(1)

Identification and Characterization of Novel Protein Kinases Involved in Developmental Processes

Isao Oishi

Department of Biology
Graduate School of Science
Osaka University

Summary

Developmental processes involve multiple cell-to-cell communication, in which protein kinases play crucial roles. In order to understand further the mechanism(s) underlying the developmental processes, I tried to identify a novel protein kinase(s) whose expression is spatio-temporally regulated during *Drosophila* development. Here I report the identification and characterization of the two novel *Drosophila* genes. One is Dpkn (*Drosophila* protein kinase related to PKN), encoding a putative protein serine-threonine kinase and the other is Dnrk (*Drosophila* neurospecific receptor kinase), encoding a putative receptor tyrosine kinase (RTK) .

The deduced amino acid sequence of the kinase domain of Dpkn exhibits a high degree of similarity to recently identified mammalian protein kinase N (PKN). The expression of Dpkn is first detected in the newly formed mesodermal cell layers and is then restricted to the developing somatic musculature, indicating a possible role of Dpkn in the development of somatic muscles in the *Drosophila* .

On the other hand, Dnrk is a typical type I membrane protein, whose cytoplasmic tyrosine kinase domain is highly related to the Trk- and Ror- families of RTKs. During *Drosophila* embryogenesis, the Dnrk gene is expressed specifically in the developing nervous system. The Dnrk protein possesses two conserved cysteine-containing domains and a kringle domain within its extracellular domain, resembling those observed in Ror-family RTKs (Ror1, Ror2 and a *Drosophila* -Ror; Dror). This protein contains the catalytic tyrosine kinase (TK) domain with two putative ATP binding motifs, resembling those observed in another *Drosophila* RTK (Dtrk) that mediates homophilic cell adhesion. The TK domain of Dnrk, expressed in bacteria or mammalian cells, exhibits apparent autophosphorylation activities *in vitro*. The TK domain lacking the distal ATP binding motif also exhibits autophosphorylation

activity, yet to a lesser extent. In addition to its TK activity, there are several putative tyrosine-containing motifs that upon phosphorylation may interact with SH2 regions of other signaling molecules. Collectively, these results suggest that Dnrk may play an important role in neural development during *Drosophila* embryogenesis.

introduction

Protein tyrosine kinases (PTKs) and protein serine-thrreonine kinases [P(S/T)Ks] play critical roles in a wide variety of cellular responses including activation, proliferation and differentiation. It has been appreciated that antigens or cytokines activate sets of receptor type or non-receptor type protein kinases, eventually leading to activation, proliferation, or differentiation of immuno-hematopoietic cells. Proliferation of T lymphocytes is triggered by the interaction of interleukin-2 (IL-2) with its specific receptor following T lymphocyte activation. The IL-2 receptor (IL-2R) consists of three subunits; the IL-2Rα, IL-2Rβ, and IL-2Rγ chains. The structures of the three distinct subunits of the IL-2R are depicted in Fig.1. IL-2Rβ and IL-2Rγ, but not IL-2Rα, belong to a superfamily of cytokine receptors, characterized by the presence of four conserved cysteines and the sequence WSXWS (the WS motif). Although none of these IL-2R subunits possess any known catalytic activity, it has been shown that both IL-2Rβ and IL-2Rγ are required to transmit the IL-2 signal to the cell interior (1-4).

The critical role of IL-2Rβ was demonstrated by cDNA expression studies using the murine hematopoietic cell lines such as BAF-B03, which is IL-2Rβ-negative but IL-2Rα- and γ-positive (1,5,6). When the human IL-2Rβ cDNA was expressed in these cells, they acquired the ability to proliferate in response to IL-2. The expression studies with deletion mutant IL-2Rβ cDNAs revealed that the membrane-proximal cytoplasmic region, the "serine-rich" region (Fig.1), is critical for the transmission of the IL-2-induced proliferative signal (1) .

Furthermore, the critical role of IL-2Rγ in IL-2 signaling was also suggested. It was shown that a mutant T cell line that has lost expression of IL-2Rγ but retains IL-2Rα and IL-2Rβ has also lost the ability to respond to IL-2 (7). The critical role of the

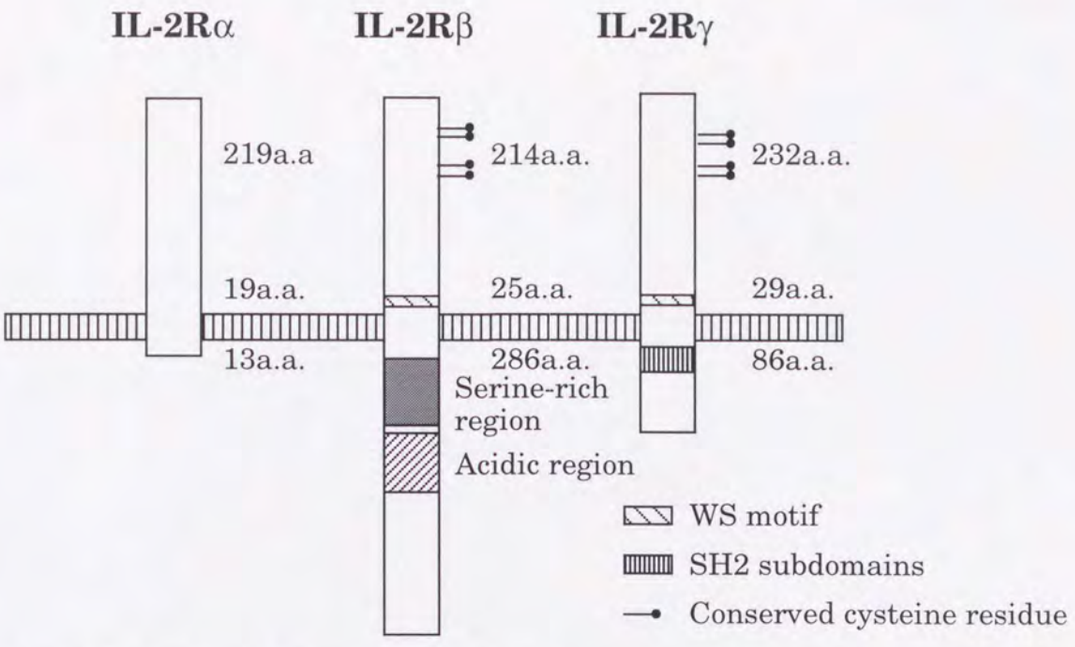


Fig. 1. A schematic diagram of the IL-2 receptor (IL-2R).
The high-affinity IL-2R consists of three distinct subunits, IL-2Rα, IL-2 Rβ and IL-2Rγ. The cytoplasmic domain of IL-2Rβ can be tentatively divided into subregions as shown in this figure.

cytoplasmic region of IL-2Rγ is also emphasized by the observation that ectopic expression of a mutant IL-2Rγ lacking most of its cytoplasmic domain inhibits the ability of the intact IL-2R to transmit the IL-2-induced proliferative signals in BAF-B03 cells (2). The critical role of the cytoplasmic region of IL-2Rγ in addition to that of IL-2Rβ led us to speculate that IL-2 signaling may require the functional cooperation between the cytoplasmic domains of IL-2Rβ and IL-2Rγ.

Despite the fact that none of IL-2R components possesses any known catalytic activity, it has been shown that IL-2 stimulation results in a rapid phosphorylation of several cellular proteins, including the receptor itself, on tyrosine as well as serine/threonine residues (8,9). Therefore, it is likely that IL-2Rβ and/or IL-2Rγ can interact with a nonreceptor-type PTK(s) and P(S/T)K(s). A panel of PTKs and of P(S/T)Ks implicated thus far in IL-2 signaling has been reported (see below). It has been shown that several P(S/T)Ks, including Raf-1 kinase, mitogen-activated protein (MAP) kinase, p70 S6 kinase, $p34^{cdk2}$ and $p34^{cdc2}$ are activated upon IL-2 stimulation

(10-18). It has also been demonstrated that $p56^{lck}$ and $p59^{fyn}$, (members of the src-family PTKs), Syk PTK (a member of the Syk/ZAP-70-family PTKs), Jak1 and Jak3 (members of the Jak-family PTKs) couple, both physically and functionally, with IL-2R (Fig.2)(19-28) (see below in details).

The src-family PTK, $p56^{lck}$ associates with the cytoplasmic "acidic" region of IL-2Rβ (see Fig.1), a region dispensable for IL-2-induced mitotic signaling (19,20). On the other hand, Syk PTK associates with the cytoplasmic "serine-rich" region of IL-2Rβ (see Fig.1), a region required for IL-2-induced cellular proliferation (22). The association of Jak1 and Jak3 with IL-2R is rather unique. Jak1 and Jak3 are structurally related kinases and belong to the same PTK family. Nonetheless, their association with the IL-2R components is selective, i.e. Jak1 associates with IL-2Rβ *via* its cytoplasmic "serine-rich" region and Jak3 associates with IL-2Rγ *via* its C-terminal region (It was shown that at least 48 C-terminal amino acids of IL-2Rγ are necessary for its association with Jak3 and that this region is deleted by nonsense mutations in many patients with X-linked severe combined immunodeficiency; XSCID.) (23,24). In addition to the physical association of IL-2R with these PTKs, it was shown that these PTKs are activated following IL-2 stimulation (23,24,27,28). Thus, it appears that this physical interaction is physiologically significant in IL-2 signaling.

Structure-function analyses of IL-2Rβ have identified critical regions in the IL-2Rβ required for activating the respective PTKs. Both the "serine-rich" and "acidic" regions of IL-2Rβ are required for activating $p56^{lck}$ upon IL-2 stimulation, indicating that the physical interaction of $p56^{lck}$ with IL-2Rβ is necessary, but not sufficient for activating $p56^{lck}$ (20). On the other hand, IL-2-induced activation of Syk PTK requires the "serine-rich" region of IL-2Rβ (22). It has been found that IL-2-induced activation of Jak3 also requires the "serine-rich" region of IL-2Rβ (28). Considering that Jak3

associates with IL-2Rγ but not with IL-2Rβ, activation of Jak3 (and possibly of Jak1) may require cooperation of both kinases associated with the IL-2R components. It has been proposed that activated Jaks phosphorylate the receptors with which they are associated, as well as various STAT (signal transducers and activators of transcription) proteins which function downstream to regulate gene expression (29,30).

Collectively, these findings raise the interesting issue of how a given cytokine receptor may function to activate many distinct signaling molecules (such as protein kinases). At present it is not clear whether there are many distinct subpopulations of IL-2Rβ (or IL-2Rγ), each of which associates with the respective signaling molecules (Fig. 2A), or if the subpopulation of IL-2Rβ (or IL-2Rγ) that associates with one signaling molecule can simultaneously associates with multiple signaling molecules (Fig.2B). In either case, it is likely that the concerted action of these signaling molecules may be required for full-scale activation of downstream signaling pathways. This phenomenon may be common to cytokine receptors, which otherwise lack any intrinsic kinase activity. Instead, the cytoplasmic domains of cytokine receptors have structurally evolved to recruit multiple signaling molecules, including PTKs and utilize them to trigger the full-scale activation of multiple downstream signaling pathways leading to cellular responses including cellular proliferation and differentiation. Furthermore, as shown in Fig. 2, this model can explain why IL-2-induced heterodimerization (see Fig.2B) or clustering (see Fig.2A) of IL-2Rβ and IL-2Rγ is required for triggering multiple downstream signaling events leading to cellular proliferation.

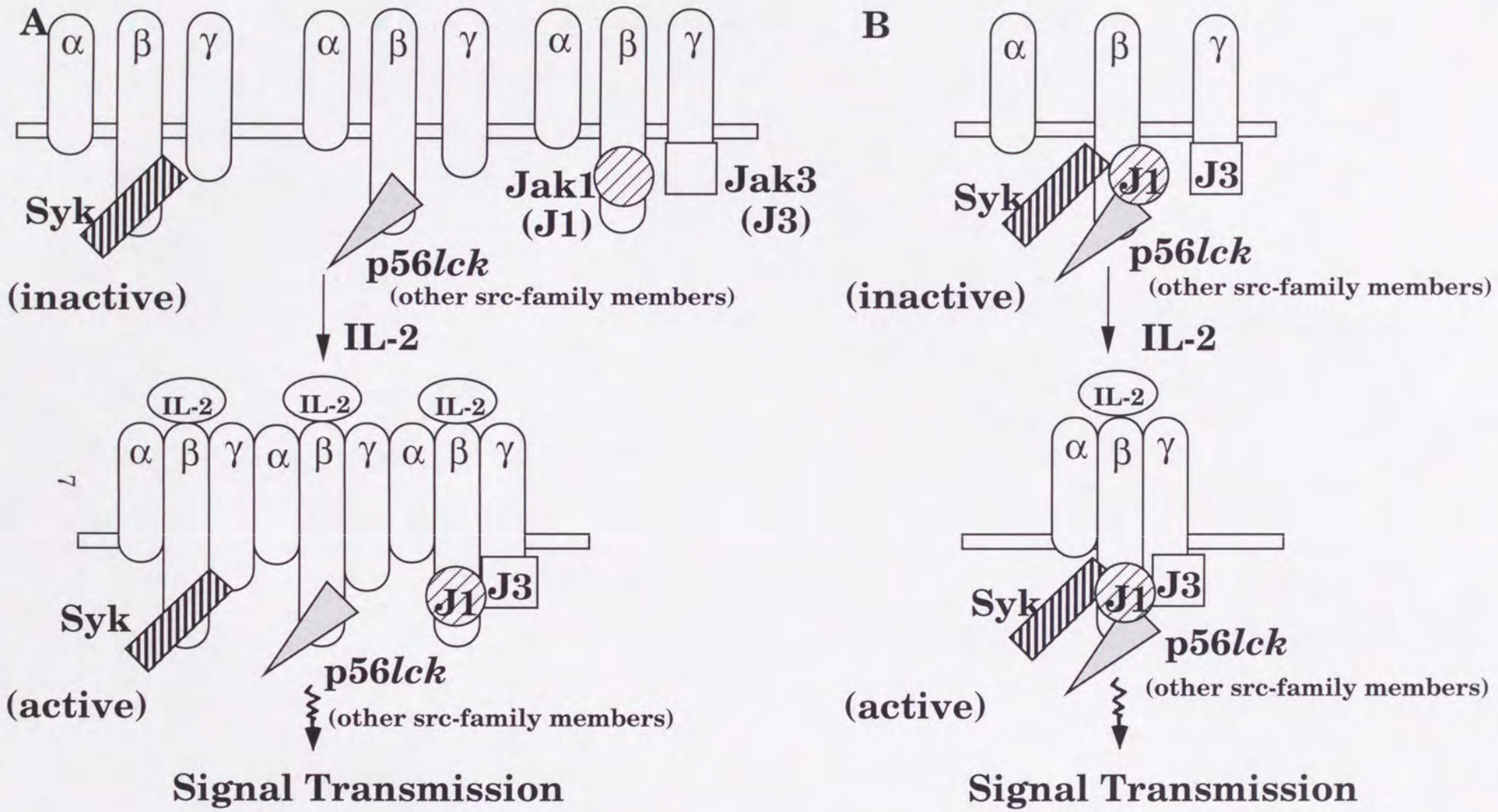


Fig. 2. Multiple kinase concert model. (A) Each IL-2Rβ (and possibly IL-2Rγ) recruits distinct kinases, including protein tyrosine kinases (PTKs). Upon IL-2 binding, these PTKs function in concert to activate multiple downstream signaling. (B) The IL-2Rβ chain can simultaneously recruit multiple kinases, including PTKs that are activated upon IL-2 binding. Whether IL-2 binding to IL-2R triggers clustering has not been experimentally demonstrated. For convenience, the exact association sites for the respective PTKs are ignored.

