A Novel Drosophila Receptor Tyrosine Kinase Expressed Specifically in the Nervous System

UNIQUE STRUCTURAL FEATURES AND IMPLICATION IN DEVELOPMENTAL SIGNALING*

(Received for publication, July 23, 1996, and in revised form, February 6, 1997)

Isao Oishi‡, Shin Sugiyama§, Zhao-Jun Liu‡, Hirohei Yamamura‡, Yasuyoshi Nishida§, and Yasuhiro Minami¶

From the ‡Department of Biochemistry, Kobe University School of Medicine, 7-5-1, Kusunoki-chou, Chuo-Ku, Kobe 650, Japan and §Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-01, Japan

We report the identification and characterization of Dnrk (Drosophila neurospecific receptor kinase), a Drosophila gene encoding a putative receptor tyrosine kinase (RTK) 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 Src homology 2 regions of other signaling molecules. Collectively, these results suggest that Dnrk may play an important role in neuronal cell survival, differentiation, and proliferation (8–17). In addition to the Trk family RTKs, Ror1 and Ror2, members of a novel family of RTKs, called the Ror family RTKs, which are related to the Trk family RTKs, have been cloned from the human neuroblastoma cell line SH-SY5Y (18). Although Ror1 and Ror2 share structural similarity with the Trk family RTKs within their cytoplasmic domain, they possess a unique domain, a kringle domain within their extracellular domain (18). Unlike the Trk family RTKs, the ligands for the Ror family RTKs have not been identified.

In Drosophila, two RTK genes (Dtrk and Dror), with some similarity to the Trk/Ror family RTKs, have been reported (19). Dtrk is expressed in both neural and nonneural embryonic tissues and has structural similarity with neural cell adhesion molecules of the immunoglobulin superfamily (19). Furthermore, it has been shown that Dtrk mediates cell adhesion in a Ca2+-dependent manner and that the adhesion process activates its tyrosine kinase activity (19). Dror shows expression restricted to neural tissues during embryogenesis (20), and its overall structure is closely related to that of the mammalian Ror family RTKs. Indeed, it possesses cysteine-rich domains as well as a kringle domain within the extracellular region, which are characteristic features of the Ror family RTKs (18).

Here we report the identification and characterization of a novel member of the Drosophila Trk/Ror family RTKs, Dnrk (Drosophila neurospecific receptor kinase), expressed specifically in the developing nervous system during embryogenesis. The extracellular domain of Dnrk exhibits a strong similarity with that of Dror, yet Dnrk contains two putative ATP-binding motifs in the cytoplasmic tyrosine kinase (TK) domain, resembling those observed in Dtrk. The cytoplasmic domain of Dnrk contains putative tyrosine-containing motifs that may interact with SH2 regions of intracellular signaling molecules upon phosphorylation. The possible significance of the two putative ATP-binding motifs and tyrosine-containing motifs in Dnrk-mediated signal transmission is discussed.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—COS cells were maintained continuously in 10% (v/v) fetal calf serum. The transient cDNA transfections into COS cells were

family (4), DFGFR, a homologue of the mammalian FGF receptor gene family (4), sevenless, a gene required for the correct determination of the R7 photoreceptor cells (6), and torso, a gene required for the formation of the anterior and posterior terminal structures of the embryo (7).

Previous studies demonstrate that in mammals, members of the Trk family RTKs (trk, trkB, and trkC) are receptors for nerve growth factor and neurotrophic factors (brain-derived neurotrophic factor and neurotrophin-3) and that this family of RTKs plays important roles in neuronal cell survival, differentiation, proliferation (8–17). In addition to the Trk family RTKs, Ror1 and Ror2, members of a novel family of RTKs, called the Ror family RTKs, which are related to the Trk family RTKs, have been cloned from the human neuroblastoma cell line SH-SY5Y (18). Although Ror1 and Ror2 share structural similarity with the Trk family RTKs within their cytoplasmic domain, they possess a unique domain, a kringle domain within their extracellular domain (18). Unlike the Trk family RTKs, the ligands for the Ror family RTKs have not been identified.
A Novel Drosophila Receptor Tyrosine Kinase

11917

Fig. 1. Nucleotide sequence of Dark cDNA and deduced amino acid sequence of the Dark gene product. The depicted nucleotide sequence corresponding to the open reading frame was derived from the longest 3.0-kb clone (pNB40-Dark). In-frame terminator codons in the 5' untranslated region were found 60, 114, and 153 base pairs upstream of the putative translation initiation start site (data not shown). Analysis of the deduced amino acid sequence of the 714-amino acid polypeptide encoded by the Dark gene revealed various structural features (see Fig. 2) that include: a putative signal peptide (amino acids 1–25; underlined), and a putative cleavage site was indicated by an open arrowhead), four consensus N-linked glycosylation sites (boxed), the -linked glycosylation sites (underlined, followed 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 astersisks.

performed as described previously (21). Mouse monoclonal antibody 12CA5 (Boehringer Mannheim) recognizes the peptide sequence (YPY-DVPDYA) derived from the human influenza hemagglutinin (HA) protein (22).

