTTCCGGTCAATTGTGTGGGATGAC 780
 241  E  R  D  A  E  D  V  K  K  Q  A  F  F  R  S  I  V  W  D  D 260

 781 CTGCTCCTGCGAAAGGTTAAACCACCATTTGTGCCGACAATTAACCACTTGGAGGATGTG 840
 261  L  L  L  R  K  V  K  P  P  F  V  P  T  I  N  H  L  E  D  V 280

 841 TCAAACTTTGACGAGGAGTTCACGTCGGAGAAGGCTCAGCTTACGCCACCGAAGAGCCGC 900
 281  S  N  F  D  E  E  F  T  S  E  K  A  Q  L  T  P  P  K  S  R 300

 901 GACACTTGA 909
 301  D  T  *  303
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the kinase domain of Dpkn gene product. The putative ATP binding motifs [GlyXGlyXXGly-----(AX)K] are underlined.

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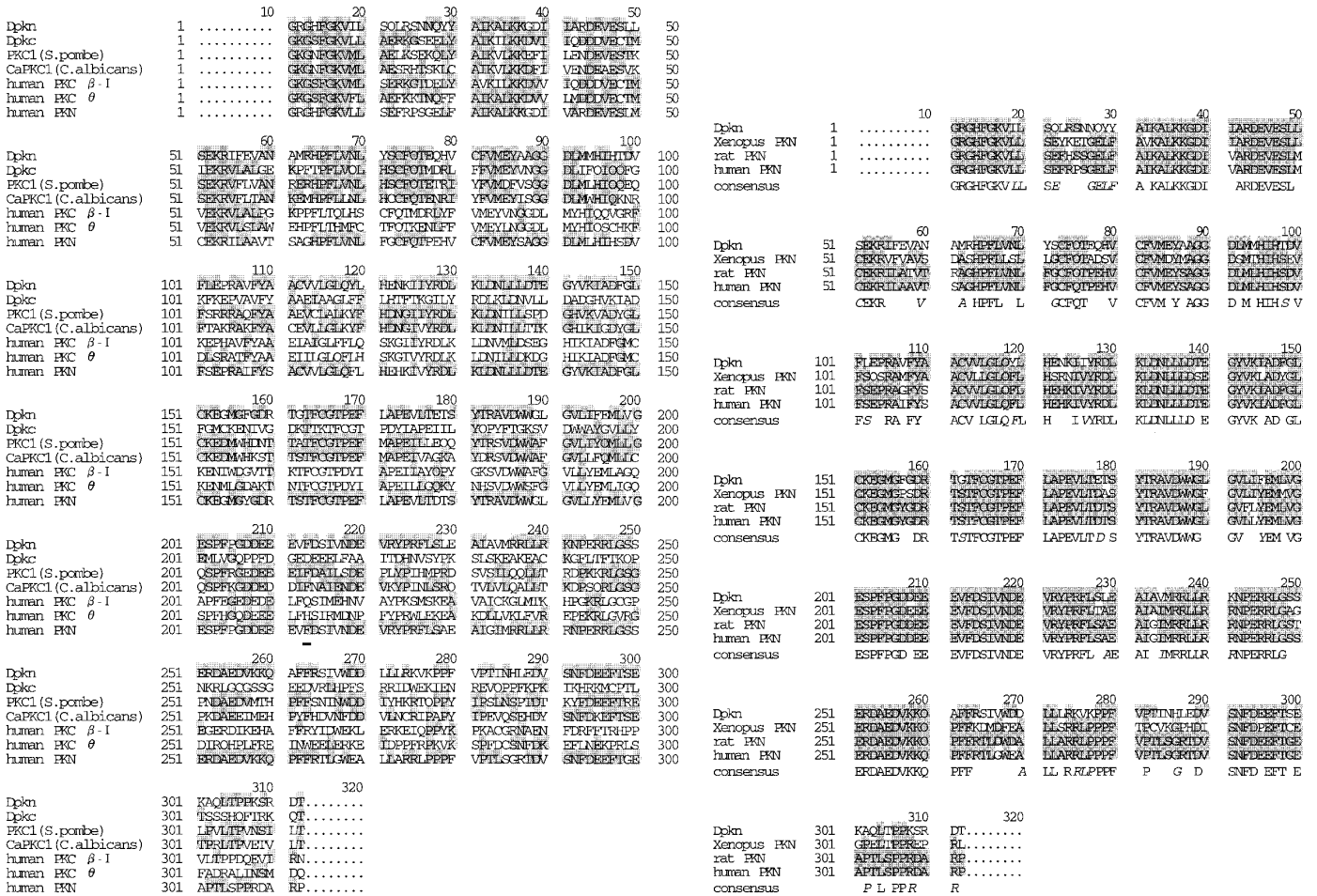