The Jak-family of PTK comprises four members, Jak1, Jak2, Jak3, and Tyk2, in mammals(31,32). It has been shown that many cytokine receptors, including IL-2R, utilize distinct sets of Jak-family PTKs to transmit their signals to the cell interior(24,31-38). Accumulating evidence indicates that Jak-family PTKs, associated with the respective cytokine receptors, are activated by stimulation with their cognate ligands, and thereby trigger the activation of STAT proteins(31-35). Furthermore, several observations have demonstrated that Jak-family PTKs play the critical roles in cytokine receptor-mediated cellular responses, including proliferation and growth inhibition. Genetic selection of cells unable to respond to IFN, the cytokine that usually induce growth inhibition, produces cell mutants lacking Jak1, Jak2 or Tyk2, and ectopic expression of the missing Jak renders mutant cells become responsive to IFN(30,31). Moreover, overexpression of the Jak3 mutant, lacking its intrinsic kinase activity, in cells responsive to IL-2 results in a drastic inhibition of the IL-2-induced cell proliferation (39). Deletions or mutations in the IL-2Rγ that disrupt Jak3 association also inactivate the receptor, producing X-linked severe combined immunodeficiency in humans(40).

Thus far, only one homologue of mammalian Jak-family PTKs has been reported in *Drosophila*(41). This Jak homologue is encoded by the *hopscotch* (*hop*) locus, whose loss-of-function alleles result in an X-linked larval/pupal zygotic lethality(41,42). The dead larvae have a normal cuticle pattern, but all larval diploid imaginal tissues are reduced in size, implying a zygotic role for *hop* in cell proliferation. In addition, it was shown that a gain-of-function *hop* mutation causes a form of "leukemia" in *Drosophila*, characterized by the formation of melanotic tumors and hypertrophy of the larval lymph glands, the hematopoietic organs(43,44). It should be noted that no such oncogenic activity has been reported for mammalian Jak-family PTKs, despite

mammalian Jaks paly an important role in cytokine-induced cell proliferation.

In addition to a zygotic role of *hop* in cell proliferation described previously, *hop* is also required maternally for the establishment of the normal array of *Drosophila* embryonic segments. In *hop* embryos, embryos derived from females lacking germ-line *hop* activity, although expression of the gap genes appear normal, there are defects in the expression patterns of the pair-rule genes (*even-skipped*, *runt*, *fushi tarazu*) as well as the segment-polarity genes (*engrailed* and *wingless*)(41). The effect of *hop* on the expression of these genes is stripe-specific. Thus, it becomes clear that *hop* also plays an important role in the development of *Drosophila*, in particular, segmentation of the embryos. The findings suggest that a mammalian Jak-family PTK(s) may also play an important role(s) in early development of mammals. With this respect, it is noteworthy that mice with gene disruption of Jak1 or Jak2 exhibit embryonic lethality.

These studies on *hop* re-emphasize an advantage of *Drosophila* as an elegant tool for the genetic analysis as well as morphological analysis of a particular functional molecule(s), including protein kinases. Furthermore, the studies suggest a role(s) of protein kinases in developmental processes. In mammals, it is sometimes difficult to study the role of a particular protein kinase(s) *in vivo* because of the well-known redundancy and/or pleiotropism of protein kinases. In fact, it has been shown that many protein kinase families (e.g. src-family PTKs) comprise many members with overlapping functions(45,46). With this respect, one can assume that protein kinases in *Drosophila* is likely to be less redundant due to its relatively small genome size and, therefore, it is suitable to analyze the function of a particular protein kinase(s) in *Drosophila*.

Developmental processes, including body patterning, tissuegenesis, organogenesis, involve multiple cell-to-cell communications, in which protein kinases appear to play

important roles(47,48). To understand further the mechanism(s) underlying these developmental processes, I tried to identify a novel protein kinase(s) involved in the development of *Drosophila*. By employing the PCR technique I have cloned five novel *Drosophila* protein kinase(s) (initially designated as DK1-DK5) that exhibit unique spatio-temporal expression patterns during the development of *Drosophila*. Among these novel kinases, I describe in details the identification and characterization of the two protein kinases, renamed as Dpkn (*Drosophila* protein kinase related to PKN) and Dnrk (*Drosophila* neurospecific receptor kinase).

Results

Cloning of novel protein kinase

The outline of the cloning strategy I employed is depicted in fig. 3. The two well conserved subdomains within the protein kinase domain were chosen to design the degenerated oligonucleotide primes for PCR. By using a PCR method and these degenerated primers, I amplified *Drosophila* genomic DNA. After subcloning of amplified products, most of them were found to encode protein kinases and among them three encoded novel protein kinases.

As shown in fig. 4, amplified products can be classified into about 10 protein kinases, and several were turned out to be amplified in a biased manner. Hence, I performed a subtractive hybridization thereafter to eliminate these frequently amplified products. As a result, I obtained two additional novel protein kinases. These five novel *Drosophila* protein kinases (DKs) were temporarily designated as DK1-DK5.

Temporal expression pattern of DKs during embryogenesis

In order to examine whether these DKs are involved in the developmental processes of *Drosophila*, first I analyzed temporal expression pattern of these genes during the embryonal development. I performed Northern blot analysis with RNA samples from embryos (0-4hr, 4-10hr, 10-22hr) (fig.5). DK1 is expressed throughout embryonal stage, yet its expression level decreased at later stages (fig.5). DK2 and DK5 are expressed at high levels in 0-4hr embryos and hardly detectable at later stages (fig.5). DK3 is expressed at high levels in 4-22hr embryos(fig.5). DK4 is expressed at high levels in 4-10hr embryos (fig.5). Thus, it was expected that these DKs are involved in the developmental processes of *Drosophila*. As described later, spatial expression patterns of DK1 and DK3 are restricted in the developing tissues. Thus, it was assumed that DK1 and DK3 are concerned in *Drosophila* tissuegenesis.

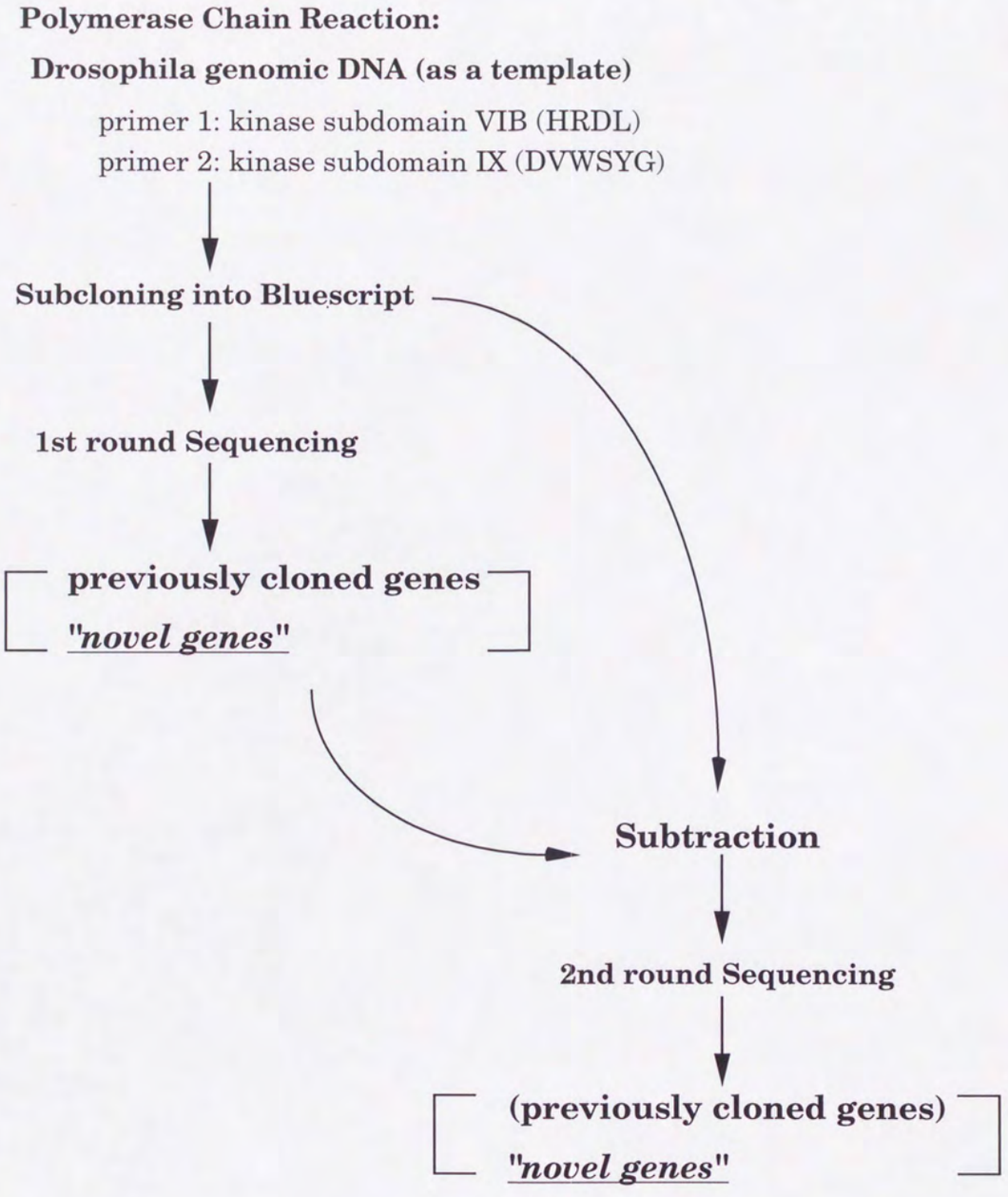


Fig.3. Strategy for cloning novel protein kinases. For PCR, degenerated primers were designed to hybridize to nucleotides coding two well conserved kinase subdomains VI B (HRDL) and IX (DVWSYG). PCR-amplification with these degenerated primers and subsequent subcloning of amplified products were performed as described in Experimental Procedures. After the 1st round sequencing, frequently amplified products were subtracted by colony hybridization.

Previously cloned genes:

		the closest mammalian counterpart
non-receptor type kinases		
DcdrK	serine/threonine kinase	cdc2
Dcdk5	serine/threonine kinase	cdk5
Dsrc	tyrosine kinase	src
Dc-src	tyrosine kinase	src
D-fps	tyrosine kinase	fps
Dash	tyrosine kinase	abl
SHARK	tyrosine kinase	Syk/ZAP-70
Hop	tyrosine kinase	Jaks
receptor type kinases		
DER (DEGFR)	tyrosine kinase	EGFR
DFGFR	tyrosine kinase	FGFR
Sevenless	tyrosine kinase	

Newly cloned genes:

non-receptor type kinases		
DK1	serine/threonine kinase	PKN
DK2	serine/threonine kinase	?
DK5	serine/threonine kinase	?
receptor type kinases		
DK3	tyrosine kinase	Trk/Ror
DK4	tyrosine kinase	Ltk

Fig.4. Cloned protein kinase genes. The obtained clones are classified into serine/threonine or tyrosine kinases on the basis of their amino acid sequence homologies with the previously reported consensus sequences for the respective protein kinases. Clones DK1-5 have not been described previously and represent putative novel protein kinases.