FIG. 2. (A) Alignment of Dpkn kinase domain with other kinase domains from members of the PKC family. Conserved residues among these kinases are shaded. (B) Alignment of Dpkn kinase domain with the previously reported *Xenopus*, rat, and human PKNs. Conserved residues among these kinases are shaded.

it has been shown that PKN is a target of Rho, a Ras-like small guanosine triphosphatase (GTPase), implicated in cytoskeletal responses to extracellular signals (12, 13), and it is activated by the binding of the active GTP-bound form of Rho (12).

Here we report a novel Drosophila gene, Dpkn, possessing a putative kinase domain exhibiting a striking homology with that of PKN. We also show the unique expression pattern of the Dpkn gene during embryogenesis of the fly.

EXPERIMENTAL PROCEDURES

DNA amplification and sequencing. For PCR, degenerated primers were designed to hybridize to nucleotides coding two well conserved kinase subdomains VI B (HRDL) and IX (DVWSYG). The primer sequences were 5′-CCGCGAATTCATCCAC(A/C)G(A/C/G/T)GA(C/T)(C/T)T-3′ and 5′-CCGCAAGCTTGCC(A/G)(A/T)A(A/G)(C/G)ACCA(C/G)AC(A/G)TC-3′ (restriction sites for EcoRI and HindIII are underlined). 100ng of genomic DNA was used as a template in 100μl PCR. The first 10 PCR cycles were 1.5min at 94°C, 2min at 55°C to 50°C (decreased 0.5°C per cycle), and 2.25min at 73°C. In the subsequent 20 cycles, samples were denatured 1.5min at 94°C, incubated 2min at 50°C and heated 2.25min at 73°C. Amplified DNA fragments of the expected size (about 200bp) were digested with EcoRI and HindIII, purified on 2% agarose gel and cloned into the EcoRI/HindIII sites of the Bluescript vector (pBS, Stratagene).

Isolation of cDNA clones. An imaginal disc cDNA library (14) was screened using probes (0.2kb EcoRI-HindIII fragment from pBS-Dpkn) radiolabeled by random priming. Probes (3×10^6 cpm/ml) were hybridized to the plasmid DNA immobilized on nitrocellulose membrane filters (Schleicher & Schuell) for 12hr at 65°C in 1× hybridization buffer (1M NaCl, 50mM Tris-HCl [pH8.0], 10mM EDTA, 0.1% [v/v] SDS), 1× Denhardt's reagent, and 100 μg/ml denatured salmon sperm DNA, and washed twice for 30 min at 65°C in 0.1× SSC, 0.1% [v/v] SDS. Clones were isolated and the longest 1.6kb clone (pNB40-Dpkn) was subjected for further analyses.

DNA sequencing and analysis. Sequencing was performed by the dideoxynucleotide chain termination method using the Thermo Sequenase core sequencing kit (Amersham) and a SQ5500 DNA sequencer (HITACHI). The final sequence was confirmed from both strands. Sequence analyses, comparison, and subsequent sequence alignment were performed using Genbank and EMBL databases through the BLASTN programs as well as the DNASIS program (Hitachi Software Engineering Co., Ltd.).

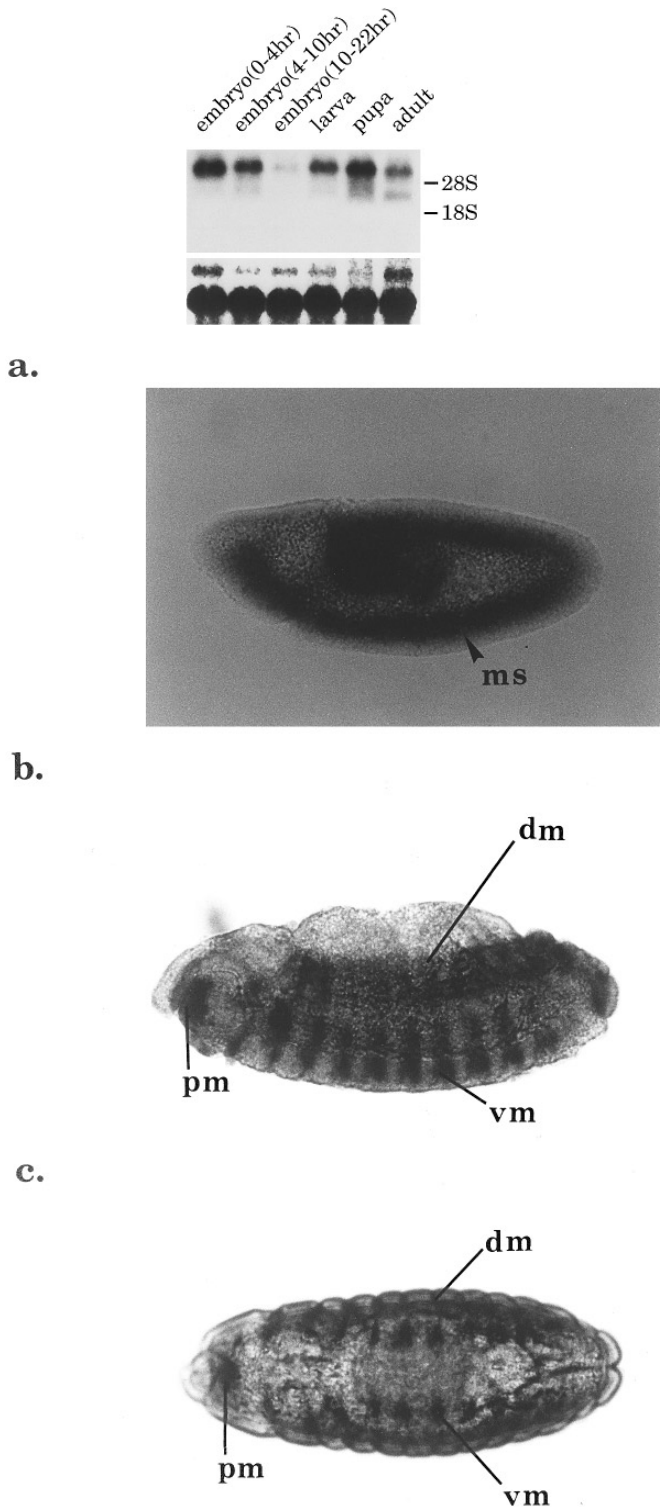

FIG. 3. (A) Developmental expression of Dpkn transcripts. Total RNA was prepared from *Drosophila* at various stages of development, separated by 1% agarose formaldehyde gels, transferred onto nylon membranes, and hybridized with radiolabeled probe for Dpkn as described in Experimental Procedures. The filters were stained with methylene blue to show total RNA [see panels indicating 18S and 28S ribosomal RNAs (control), stained with methylene blue]. Amount of RNA loaded in each lane was also normalized by rehybridization with labeled probe for rp49 (data not shown). (B) Localization of Dpkn transcripts in embryos detected by whole mount *in situ* hybridization. (a) Lateral view of embryo at gastrulation (stage 9). Expression is restricted to mesodermal cell layers. (b) Lateral view of developing embryo (stage 14). Expression of Dpkn was retained in the developing somatic muscular system throughout embryogenesis. (c) Ventral view of developing embryo (stage 14-15). Anterior to left in all embryos. Dorsal to the top in a and b. ms, mesoderm; pm, pharyngeal musculature; dm, dorsal musculature; vm, ventral musculature.