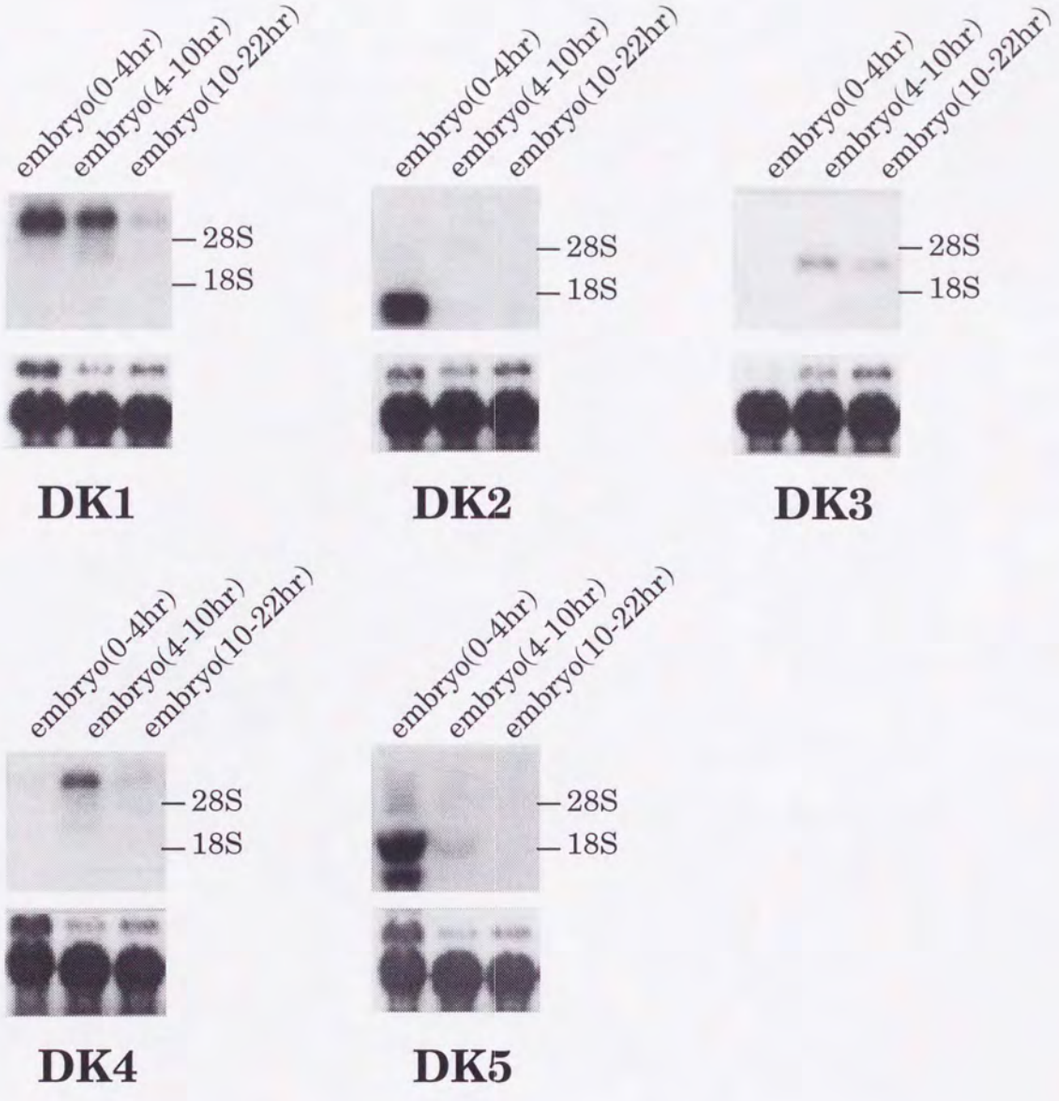


Fig.5 Temporal expression pattern of DK1-5 transcripts. Total RNA was prepared from *Drosophila* embryos (0-4hr, 4-10hr, 10-22hr), separated by 1% agarose formaldehyde gels, transferred onto nylon membranes, and hybridized with radiolabeled probes for the respective transcripts. The filters were stained with methylene blue to show total RNA [see panels indicating 18S and 28S ribosomal RNAs (control), stained with methylene blue].

Cloning of Dpkn cDNAs

Using the subcloned region of DK1 as a probe, I screened a cDNA library from *Drosophila* imaginal discs (see Experimental Procedures). Among cDNA clones isolated, the cDNA clone containing the longest insert (~1.6 kb) for Dpkn (*Drosophila* protein kinase related to PKN) was sequenced.

Dpkn cDNA encoded an amino acid sequence of a typical protein kinase domain, including a putative ATP binding motif (Fig.6). In Dpkn, the amino acid residues conserved within the serine/threonine protein kinase family are found, indicating that Dpkn is a member of this family (49,50). Comparative sequence analysis revealed that the kinase domain of Dpkn (~300 amino acids) is highly homologous to the corresponding domains of the protein kinase C family (Fig.7A). The highest homology (~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.7B, 51,52).

Spatio-temporal expression pattern of Dpkn

To characterize the temporal expression pattern of the Dpkn gene, I performed Northern blot analysis with RNA samples from embryos (0-4hr, 4-10hr, 10-22hr), larvae, pupae, and adult flies. The 1.6kb fragment of the Dpkn cDNA was used as a probe. Dnrk probe detected a major band of about 7 kb in size (Fig.8A). As shown in Fig.8A, Dpkn was expressed throughout *Drosophila* development, yet its expression level decreased at later stages of embryogenesis .

```
  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
```

Fig. 6. Nucleotide sequence and deduced amino acid sequence of the kinase domain of Dpkn gene product. The putative ATP binding motifs [GXGXXG-----(AX)K] are underlined.

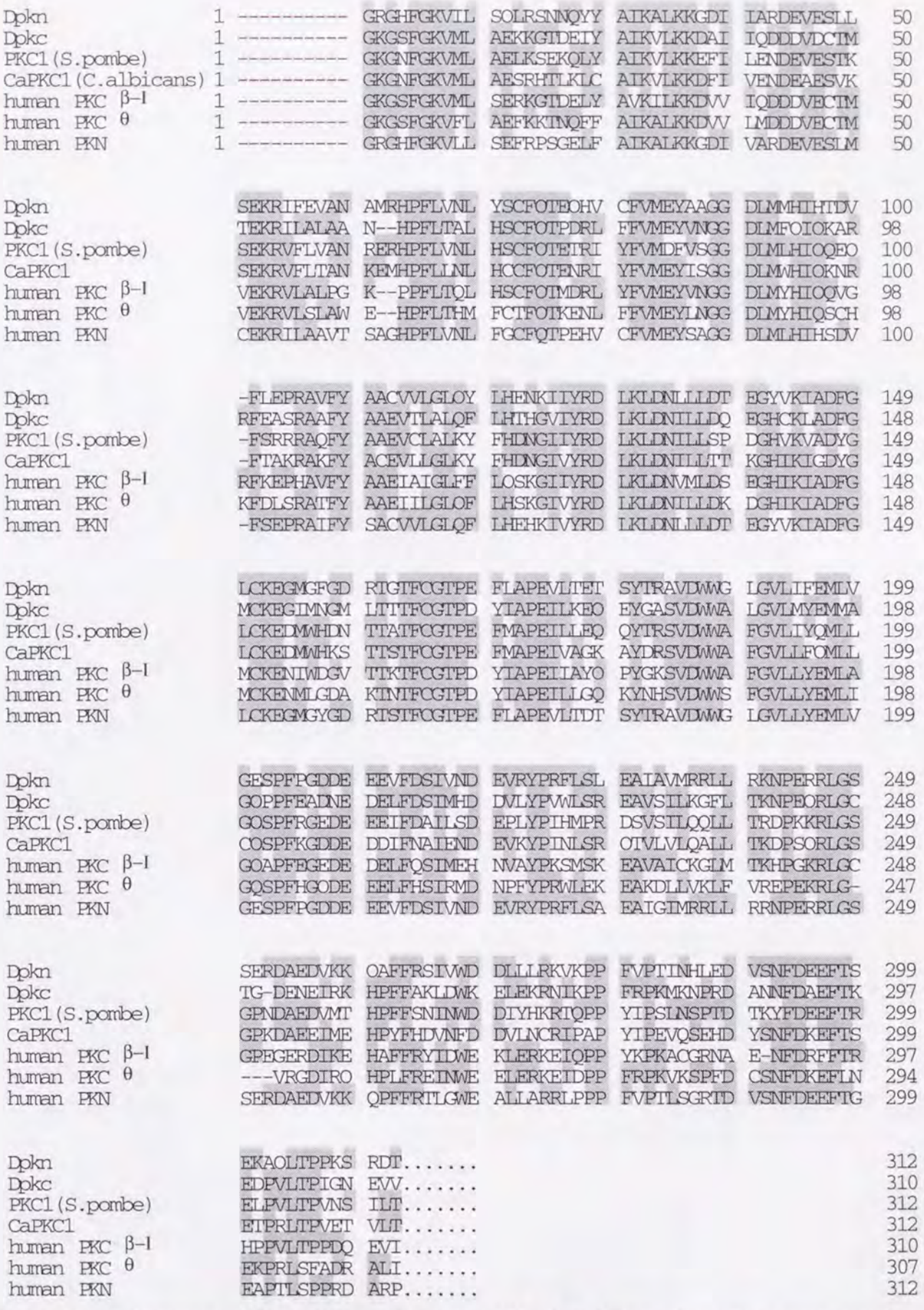


Fig. 7. (A) Alignment of Dpkn kinase domain with other kinase domains from members of the PKC family . Conserved residues among these kinases are shaded.

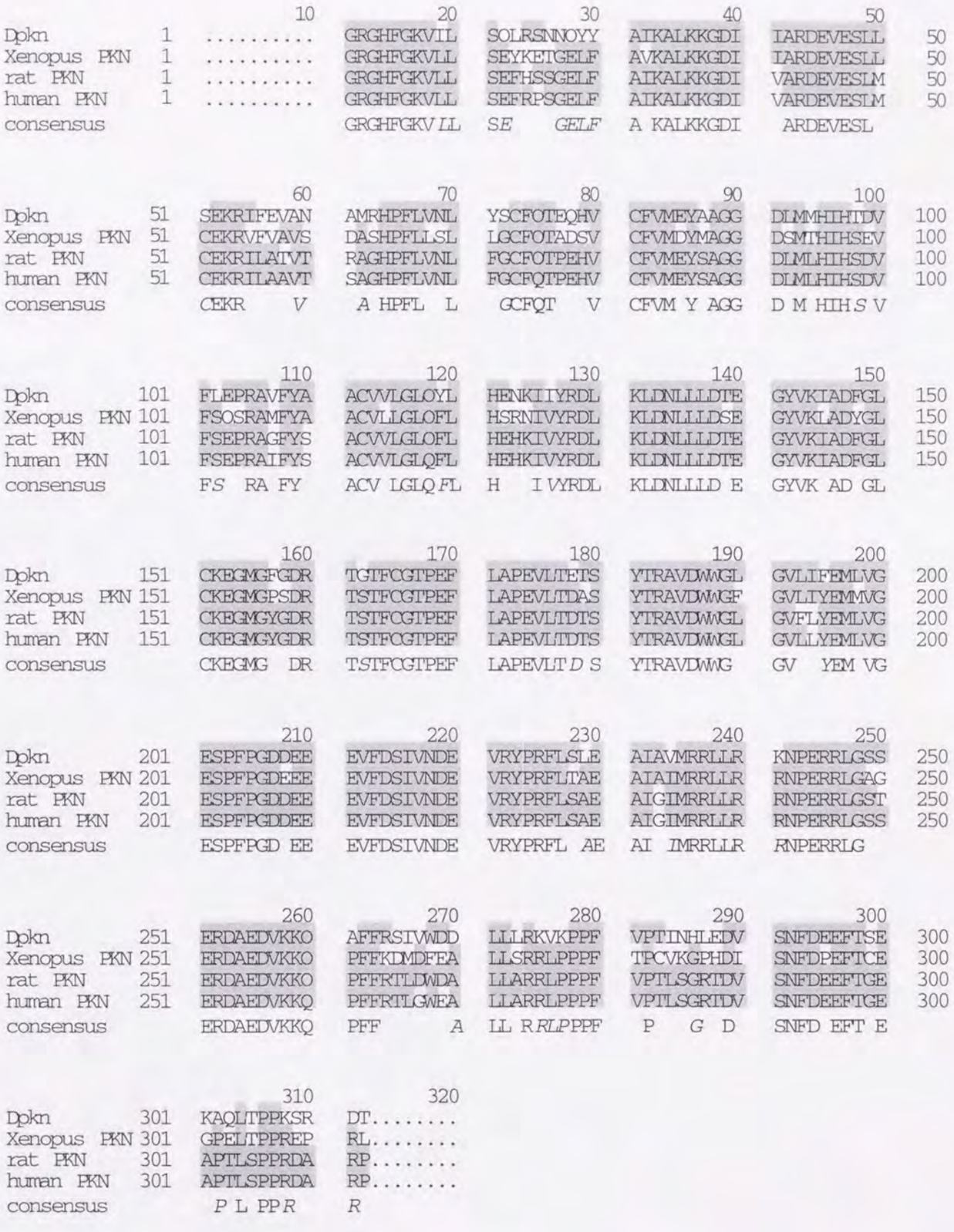


Fig.7 (B) Alignment of Dpkn kinase domain with the previously reported Xenopus, rat, and human PKNs. Conserved residues among these kinases are shaded.

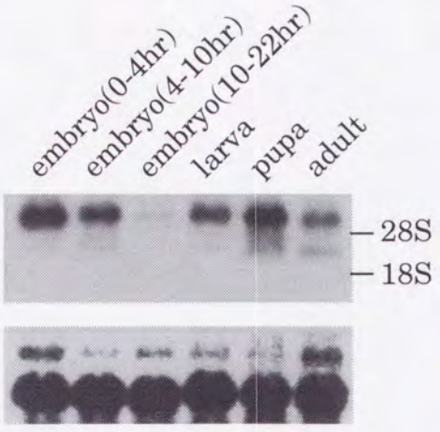
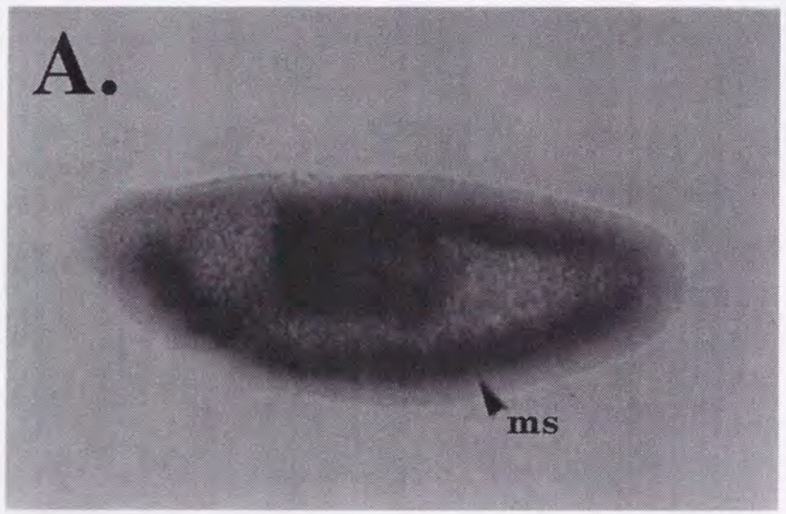
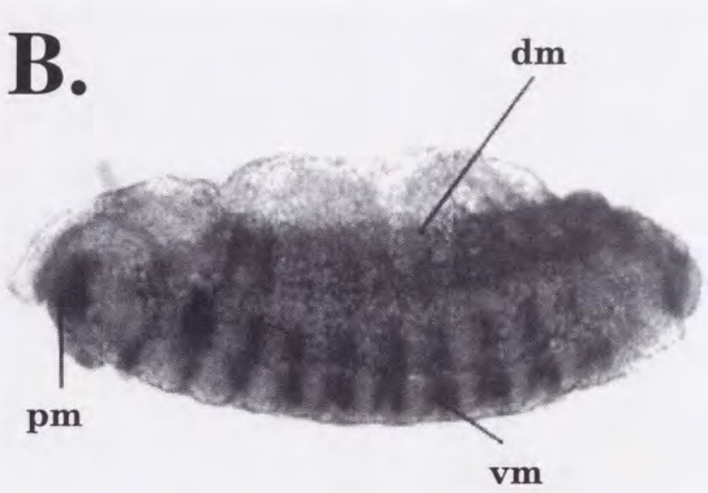


Fig. 8. (A) Developmental expression of Dpkn transcripts. Total RNA was prepared from Drosophila at various stages of development, separated by 1% agarose formaldehyde gels, transferred onto nylon membranes, and hybridized with radiolabeled probe for Dpkn as described in Experimental Procedures. The 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).

To determine the spatial distribution of Dpkn transcripts during embryogenesis, I performed *in situ* hybridization on whole-mount embryos (see Experimental Procedures). Distinct expression of Dpkn was detected primarily in the mesodermal layer (Fig.8B, panel A.). Expression of Dpkn was then restricted to the somatic musculature (Fig.8B, 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).





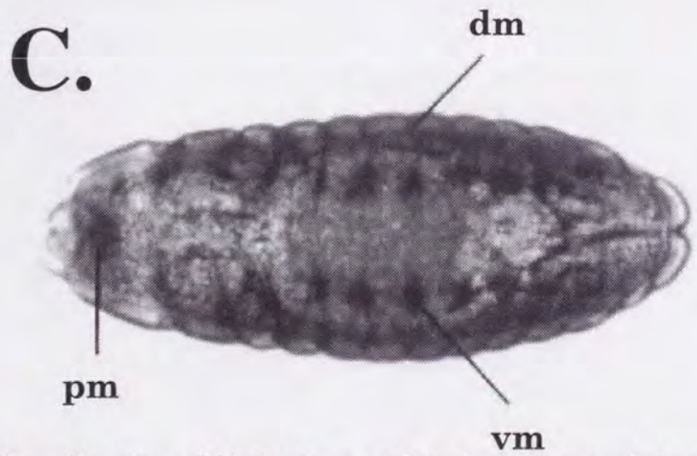


Fig.8. (B) Localization of Dpkn transcripts in embryos detected by whole mount in situ hybridization. A: Lateral view of embryo at gastrulation (stage 9). Expression is restricted to mesodermal cell layers. B: Lateral view of developing embryo (stage 14). Expression of Dpkn was retained in the developing somatic muscular system throughout embryogenesis. C: Ventral view of developing embryo (stage 1 4-15). 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.

Cloning of Dnrk cDNAs

Using the subcloned region of DK3 as a probe, I screened cDNA libraries from *Drosophila* imaginal discs (see Experimental Procedures). cDNA clones were isolated from the cDNA library and the longest cDNA insert (~3.4 kb) for Dnrk(*Drosophila* neurospecific receptor kinase) was further characterized. The nucleotide sequence of this Dnrk clone contains one long open reading frame with in-frame stop codons preceding the first ATG (Fig.9). In addition, sequences upstream of the the putative translation start site (the first ATG) matched the *Drosophila* consensus for translational initiation (53,54). The initiating methionine is followed by a hydrophobic domain of 25 residues that may serve as a signal peptide (55,56). By analogy with other signal peptides, this sequence would be cleaved between residues 25 and 26 at the Ala-Asn junction (Fig.9, 55,56). Accordingly, the mature Dnrk protein would contain 689 amino acids (aa) and resembles a typical RTK. Within this protein, the amino-terminal 275 aa residues constitute the extracellular domain, the membrane spanning domain (26 aa), and the carboxy-terminal 388 aa residues, the cytoplasmic domain.

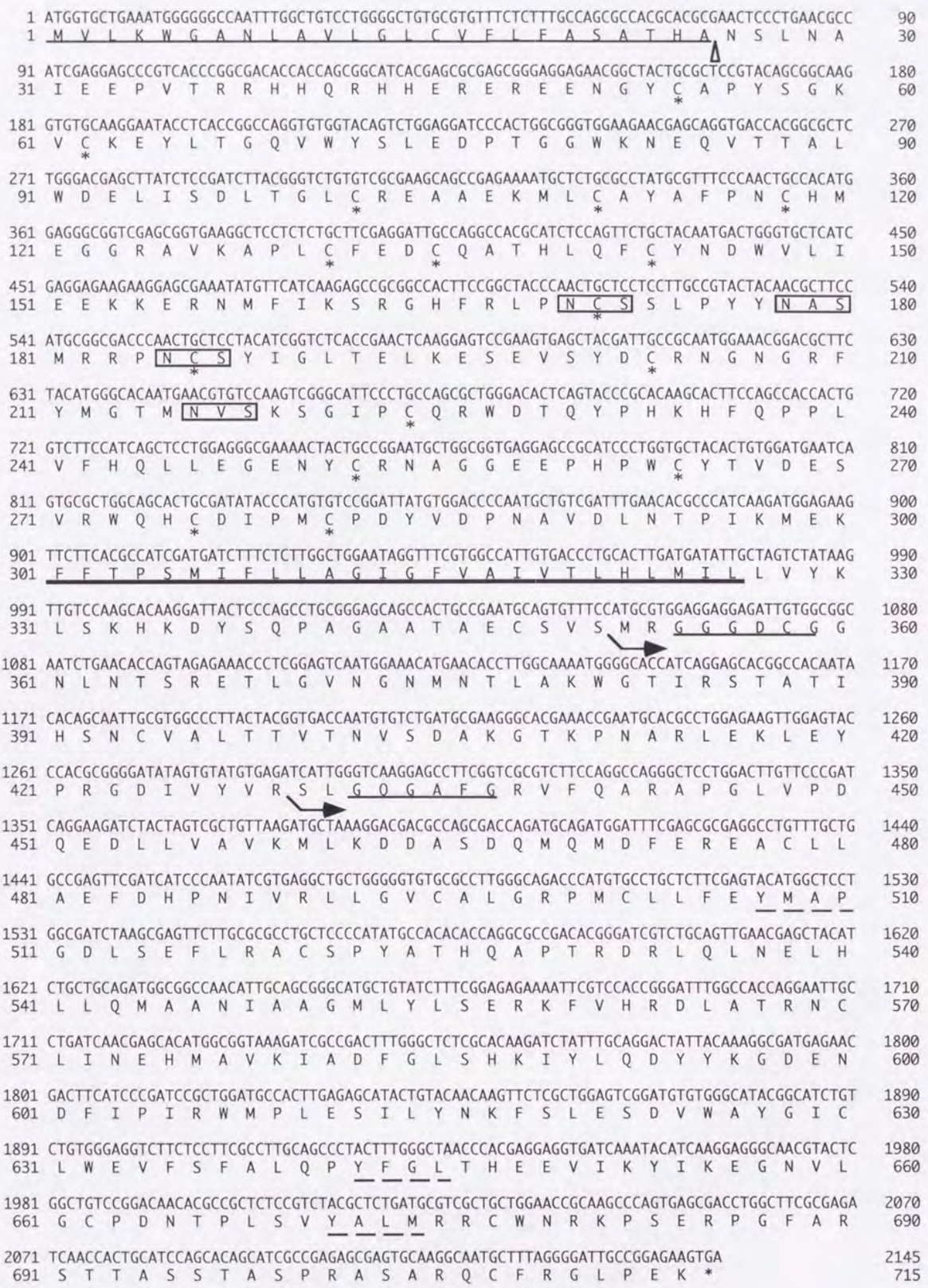


Fig. 9. Nucleotide sequence of Dnrk cDNA and deduced amino acid sequence of the Dnrk gene product. (To be continued)

(Fig9. continued) The depicted nucleotide sequence corresponding to the open reading frame was derived from the longest 3.0kb clone (pNB40-Dnrk). In-frame terminator codons in the 5' untranslated region were found 60, 114, and 1 53bp upstream of the putative translation initiation start site (data not shown). Analysis of the deduced amino acid sequence of the 714 amino acid (aa) polypeptide encoded by the Dnrk gene revealed various structural features (see Fig.10a &b) that include: a putative signal peptide (aa 1-25; underlined, and a putative cleavage site was indicated by an open arrowhead), four consensus N-linked glycosylation sites (boxed), the putative transmembrane region (aa 301-3 26; underlined by a solid bar), two putative ATP binding motifs (indicated by arrows followed by underlined GlyXGlyXXGly sequences, see Text) and three putative tyrosine-containing motifs (indicated by dotted lines) that may interact with SH2 regions of signaling molecules upon tyrosine phosphorylation (see Text). Cysteine residues within the extracellular domain are indicated by asterisks.

Structural Features of Dnrk

The putative TK domain of Dnrk is most similar to those of the vertebrate Trk- and Ror-family RTKs (57-61) as well as to the previously cloned *Drosophila* RTK, Dror (61) (Fig.10a). It reveals about 40-45% identity to the corresponding domains of TrkB, Ror1, Ror2, and Dror (Fig.10a)(57,61,62). The TK domain of Dnrk also shares somewhat lower levels of similarity with those of the Trk-related *Drosophila* RTK, Dtrk, and the muscle-specific RTKs, the Torpedo RTK and the mammalian MuSKs (muscle-specific kinases) (62,63). Like the previously reported Trk- and Ror-family RTKs, Dnrk contains the TyrXXAspTyrTyr sequence motif (aa 590-595, Fig.9 & 10a), corresponding to the autophosphorylation site of insulin receptor(s) (60,64). Interestingly, Dnrk possesses the two putative ATP binding motifs (Gly354XGlyXX Gly359/Lys380 and Gly432XGlyXXGly437/Lys459, Fig.9) within its TK domains, that is a characteristic feature found in the Dtrk protein (65). Furthermore, there are several putative tyrosine-containing motifs (Tyr507MetAlaPro510, Tyr642PheGly Leu645, Tyr671AlaLeuMet674; see Fig.9) that may interact with Src Homology 2 (SH2) regions of cellular signaling molecules [SH2 of Vav for PO_4-Tyr507MetAla Pro510, SH2 of PTP1C (possibly corkscrew, csw) forPO_4-Tyr642PheGlyLeu645, and

SH2s of Shc & Csk for PO_4-Tyr671AlaLeuMet674] upon tyrosine phosphorylation (66,67), although thus far the *Drosophila* homologues of mammalian Vav and Csk have not been reported.