Northern blot analysis. Total RNA from embryo, larva, pupa and adult flies were prepared by using ISOGEN (WAKO). For RNA blot analysis, 5μg of total RNA was electrophoresed on 1% agarose formaldehyde gels, and transferred onto nylon membranes. The probe DNA was prepared from pBS-Dpkn by digestion with EcoRI and HindIII, and labeled with [α-^{32}P]dCTP (Amersham, 3000Ci/mmol) using the Multiprime labeling kit (Amersham) and hybridized as described previously (15). Specific activity was $\sim 1\times10^6$ cpm/ng for all the probe DNAs.

In situ hybridization of whole mount embryos. *In situ* hybridization to whole mount embryos using digoxigenin-labeled RNA probes was performed as described (16) with minor modifications (17). Single strand antisense or sense RNA probes were synthesized *in vitro* using T7 or T3 RNA polymerases, DIG RNA Labeling Mix (Boehringer Mannheim), and the pBS-Dpkn as a template following the manufacturer's recommended protocol.

RESULTS AND DISCUSSION

Cloning of Dpkn cDNAs. Using degenerate oligonucleotide primers to well-conserved stretches of amino acids in protein kinases, we PCR-amplified *Drosophila* genomic DNA. After subcloning of amplified products, several were found to encode kinase domains and among them one encoded kinase subdomains related to those of members of the PKC family. Using this amplified region as a probe, we screened a cDNA library from *Drosophila* imaginal discs (see Experimental Procedures) and the cDNA clone with the longest insert ($\sim$1.6 kb) was sequenced.

One of the frames of the Dpkn cDNA encoded an amino acid sequence of a typical protein kinase domain, including a putative ATP binding motif (Fig.1). The amino acid residues conserved within the serine/threonine protein kinase family are found in Dpkn, indicating that Dpkn is a member of this family (18, 19). Comparative sequence analysis revealed that the kinase domain of Dpkn ($\sim$300 amino acids) is highly homologous to the corresponding domains of the protein kinase C family (Fig.2A). The highest homology ($\sim$80%) was seen between the kinase domain of Dpkn and that of a recently identified novel protein kinase, designated PKN, from *Xenopus*, rat and human (Fig.2B, 10, 11).

It has been shown that PKN is a target of Rho, a Ras-like small GTPase and that the Rho-binding site is localized within the N-terminal portion of PKN, that has been assumed to be a regulatory domain of PKN (12). Thus, the entire sequence of Dpkn is required to elucidate whether Dpkn is indeed a *Drosophila* homologue of mammalian PKNs.

Expression of Dpkn. To characterize the expression pattern of the Dpkn gene, we first performed Northern blot analysis with RNA samples from embryos (0-4hr, 4-10hr, 10-22hr), larvae, pupae, and adult flies. The 1.6 kb fragment of the Dpkn cDNA was used as a probe. Dnrk probe detected a major band of about 7 kb in size (Fig.3A). As shown in Fig.3A, Dpkn was expressed throughout *Drosophila* development, yet its expression level decreased at later stages of embryogenesis (Fig.3A).

To determine the tissue specificity of Dpkn transcripts during embryogenesis, we performed *in situ* hybridization on whole-mount embryos (see Experimental Procedures). Distinct expression of Dpkn was detected primarily in the mesodermal layer (Fig.3B, panel a.). Expression of Dpkn was then restricted to the somatic musculature (Fig.3, panels b. & c.). This expression appeared to be sustained in a subset of the muscular cell lineage throughout the remainder of embryogenesis (data not shown). In this regard, it is important to note that mammalian PKNs identified thus far are expressed rather ubiquitously, although a higher degree of expression is detected in heart and skeletal muscle (11). These results indicated that Dpkn may play a role in the development and function of somatic muscles in *Drosophila*. A functional characterization of Dpkn awaits isolation of mutations in the Dpkn gene. The existence of such mutants will unravel the possible roles of Dpkn in the development and function of somatic muscles in *Drosophila*.

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