The extracellular domain of Dnrk exhibits a high degree of homology with those of Dror and human Rors. Sequence alignment of the Dnrk, Dror and human Rors extracellular domains is indicated (Fig.10b). The most notable feature is that all 16 cysteines in Dnrk are also found in equivalent positions in Dror, Ror1 and Ror2. The Dnrk extracellular domain also contains four potential N-linked glycosylation sites (Fig.9). As shown in Fig.10b, the Dnrk extracellular domain contains two cysteine-rich domains (cys domain) and a membrane-proximal kringle domain, that are characteristic features of the Ror-family RTKs (Fig.10b). The kringle domain is a highly folded structure, rich in cysteines and is found in certain blood coagulation proteins, apolipoprotein, and hepatocyte growth factor (68-71). For receptor-type proteins, the kringle motif has been reported for the Ror-family RTKs, Rors and Dror, and for the Torpedo RTK (60-62). Although the functional role of the kringle domain in these RTKs remains unclear, it is believed to be involved in mediating protein-protein interactions (61,68). However, unlike human Rors, both Dnrk and Dror lack the N-terminal immunoglobulin-like (Ig-like) domain. Intriguingly, the extracellular domain of Dnrk also displays some degree of similarity with those of muscle-specific RTKs, the Torpedo RTK and the mammalian MuSKs (data not shown).

```
Dnrk              425 IVYVRSLGQG AFGRVFQARA PGLVPDQEDL LVAVKMLKDD ASDQMQMDFE     474
Trk B  (mouse)    537 IVLKRELGEG AFGKVFLAEC YNLCPEQDKI LVAVKTLKD- ASDNARKDFH     585
Ror 1  (human)    473 VRFMEELGEC AFGKIYKGHL YLPGM-DHAQ LVAIKTLKDY NNPQQWMEFQ     521
Ror 2  (human)    473 VRFMEELGED RFGKVYKGHL FGPAPGEQTQ AVAIKTLKDK AEGPLREEFR     522
Dror              410 VEFLEELGEG AFGKVYKGQL LQPNK--TTI TVAIKALKEN ASVKTQQDFK     457

Dnrk                  REACLLAEFD HPNIVRLLGV CALGRPMCLL FEYMAPGDLS EFLRACSPY-     523
Trk B  (mouse)        REAELLTNLQ HEHIVKFYGV CVEGDPLIMV FEYMKHGDLN KFLRAHGPD-     634
Ror 1  (human)        QEASLMAELH HPNIVCLLGA VTQEQPVCML FEYINQGDLH EFLIMRSPHS     571
Ror 2  (human)        HEAMLRARLQ HPNVVCLLGV VTKDQPLSMI FSYCSHGDLH EFLVMRSPHS     572
Dror                  REIELISDLK HQNIVCILGV VLNKEPYCML FEYMANGDLH EFLISNSPT-     506

Dnrk                  ---ATHQAPT RDRLQLNELH LLQMAANIAA GMLYLSERKF VHRDLATRNC     570
Trk B  (mouse)        ---AVLMAEG NPPTELTQSQ MLHIAQQIAA GMVYLASQHF VHRDLATRNC     681
Ror 1  (human)        DVGCSSDEDG TVKSSLDHGD FLHIAIQIAA GMEYLSSHFF VHKDLAARNI     621
Ror 2  (human)        DVG-STDDDR TVKSALEPPD FVHLVAQIAA GMEYLSSHHV VHKDLATRNV     621
Dror                  ---------- -EGKSLSQLE FLQIALQISE GMQYLSAHHY VHRDLAARNC     545

Dnrk                  LINEHMAVKI ADFGLSHKIY LQDYYKGDEN DFIPIRWMPL ESILYNKFSL     620
Trk B  (mouse)        LVGENLLVKI GDFGMSRDVY STDYYRVGGH TMLPIRWMPP ESIMYRKFTT     731
Ror 1  (human)        LIGEQLHVKI SDLGLSREIY SADYYRVQSK SLLPIRWMPP EAIMYGKFSS     671
Ror 2  (human)        LVYDKLNVKI SDLGLFREVY AADYYKLLGN SLLPIRWMAP EAIMYGKFSI     671
Dror                  LVNEGLVVKI SDFGLSRDIY SSDYYRVQSK SLLPVRWMPS ESILYGKFTT     595

Dnrk                  ESDVWAYGIC LWEVFSFALQ PYFGLTHEEV IKYIKEGNVL GCPDNTPLSV     670
Trk B  (mouse)        ESDVWSLGVV LWEIFTYGKQ PWYQLSNNEV IECITQGRVL QRPRTCPQEV     781
Ror 1  (human)        DSDIWSFGVV LWEIFSFGLQ PYYGFSNQEV IEMVRKRQLL PCSEDCPPRM     721
Ror 2  (human)        DSDIWSYGVV LWEVFSYGLQ PYCGYSNQDV VEMIRNRQVL PCPDDCPAWV     721
Dror                  ESDVWSFGVV LWEIYSYGMQ PYYGFSNQEV INLIRSRQLL SAPENCPTAV     645

Dnrk                  YALMRRCWNR KPSERPGFAR STTASSTASP RASARQCFRG LPEK           714
Trk B  (mouse)        YELMLGCWQR EPHTRKNIKS IHTLLQNLAK ASPVYLDILG               821
Ror 1  (human)        YSLMTECWNE IPSRRPRFKD IHVRLRSWEG LSSHTSSTTP SGGNATT...    768
Ror 2  (human)        YALMIECWNE FPSRRPRFKD IHSRLRAWGN LSNYNSSAQT SGASNTT...    768
Dror                  YSLMIECWHE QSVKRPTFTD ISNRLKTWHE GHFKASNPEM               685
```

Fig.10. (a) Alignment of Dnrk TK domain with other TK domains from members of the Trk-/Ror-family RTKs. Residues that are highly conserved among the five RTKs are shaded. Dashed line indicates gaps inserted for optimal alignment.

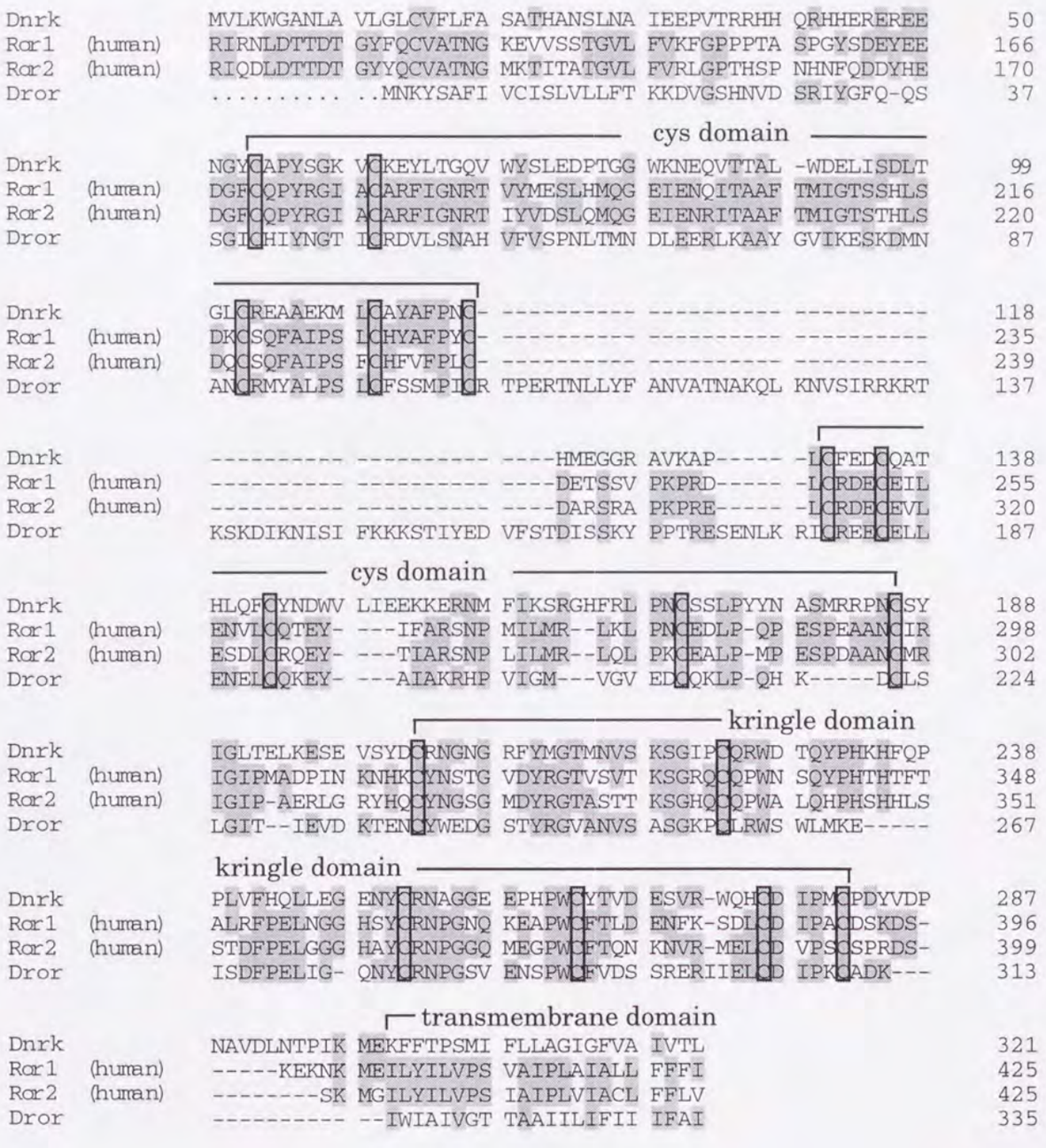


Fig.10. (b) Alignment of Dnrk extracellular domains with the previously reported Ror-family RTKs [Ror1, 2 (Human), Dror (*Drosophila*)]. The shared cysteine-cont aining domain (cys domain), kringle domain, and amino-terminal portion of the transmembrane domain are marked by brackets. Conserved cysteine residues are boxed. Residues that are highly conserved among the four RTKs are shaded. Dashes indicate gaps inserted for optimal alignment.

Chromosomal Mapping and Expression of Dnrk

To determine the cytological location of Dnrk gene, a cDNA probe was hybridized to polytene chromosomes (see Experimental Procedures). Chromosome *in situ* hybridization showed that Dnrk maps to the 49f region on the right arm of the second chrosome (Fig.11a, 72).

To characterize the temporal expression pattern of the Dnrk gene, I performed Northern blot analysis with RNA samples from embryos (0-4hr, 4-10hr, 10-22hr), larva, pupa, and adult flies. Radiolabeled cDNA, covering the entire open reading frame, was used as a probe. Dnrk probe detected a major band about 3.5-kb in size (Fig.11b). Dnrk is expressed at high levels in 4-22hr embryos, larva, pupa, with maximal expression in pupa where the restructuring of the nervous system occurs (Fig.11b). It is noteworthy that during embryogenesis the level of Dnrk expression accumulated maximum during 4-10hr and gradually declined thereafter (see Fig.11b). A decreased level of expression was observed in adult flies.

I next performed *in situ* hybridization experiments to whole-mount embryos to determine the tissue specificity of Dnrk transcripts during embryogenesis (see Experimental Procedures). Distinct expression of Dnrk was not detected at stages preceding germ band elongation. Weak expression was observed at stages 10 in the ventral area of the germ band corresponding to the neurogenic ectoderm (data not shown). This expression became stronger and clearer at stage 11 and was restricted to the layer of neural progenitor cells between the epidermal and mesodermal cell layers (Fig.12A). This expression appeared to be sustained in the neural cell lineage throughout the remainder of embryogenesis resulting in expression in the brain and ventral nerve cord (Fig.12B). The distribution of transcripts after germ band shortening (stage 13, Fig.12C) matched the profile of developing commisures and

connectives. Expression of Dnrk was also observed in the peripheral nervous system at stage 17 when larval sensory cells have differentiated (Fig.12D). No mutations having defects in neural development are known to be located at the 49f chromosomal region where Dnrk mapped to.

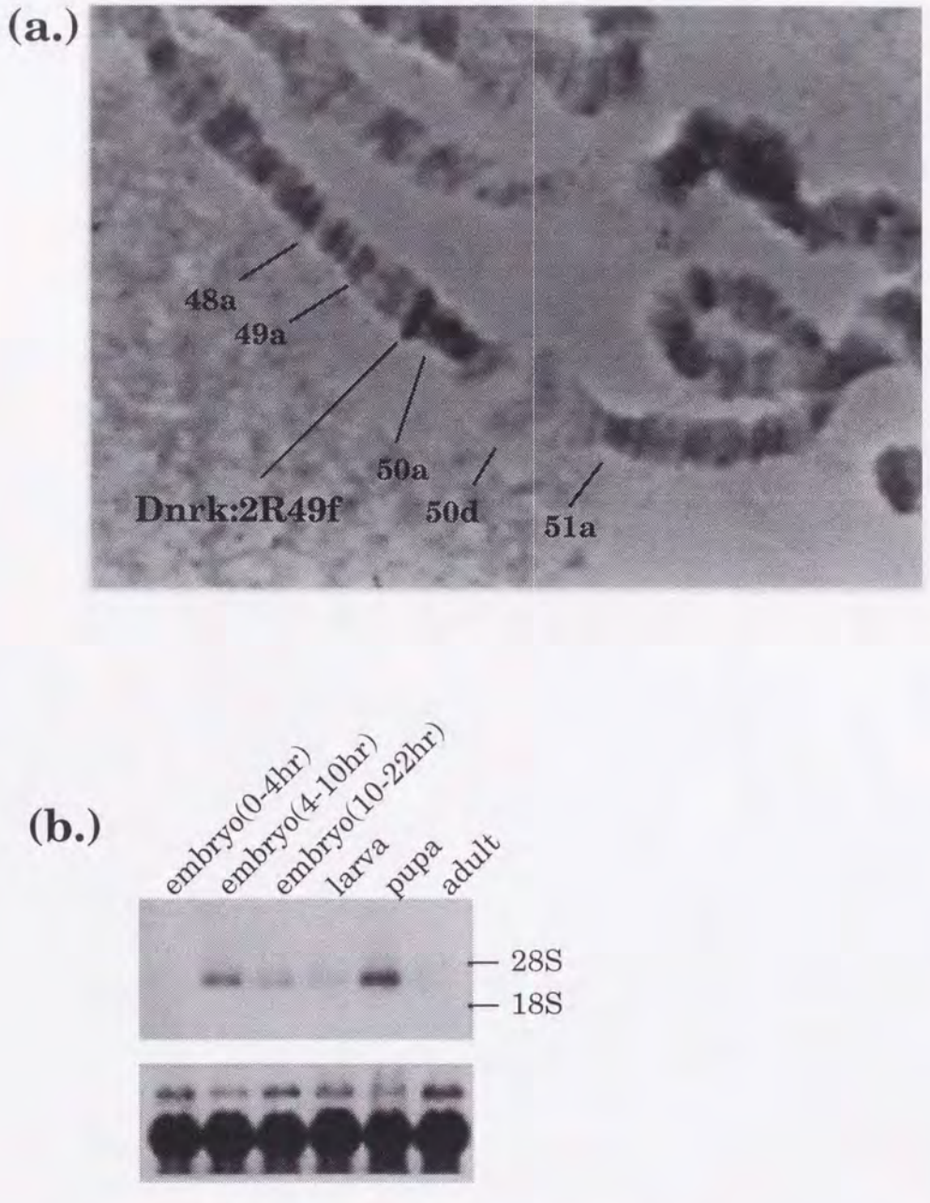


Figure 11. (a) Cytological position of Dnrk determined by in situ hybridization to salivary gland chromosomes. A signal is detected at the 49f band on the right arm of the second chromosome close to a prominent puff at 50d.
(b) Developmental expression of Dnrk transcripts. Total RNA was prepared from Drosophila at various stages of development, separated by 1% agarose formaldehyde gels, transferred onto nylon membranes, and hybridized with radiolabeled probe for Dnrk 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, although a relatively small amount of transcripts detected by rp49 probe was observed for RNA from pupa (data not shown).

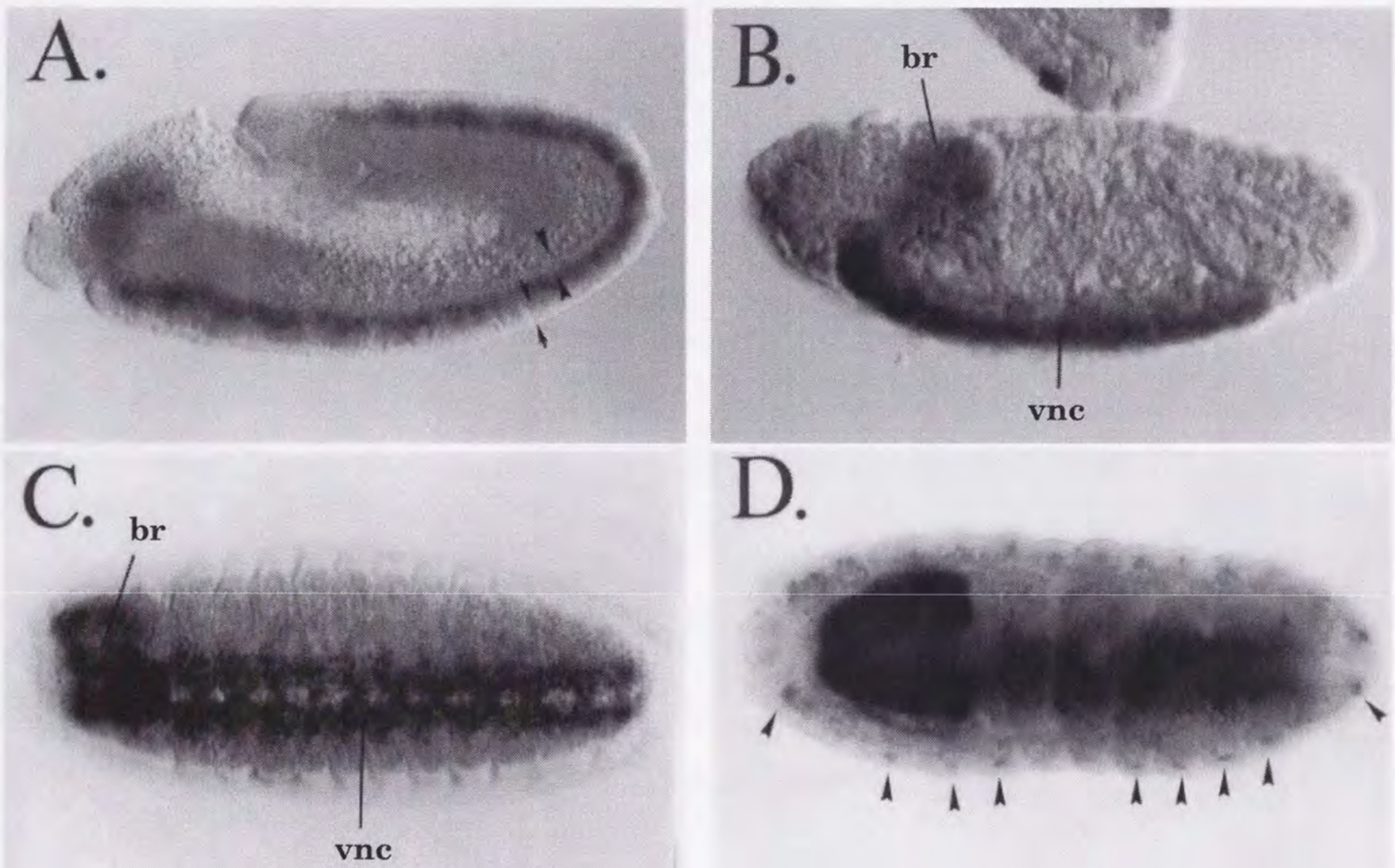


Fig.12. Localization of Dnrk transcripts in embryos detected by whole mount in situ hybridization. A: Lateral view of elongated germ band embryo (stage 11). Expression is restricted to neurogenic cell layer and not mesodermal (arrow heads) nor epidermal (arrows) cell layers. B: Lateral view of fully developed embryo (stage 17). Expression of Dnrk was retained in the developing central nervous system throughout embryogenesis. C: Ventro-lateral view of embryo after germ band shortening (stage 13). Expression of Dnrk transcripts matches the distribution of the commisures and connectives in the ventral nerve cord. D: Ventral view of fully developed embryo (stage 1 7). Dnrk transcripts are detected in larval sensory organs (Examples indicated by arrowheads). Anterior to left in all embryos. Dorsal to the top in A and B. br: brain, vnc: ventral nerve cord.

Protein Kinase Activity of Dnrk

To test the catalytic activity of the putative TK domain of Dnrk, I constructed expression vectors encoding the HA-tagged cytoplasmic kinase domains of Dnrk (see Experimental Procedues). Since the TK domain of Dnrk possesses two putative ATP binding motifs (distal to the following kinase subdomains, proximal to the following kinase subdomains), the two different HA-tagged kinase domains of Dnrk, HA-DnrkS (aa 404-714 of Dnrk) and HA-DnrkL (aa 337-714 of Dnrk) were expressed transiently in COS cells (see Experimental Procedures). The HA-DnrkL protein possesses the two ATP binding motifs, while the HA-DnrkS protein lacks the region (aa 337-403) containing the distal ATP binding motif (Fig.13a).

Expression of HA-DnrkS and HA-DnrkL was first assessed by anti-HA immunoblotting of whole cell lysates from COS cells transfected with expression vectors encoding either HA-DnrkS or HA-DnrkL. As shown in Fig.13b, anti-HA antibody clearly detected HA-DnrkS and HA-DnrkL with expected molecular masses (38kDa and 45kDa, respectively). Expression of HA-DnrkS was constantly higher (~1.5-fold) than that of HA-DnrkL. As expected, when anti-HA immunoprecipitates of whole cell lysates from COS cells, expressing the respective HA-tagged proteins, were subjected to anti-HA immunoblotting, relatively high expression (~1.5-fold) of HA-DnrkS was also observed when compared to HA-DnrkL (data not shown).

To examine the catalytic activities of HA-DnrkS and HA-DnrkL, anti-HA immunoprecipitates from cells, expressing the respective proteins, were incubated in the presence of [γ-^{32}P] ATP. Specific phosphorylation of DnrkL with an expected molecular mass was observed (Fig.13c). DnrkS lacking the distal ATP binding motif was also specifically phosphorylated *in vitro*, yet to a lesser extent (about 1/3

compared to DnrkL) (Fig.13c). Since the amount of the HA-DnrkS immunoprecipitated with anti-HA antibody is higher (~1.5-fold) than that of DnrkL, our result suggests that DnrkL exhibits a higher specific activity (4-5 fold) when compared to DnrkS. Phosphoamino acid analysis revealed that phosphorylations of DnrkL and DnrkS occurred on tyrosine as well as serine residues (data not shown). The extents of tyrosine versus serine phosphorylation on DnrkL (containing 24 serine and 14 tyrosine residues) and DnrkS (containing 18 serine and 13 tyrosine residues) were comparable (data not shown). It is likely that phosphorylation of DnrkL and DnrkS on serine residues was due to a contaminating serine kinase, however, I cannot entirely rule out the possibility that phosphorylation of DnrkL and DnrkS on tyrosine residues is mediated by a contaminating tyrosine kinase. In this respect, it should be noted that bacterially expressed fusion proteins, DnrkS and DnrkL respectively fused to glutathione S-transferase (GST), were phosphorylated on tyrosine residues (data not shown). Since DnrkS exhibited autophosphorylation activity, yet to a lesser extent compared to DnrkL, it was indicated that the proximal ATP binding motif alone is sufficient for the kinase activity of Dnrk. Furthermore, our results suggest that the distal ATP binding motif may be required for the full-scale kinase activity of Dnrk, although further study is required to elucidate the exact role of the distal ATP binding motif.

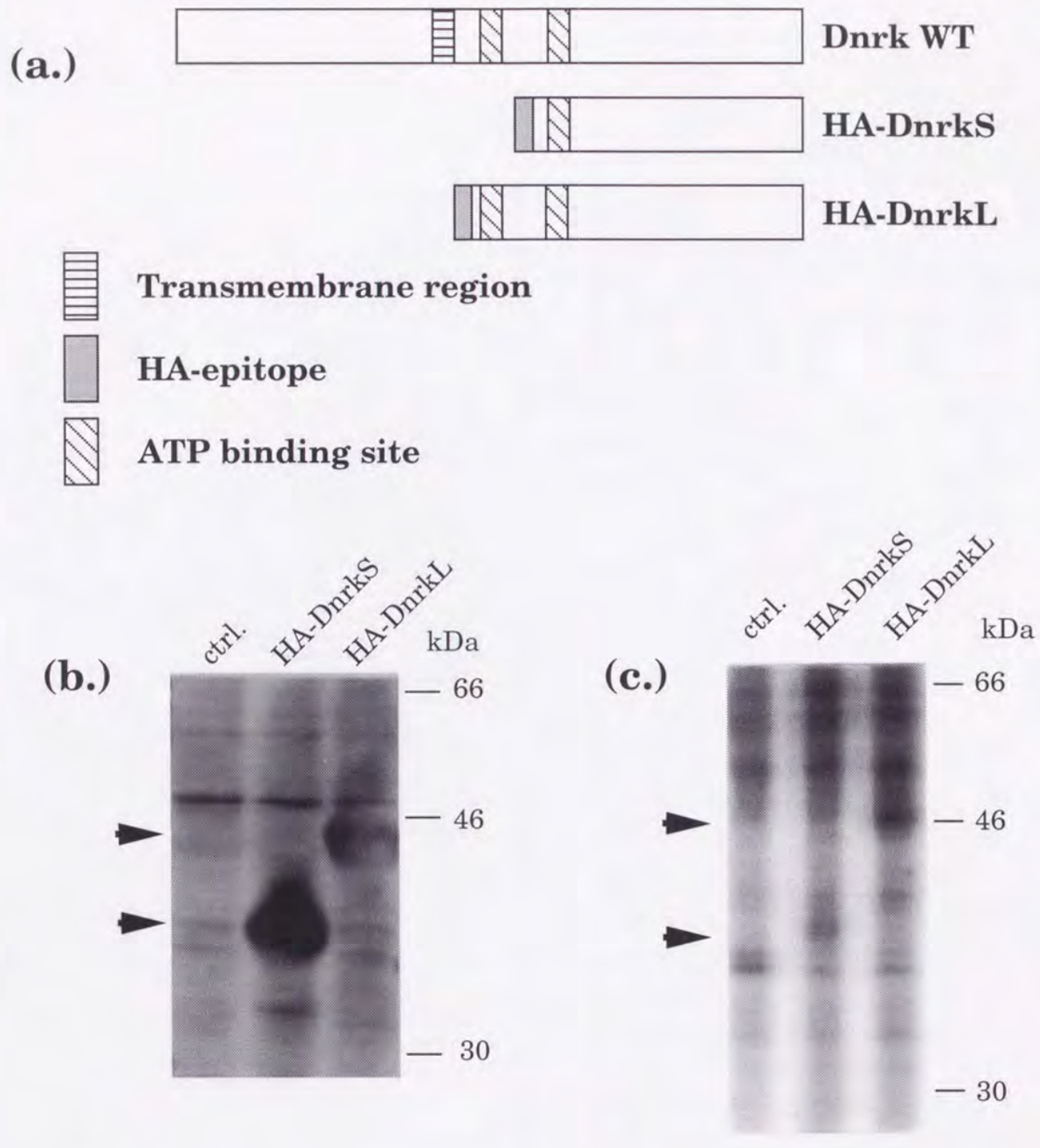


Fig.13. (a) A schematic diagram of the HA-tagged TK domains of Dnrk, HA-DnrkS and HA-DnrkL. (b) Expression of HA-DnrkS and HA-DnrkL. Cell lysates (equivalent cell numbers) were prepared from COS cells; COS cells transfected with control vector (pEF), HA-DnrkS (aa404-714 of Dnrk) (pEF-HA-DnrkS), HA-DnrkL (aa337-714 of Dnrk) (pEF-HA-DnrkL). The whole cell lysates (equivalent cell numbers) were analyzed by anti-HA immunoblotting as described in Experimental Procedures. Expression of HA-DnrkS detected by immunoblotting was constantly higher (1.5~2-fold) than that of HA-Dnrk (see Text). (c) In vitro kinase activities of HA-DnrkS and HA-DnrkL. Cell lysates (equivalent cell numbers, see Fig.13b) from COS cells transfected with pEF, pEF-HA-DnrkS, and pEF-HA-DnrkL, respectively, were immunoprecipitated with anti-HA antibody followed by anti-HA immunoblotting (see Experimental Procedures). In vitro kinase assay was performed as described in Experimental Procedures. About 3-fold higher autophosphorylation of HA-DnrkL was observed when compared to HA-DnrkS, although amounts of HA-DnrkL immunoprecipitated with anti-HA antibody was lower (about 2/3) than that of HA-DnrkS (data not shown, see Fig.13b).

Discussion

Dpkn

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 (73-75). 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 (75-78). Thus far, three PKC genes in the fruit fly *Drosophila* melanogaster have been identified (79-81). They include DPKC53E, a homologue of the mammalian PKCα (80), DPKC98E, a homologue of the mammalian PKCδ (81), and eye-PKC, a mammalian PKCa homologue expressed exclusively in photoreceptor cells of the visual system (81). 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 (51,52).

I 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).

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 (82,83), and is activated by the binding of the active GTP-bound form of Rho (82). The Rho-binding site is localized within the N-terminal portion of PKN, that has been assumed to be a regulatory domain of PKN (82). Thus, the entire sequence of Dpkn is required to elucidate whether Dpkn is indeed a *Drosophila* homologue of mammalian PKNs.

The expression of Dpkn is restricted to mesodermal cell layers in early embryo and retained in the developing somatic muscular system throughout embryogenesis.

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 (52). These results indicated that Dpkn may play a role in the development and function of somatic muscles in *Drosophila*. Considerring the fact that apparent expression of Dpkn is observed in mesodermal cell layers during early embryogenesis, Dpkn may also play a role in the movement of mesodermal cells. 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*.

Dnrk

Using a PCR-based approach, I have cloned a cDNA encoding a novel *Drosophila* RTK, Dnrk, that is expressed exclusively in the nervous system during embryogenesis. Dnrk possesses unique structural features that have been reported for the Trk- and Ror-family RTKs, in particular for Ror-family RTKs. The cytoplasmic tyrosine kinase (TK) domain of Dnrk exhibits a high degree of homology with those of the Trk- and Ror-family RTKs (57-61) (Fig.10a). Interestingly, like Dtrk, the Trk-related *Drosophila* RTK (60), the cytoplasmic TK domain of Dnrk contains two tandemly repeated putative ATP binding motifs at the membrane proximal portion (Fig.9). Within the extracellular domain of Dnrk, there are two cysteine-rich domains and a membrane-proximal kringle domain, unique domain(s) shared with the Ror-family RTKs (mammalian Rors, *Drosophila* Dror) and muscle-specific RTKs (mammalian MuSKs, Torpedo RTK) (60-63) (Fig.10b, data not shown). Considering the fact that both Dnrk and Dror display structural similarities with human Ror1 and

Ror2, and that expression of these RTKs (Dnrk, Dror, Ror1, and Ror2) was restricted to the developing nervous system, it is possible that Dnrk as well as Dror are *Drosophila* homologues of mammalian Rors (Ror1 and Ror2) (see below). Thus far, ligands for these neuronal RTKs as well as muscle-specific RTKs have not been reported. Since these RTKs, including Dnrk, share particular similarity within their extracellular domains, it is likely that their cognate ligands are also structurally related. At present, it is unclear whether their ligands are soluble, like neurotrophins, or cell surface molecules.

Similar to Dror, distinct expression of Dnrk was not detected before the extended germ-band stage (Fig.12). In addition, like Dror, Dnrk is exclusively expressed in the nervous system. With this respect, it is important to note that Dtrk, another *Drosophila* RTK related to the Trk-family RTKs, is expressed in the nervous system as well as outside the nervous system (62). The determination and differentiation of neuroprecursor cells in both the CNS and PNS begin at stages preceding those that the expression of Dnrk was first seen in these tissues. Thus, the role of Dnrk would be expected to be one involving the subsequent differentiation or organization of the cells of the CNS and PNS.

In addition to structural features of Dnrk, our *in vitro* kinase analysis of Dnrk revealed that Dnrk indeed possesses tyrosine kinase activity (Fig.13c). As described, Dnrk contains two putative ATP binding motifs within its TK domain. Therefore, it was interesting to examine the possible roles of the respective ATP binding motifs in TK activity of Dnrk. Since antibody against the Dnrk protein is not currently available, I constructed and expressed the HA-tagged cytoplasmic TK domains of Dnrk in COS cells. The HA-DnrkS, lacking the distal ATP binding motif, still exhibits autophosphorylation activity *in vitro*, albeit to a lesser extent when compared to the HA-DnrkL that possesses both ATP binding motifs (Fig.13c). Thus, it became evident

that the proximal ATP binding motif itself is sufficient for the catalytic activity of Dnrk. Our results also suggest that the distal ATP binding motif may be required for the full-scale activity of Dnrk, although further studies will be required to clarify this issue. To date, among previously reported RTKs, only Dnrk and Dtrk possess two putative ATP binding motifs within their TK domains. However, it remains unclear whether or not this unique feature reflects the phylogenical relationship of Dnrk and Dtrk.

Previous studies demonstrate that activation of RTKs result in auto-phosphorylation of RTKs, thereby creating binding sites for SH2 region containing cytosolic proteins, including phosphoinositide 3-kinase, phospholipase Cγ, Grb2, and Shc (66,67). This molecular interaction triggers subsequent signaling cascades, that eventually reach the nucleus. Recently, the use of degenerate phosphopeptide libraries have revealed the substrate specificities of SH2 domains and the phosphotyrosine-containing motifs within RTKs as well as cytosolic tyrosine kinases (reviewed in 67). On the basis of the previously reported prediction, I found several tyrosine-containing motifs (Tyr507MetAlaPro510, Tyr642PheGlyLeu645, Tyr671AlaLeuMet674) within the catalytic TK domain of Dnrk, that are candidate sites for interaction with SH2 region-containing cytoplasmic signaling molecules. Upon tyrosine phosphorylation, for example, Tyr507MetAlaPro510, Tyr642PheGlyLeu645, Tyr671AlaLeuMet674 are assumed to be able to interact with SH2 regions of mammalian proteins, Vav, PTP1C, Shc & Csk, respectively. Thus far, csw and dShc, the *Drosophila* homologues of PTP1C and Shc, have been reported (84,85). Such putative tyrosine-containing motifs were not found in the corresponding domain of Dror. At the present time I do not know whether some if not all of the tyrosine residues within these putative motifs are phosphorylated upon activation of Dnrk. Nonetheless, these preliminary observations raise an interesting possibility to

be tested that Dnrk may interact with cytoplasmic signaling molecules by utilizing such motifs.

Like Dror, expression of Dnrk is restricted to the nervous system during embryogenesis (Fig.12). Furthermore, the peak expression of Dnrk as well as Dror occurs when early processes of neuronal differentiation, including axonogenesis, occur. Recently, to perform the genetic and developmental analysis, I have also cloned mouse homologues of Dnrk and Dror, mouse Ror1 and Ror2, that expressed in the head of developing embryos (data not shown). In addition, it has been reported that Rors are also expressed relatively early during development and that their expression declines drastically at a later stage in rat embryo(60). Thus, these Ror-family RTKs (Dnrk, Dror, and mammalian Rors) may play an important role in early neuronal development.

Like human Rors, mouse Ror proteins possess an Ig-like domain, two conserved cysteine-containing domains and a kringle domain within their extracellular domain (Fig14). Unlike Dnrk, the cytoplasmic regions of mouse Rors lack a putative tyrosine-containing motif that may interact with SH2-containing signaling molecules, instead they possess proline-rich sequences that may interact with SH3 and/or WW domains in addition to their catalytic tyrosine kinase domain(86,87).

Recently, it has been reported that the muscle-specific RTK, MuSK, related to Ror-family RTKs, is specifically expressed in early myotomes and developing muscle, and becomes selectively localized to the postsynaptic muscle surface at neuromuscular junctions upon muscular maturation (63). Moreover, it has been shown that a targeted disruption of the MuSK gene results in a failure in the formation of neuromuscular synapses (88,89). These results demonstrate a critical role(s) of MuSK in synapse formation at neuromuscular junctions. Considering related features (i.e. structure and expression pattern) of the Ror-family RTKs (Dnrk,

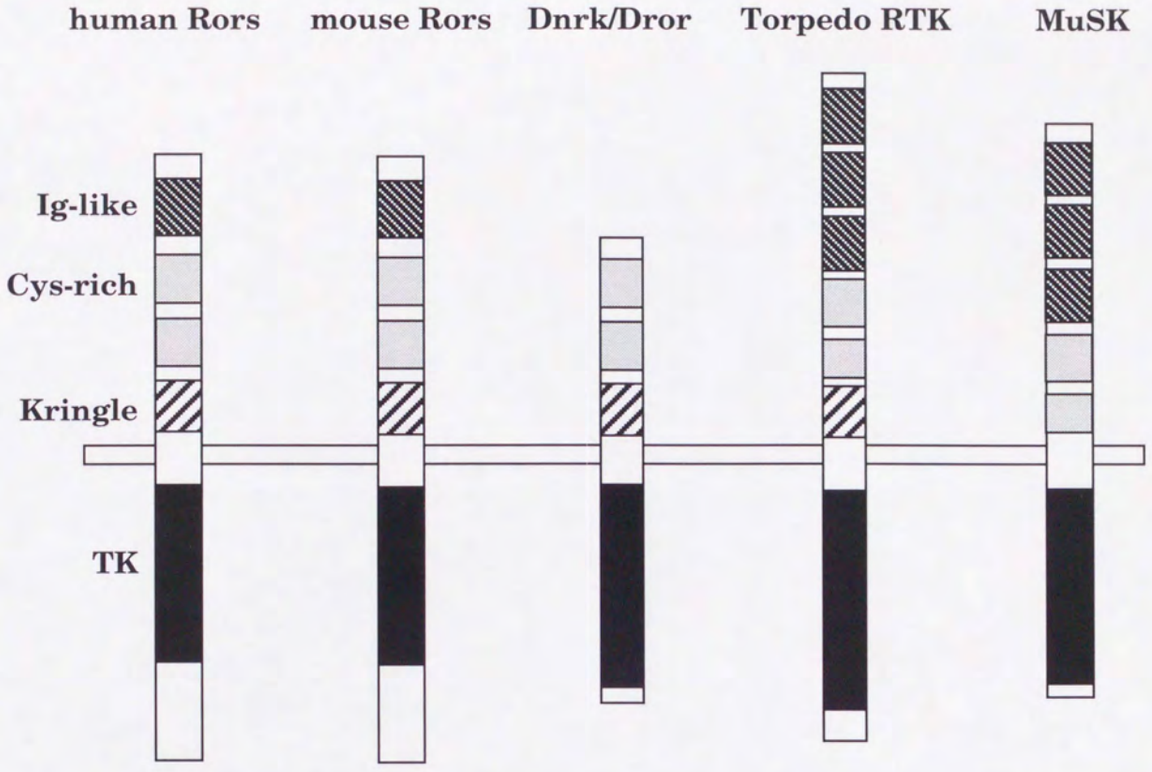


Fig.14. Structual features of Ror and MuSK family RTKs.

Dror, and mammalian Rors) with MuSK, it is possible that these Ror-family RTKs may play an important role(s) in synapse formation in developing nervous system. Further studies are required to address this important issue.

Experimental Procedures

Cells and Antibodies

COS cells were maintained continuously in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) FCS. The transient cDNA transfections into COS cells were performed as described previously (22). Mouse monoclonal antibody 12CA5 (Boehringer Mannheim) recognizes the peptide sequence (YPYDVPDYA) derived from the human influenza hemagglutinin (HA) protein (90).

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 100ml 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, annealed 2min at 50°C and incubated 2.25min at 73°C. Amplified DNA fragments with 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 (91) was screened using probes (0.2kb EcoRI-HindIII fragment from pBS-Dpkn or pBS-Dnrk) radiolabeled by random priming. Probes ($3x10^6$ cpm/ml) were hybridized to the plasmid DNA immobilized on

nitrocellulose membrane filters (Schleicher & Schuell) for 12hr at 65°C in 1x hybridization buffer (1M NaCl, 50mM Tris-HCl [pH8.0], 10mM EDTA, 0.1% [v/v] SDS), 1x Denhardt's reagent, and 100 mg/ml denatured salmon sperm DNA, and washed twice for 30 min at 65°C in 0.1x SSC, 0.1% [v/v] SDS. Clones were isolated and the longest 3.0kb clone (pNB40-Dnrk) or 1.6kb clone (pNB40-Dnrk) 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.).

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 DNAs were prepared from pBS-DKs 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 (16). Specific activity was ~$1x10^6$ cpm/ng for all the probe DNAs.

In situ Chromosomal Mapping of the Dnrk Gene

In situ hybridization on squashes of polytene chromosome was performed as

described previously (92). The digoxigenin-labeled DNA probe was prepared using the DIG DNA Labeling kit following the manufacturer's recommended protocol (Boehringer Mannheim). Developmental stages were determined as described previously (93).

In situ Hybridization of Whole Mount Embryos

In situ hybridization to Whole Mount Embryos using digoxigenin-labeled RNA probes was performed as described (94) with minor modifications (95). 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 or pBS -Dnrk as a template following the manufacturer's recommended protocol.

Expression of HA-tagged cytoplasmic kinase domains of Dnrk

Expression vectors encoding the HA-tagged cytoplasmic kinase domains of Dnrk [pEF-HA-DnrkS (aa 404-714 of Dnrk) , and pEF-HA-DnrkL (aa 337-714 of Dnrk)] were constructed. The constructs were made to add two tandemly repeated HA epitopes at the N terminus of the respective cytoplasmic kinase domains (DnrkS and DnrkL). The cDNA fragments corresponding to DnrkS and DnrkL were obtained by PCR using a combination of specific primers that create EcoRI sites at the end of the cDNA fragments. EcoRI-digested PCR products were cloned into the Bluescript at EcoRI site, and the sequence of the respective PCR products was confirmed. Subsequently, the cDNA fragments for DnrkS and DnrkL with EcoRI sites were ligated to the EcoRI-cleaved backbone fragment of the pEF expression vector (96), with an additional nucleotide sequence that encodes the two tandemly repeated HA epitopes, at the 5' end of the EcoRI-cleaved vector.

In vitro Kinase Assay

The transient cDNA transfection into COS cells was performed using the calcium phosphate method as described previously (22). COS cells were solubilized with lysis buffer (50 mM Tris-HCl[pH7.4], 0.5%[v/v] Nonidet P-40 [NP-40],150mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4, 1mM phenylmethyl sulphonyl fluoride [PMSF], 10mg/ml leupeptin, 10mg/ml aprotinin) for 30min at 4°C. The lysates were centrifuged to remove insoluble materials and resultant supernatants were precleared for 1hr at 4°C with protein A-Sepharose. The precleared supernatants were then immunoprecipitated with the anti-HA antibody and protein A-Sepharose for 3hr at 4°C. Immunoprecipitates were washed once with kinase buffer (25mM Tris-HCl [pH7.4], 0.1%[v/v] NP-40, 10mM $MgCl_2$, 3mM $MnCl_2$, 30mM Na_3VO_4) and resuspend in 30 ml of the kinase buffer. Reactions were initiated by the addition of [γ–^{32}P]ATP (10mCi of [γ-^{32}P]ATP/sample, 5000 Ci/mmol; Amersham) and incubated for 20min at 37°C. The reactions were terminated by the addition of an equal volume of 2xLeammli buffer. Subsequently, samples were separated by SDS-PAGE (13%) under reducing conditions and the gel was subjected to autoradiography. Band intensities were quantitated using a Fujix imaging analyzer (BAS2000).

Immunoblotting Analysis

For immunoblotting analysis, whole cell lysates or anti-HA immunoprecipitates from the transfected COS cells were subjected to SDS-PAGE (13%), and electrophoretically transferred onto PVDF membrane filters. After blocking with TBST-milk (10mM Tris-HCl [pH 8.0] , 150mM NaCl, 0.5% [v/v] Tween 20, 5% nonfat dry milk), membrane filters were incubated with anti-HA antibody in TBST for 1hr at room temperature. Then filters were washed with TBST and incubated with

HRP-conjugated goat anti-mouse IgG antibodies (Bio Rad) for 1hr at room temperature. After the washes, the immunoreactive proteins were visualized by using a chemiluminescence reagent (Renaissance, NEN).

Acknowledgments

I express my appreciation to Prof. T. Taniguchi, Prof. H. Yamamura, and Dr. Y.Minami for their helpful guidance and continuous encouragement. I also thank all my colleagues of Taniguchi laboratory and Yamamura laboratory for their help, valuable discussions, and encouragement.

References

1. Hatakeyama M, Mori H, Doi T, Taniguchi T:(1989) Cell, 59,837-845.

2. Kawahara A, Minami Y, Taniguchi T:(1994), Mol.Cell. Biol., 14,5433-5440.

3. Nakamura Y, Russell SM, Mess SA, Friedmann M, Erdos M, Francois C, Jacques Y, Adelstein S, Leonard WJ:(1994) Nature, 369,330-333.

4. Nelson BH, Lord JD, Greenberg PD: (1994) Nature, 369,333-336.

5. Doi T, Hatakeyama M, Minamoto S, Kono T, Mori H, Taniguchi T: (1989) Eur.J.Immunol 1989, 19,2375-2378.

6. Otani H, Siegel JP, Erdos M, Gnarra JR, Toledano MB, Sharon M, Mostowski H, Feinberg MB, Pierce JH, Leonard WJ: (1994) Proc.Natl.Acad.Sci.U.S.A., 89,2789-2793.

7. Arima N, Kamio M, Imada K, Hori T, Hattori T, Tsudo M, Okuma M, Uchiyama T: (1992) J.Exp.Med., 176,1265-1272.

8. Minami Y, Kono T, Miyazaki T, Taniguchi T: (1993) Annu.Rev.Immunol. , 11,245-267.

9. Minami Y, Kono T, Yamada K, Taniguchi T: (1992) Biochem.Biophys.Acta., 1114,163-177.

10. Turner B, Rapp U, App H, Greene M, Dobashi K, Reed J: (1991) Proc.Natl.Acad.Sci .U.S.A. , 88,1227-1231.

11. Zmuidzinas A, Mamon HJ, Roberts TM, Smith KA: (1991) Mol.Cell.Biol., 11, 2794-2703.

12. Maslinski W, Remillard B, Tsudo M, Strom TB: (1992) J.Biol.Chem., 267,15281-15284.

13. Turner BC, Tonks NK, Rapp UR, Reed J: (1993) Proc.Natl.Acad.Sci.U.S.A., 90,5544-5548.

14. Perkins GR, Marvel J, Collins MKL: (1993) J.Exp.Med. , 178,1429-1434.

15. Watts JD, Welham MJ, Kalt L, Schrader JW, Aebersold R: (1993) J.Immunol., 151,6862-6871.

16. Minami Y, Oishi I, Liu Z-J, Nakagawa S, Miyazaki T, Taniguchi T: (1994) J.Immunol. , 152,5680-5690.

17. Kuo CJ, Chung J, Fiorentino DF, Flanagan WM, Blenis J, Crabtree GR: (1992) Nature, 358,70-73.

18. Morice WG, Wiederrecht G, Brunn GJ, Siekierka JJ, Abraham RT: (1993) J.Biol.Chem., 268,22737-22745.

19. Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin SD, Perlmutter RM, Taniguchi T:(1991) Science, 252,1523-1528.

20. Minami Y, Kono T, Yamada K, Kobayashi N, Kawahara A, Perlmutter RM, Taniguchi T: (1993) EMBO J., 12,759-768.

21. Kobayashi N, Kono T, Hatakeyama M, Minami Y, Miyazaki T, Perlmutter RM, Taniguchi T: (1993) Proc Natl Acad Sci USA, 90,4201-4205.

22. Minami Y, Nakagawa Y, Kawahara A, Miyazaki T, Sada K, Yamamura H, Taniguchi T: (1995) Immunity,2,89-100.

23. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, Goldman AS, Schmalstieg FC, Ihle JN, O'Shea JJ, Leonard WJ: (1994) Science, 266,1042-1045.

24. Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu Z-J, Oishi I, Silvennoinen O, Witthuhn BA, Ihle JN, Taniguchi T: (1994) Science, 266,1045-1047.

25. Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA, Bolen JB: (1991) Proc.Natl.Acad.Sci.U.S.A ., 88,1996-2000.

26. Qin S, Inazu T, Yang C, Sada K, Taniguchi T, Yamamura H: (1994) FEBS lett. , 345,233-236.

27. Johnston JA, Kawamura M, Kirken RA, Chen Y-Q, Blake TB, Shibuya K, Ortaldo JR, McVicar DW, O'Shea JJ: (1994) Nature, 370,151-153.

28. Witthuhn BA, Silvennoinen O, Miura O, Lai KS, Cwik C, Liu ET, Ihle JN: (1994) Nature , 370,153-157.

29. Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvennoinen O: (1994) TIBS, 19,222-227.

30. Darnell JE,Kerr IM, Stark GR: (1994) Science , 264,1415-21

31. Ihle JN, Kerr IM : (1995) Trends.Genet. ,11,69-74.

32. Ziemiecki A; Harpur AG; Wilks AF: (1994) Trends cell Biol.4,207-212.

33. Schindler C,Darnell JE :(1995) Annual Review of Biochemistry,64 , 621-651.

34. Ihle JN: (1995) Nature, 377 , 591-594.

35. Taniguchi T : (1995) Science,268, 251-5.

36. Taniguchi T,Minami Y: (1993) Cell, 73,5-8.

37. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, et al: (1994) Science, 263,92-95.

38. Narazaki M, Witthuhn BA, Yoshida K, Silvennoinen O, Yasukawa K, Ihle JN, Kishimoto T, Taga T: (1994) Proc.Natl.Acad.Sci.USA. , 91,2285-2289.

39. Kawahara A, Minami Y, Miyazaki T, Ihle JN, Taniguchi T: (1995) Proc. Natl. Acad. Sci. USA 92, 8724-8728.

40. Leonard WJ, Noguchi M, Russell SM, McBride OW: (1994) Immunol.Rev.,138: 61-86

41. Binari R, Perrimon N: (1994) Genes.Dev. , 8, 300-312

42. Perrimon N, Mahowald AP Dev.Biol. 1986 Nov; 118(1): 28-41.

43. Harrison DA,Binari R, Nahreini TS, Gilman M, Perrimon N: (1995) EMBO J. 14,2857-2865

44. Luo H, HanrattyWP,Dearolf CR: (1995) EMBO J. ,14, 1412-1420.

45. Mustelin T, Burn P: (1993) Trends.Biochem.Sci.,18,215-220.

46.Karnitz LM, Abraham RT: (1995) Curr.Opin.Immunol. ,7,320-326.

47. Amaya E, Musci TJ, Kirschner MW: (1991) Cell.,66,257-270.

48. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C: (1995) Nature., 376, 768-71

49. Hanks SK, Quinn AM, and Hunter T: (1988) Science 241, 42-52.

50. Hanks SK, and Quinn AM : (1991) Methods Enzymol. 200, 38-62.

51. Mukai H, Mori K, Takanaga H, Kitagawa M, Shibata, H Shimakawa M, Miyahara M, and Ono Y: (1995) Biochim.Biophys. Acta 1261, 296-300.

52. Mukai H, and Ono Y: (1994) Biochem. Biophys. Res. Commun. 199, 897-904.

53. Cavener DR : (1987) Nucl. Acids Res.. 15, 1353-1361.

54. Cavener DR, Ray SC : (1991) Nucl. Acids Res. 19, 3185-3192.

55. von Heijne G :(1983) Eur. J. Biochem. 133, 17-21.

56. von Heijne G :(1986) Nucl. Acids Res. 14, 4683-4690.

57. Klein R, Parada LF, Coulier F, and Barbacid M : (1989) EMBO J., 8, 3701-3709

58. Martin-Zanca D, Oskam R, Mitra G, Copeland T, and Barbacid M : (1989) Mol. Cell. Biol. ,9, 24-33

59. Lamballe F, Klein R, Barbacid M : (1991) Cell, 66, 967-979.

60. Masiakowski P, and Carroll RD : (1992) J. Biol. Chem. 267, 26181-26190.

61. Wilson C, Goberdhan DCI, and Steller H: (1993) Proc. Natl. Acad.Sci. USA 90, 7109-7113

62. Jennings CGB, Dyer SM, and Burden SJ : (1993) Proc. Natl. Acad. Sci. USA 90, 2895-2899.

63. Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, Nunez L, Park JS, Stark JL, Gies DR, Thomas S, Beau MML, Fernald AA, Copeland NG, Jenkins NA, Burden SJ, Glass DJ, and Yancopoulos GD : (1995) Neuron 15, 573- 584.

64. Pearson RB, and Kemp BE : (1991) Methods Enzymol. 100, 62-81.

65. Pulido D, Campuzano S, Koda T, Modolell J, and Barbacid M : (1992) EMBO J. 11, 391-404.

66. Songyang Z, Shoelson SE, McGlade J, Oliver P, Pawson T, Bustelo XR, Barbacid M, Sabe H, Hanafusa H, Yi T, Ren R,Baltimore D, Ratnofsky S, Feldman RA, Cantley LC : (1994) Mol. Cell. Biol. 14, 2777-2785.

67. Songyang Z, and Cantley LC : (1995) TIBS 20, 470-475.

68. Patthy L, Trexler M, Vali Z, Banyai L, and Varadi A (1984) FEBS Lett. 171, 131-136.

69. Patthy L :(1985) Cell 41, 657-663.

70. McLean JW, Tomlinson JE, Kuang W-J, Eaton DL, Chen EY, Fless GM, Scanu AM, and Lawn RM : (1987) Nature,300, 132-137.

71. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, and Shimizu S : (1989) Nature,342, 440-443.

72. Sorsa V: (1988) Chromosome maps of Drosophila, Volume II. CRC Press, Boca Raton, Florida.

73. Nishizuka Y : (1992) Science, 258, 607-614.

74. Dekker LV, and Parker PJ : (1992) TIBS, 19, 73-77.

75. Nishizuka Y :(1995) FASEB J. ,9, 484-496.

76. Hardie G, and Hanks S : (1995) The Protein Kinase FactsBook vol.I (Academic Press), p80-88.

77. Ohno S, Kawasaki H, Imajoh S, Suzuki K, Inagaki M, Yokokura H, Sakoh T, and Hidaka H : (1987) Nature ,325, 161-166 .

78. Baier G, Telford D, Giampa L, Coggeshall K M, Baier-Bitterlich G, Isakov N, and Altman A : (1993) J. Biol. Chem. ,268, 4997-5004.

79. Hardie G, and Hanks S : (1995) The Protein Kinase FactsBook vol.I (Academic Press), p89-90

80. Rosenthal A, Rhee L, Yadegari R, Paro R, Ullrich A, and Goeddel DV : (1987) EMBO J., 6, 433-441.

81. Schaeffer E, Smith D, Mardon G, Quinn W, and Zuker C : (1989) Cell, 57, 403-412.

82. Watanabe G, Saito Y, Madaule P, Ishizaki T, Fujisawa K, Morii N, Mukai H, Ono Y, Kakizuka A, and Narumiya S : (1996) Science, 271, 645-648.

83. Amano M, Mukai H, Ono Y, Chihara K, Matsui T, Hamajima Y, Okawa K, Iwamatsu A, and Kaibuchi K : (1996) Science ,271, 648-650

84. Perkins L A, Larsen I, and Perrimon N : (1992) Cell ,70, 225-236

85. Lai K-MV, Olivier JP, Gish GD, Henkemeyer M, McGlade J, and Pawson T : (1995) Mol. Cell. Biol. ,15, 4810-4818

86. Sudol M :(1996) TIBS,21 ,161-163.

87. Bork P,Sudol M: (1994) Trends.Biochem.Sci. 19,531-533

88. DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT,

Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glassm DJ ,Burden, SJ andYancopoulos, GD : (1996) Cell, 85 ,501-512

89. Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR, Shah S, Mattsson K, Burden SJ, DiStefano P S, Valenzuela DM, DeChiara TM, and Yancopoulos GD (1996) : Cell ,85, 513-523

90. Wilson IA, Niman H L, Houghten RA, Cherenson AR, Connolly ML, and Lerner RA: (1984) Cell 37, 767-778

91. Brown NH, and Kafatos FC : (1988) J Mol. Biol. 203, 425-437

92. Nishida Y, Hata M, Ayaki T, Ryo H, Yamagata M, Shimizu K ,and Nishizuka Y : (1988) EMBO J. 7 ,775-781

93. Campos-Ortega JA, and Hartenstein V: (1985) The embryonic development of Drosophila melanogaster, Springer-Verlag, Heidelberg

94. Tautz D, and Pfeifle C : (1989) Chromosoma 98, 81-85

95. Kobayashi S, Saito H, and Okada M:(1994) Develop. Growth & Differ. 36, 629-632

96. Okamoto Y, Minamoto S, Shimizu K, Mogami H, and Taniguchi T : (1990) Proc. Natl. Acad. Sci. USA 87, 6584-6588