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'–CCCGAATTCTTACAAC(A/C)G(A/T)GCG/CTT/CT/TT/3' and 5'–CCGCAGGTTGCG(A/G)(A/T)AC(A/G)(C/G)AC/GAC/ATC-T-3' (restriction sites for EcoRI and HindIII are underlined). 100 ng of genomic DNA were used as a template in 100 μl of PCR, and a putative cleavage site was indicated by an open arrowhead), four consensus N-linked glycosylation sites (boxed), the -linked glycosylation sites (underlined, followed 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 astersisks.

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'–CCCGAATTCTTACAAC(A/C)G(A/T)GCG/CTT/CT/TT/3' and 5'–CCGCAGGTTGCG(A/G)(A/T)AC(A/G)(C/G)AC/GAC/ATC-T-3' (restriction sites for EcoRI and HindIII are underlined). 100 ng of genomic DNA were used as a template in 100 μl of PCR, and a putative cleavage site was indicated by an open arrowhead), four consensus N-linked glycosylation sites (boxed), the -linked glycosylation sites (underlined, followed 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 astersisks.

Northern Blot Analysis—Total RNA from embryo, larva, pupa, and adult flies were prepared by using ISOGEN (WAKO). For RNA blot analysis on squashes of polytene chromosome was performed as described previously (25). 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 on squashes of polytene chromosome was performed as described previously (25). 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 (25).

In Situ Hybridization of Whole-mount Embryos—In situ hybridization to whole-mount embryos using digoxigenin-labeled RNA probes was performed as described previously (21). Mouse monoclonal antibody 12CA5 (Boehringer Mannheim) recognizes the peptide sequence (YPY-DVPDYA) derived from the human influenza hemagglutinin (HA) protein (22).

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'–CCCGAATTCTTACAAC(A/C)G(A/T)GCG/CTT/CT/TT/3' and 5'–CCGCAGGTTGCG(A/G)(A/T)AC(A/G)(C/G)AC/GAC/ATC-T-3' (restriction sites for EcoRI and HindIII are underlined). 100 ng of genomic DNA were used as a template in 100 μl of PCR, and a putative cleavage site was indicated by an open arrowhead), four consensus N-linked glycosylation sites (boxed), the -linked glycosylation sites (underlined, followed 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 astersisks.

Downloaded from http://www.jbc.org/ on July 24, 2018
FIG. 2. A, alignment of the Dnrl 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, gaps inserted for optimal alignment. B, alignment of Dnrl extracellular domains with
A Novel Drosophila Receptor Tyrosine Kinase

Expression of HA-tagged Cytoplasmic Kinase Domains of Dnrk—Expression vectors encoding the HA-tagged cytoplasmic kinase domains of Dnrk (pEF-HA-DnrkS (amino acids 404–714 of Dnrk) and pEF-HA-DnrkL) 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 (29), 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 (21). COS cells were solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 30 min at 4 °C. The lysates were centrifuged to remove insoluble materials, and resultant supernatants were precleared for 1 h at 4 °C with protein A-Sepharose. The precleared supernatants were then immunoprecipitated with the anti-HA antibody and protein A-Sepharose for 3 h at 4 °C. Immunoprecipitates were washed once with kinase buffer (25 mM Tris-HCl, pH 7.4, 0.1% (v/v) Nonidet P-40, 10 mM MgCl2, 3 mM MnCl2, and 30 μM Na3VO4) and resuspended in 30 μl of the kinase buffer.

RESULTS

Cloning of Dnrk cDNAs—Using a PCR strategy and degenerate oligonucleotide primers to well-conserved stretches of amino acids in TKs, we amplified Drosophila genomic DNA. After subcloning of amplified products, several were found to encode TKs, and among them, one encoded a TK region with greatest sequence similarity to the Trk/Ror family RTKs. Using this amplified region as a probe, we 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 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. 1). In addition, sequences upstream of the putative translation start site (the first ATG) matched the Drosophila consensus for translational initiation (31, 32). The initiating methionine is followed by a hydrophobic domain of 25 residues that may serve as a signal peptide (33, 34). By analogy with other signal peptides, this sequence would be cleaved between residues 25 and 26 at the Ala-Asn junction (Fig. 1; Refs. 33 and 34). Accordingly, the mature Dnrk protein would contain 689 amino acids and resembles a typical RTK. Within this protein, the N-terminal 275-amino acid residues constitute the extracellular domain, the membrane spanning domain (26 amino acids), and the C-terminal 388-amino acid residues, the cytoplasmic domain.

Structural Features of Dnrk—The putative TK domain of Dnrk is most similar to those of the vertebrate Trk and Ror family RTKs (8–10, 18) as well as to the previously cloned family RTKs (8–10, 18) as well as to the previously reported Ror family RTKs (Ror 1 and Ror 2 (human) and Dror (Drosophila)). The shared cysteine-containing domain (cys domain), kringle domain, and N-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, gaps inserted for optimal alignment.
Drosophila RTK, Dror (Fig. 2A). It reveals about 40–45% identity to the corresponding domains of TrkB, Ror1, Ror2, and Dror (Fig. 2A). The TK domain of Dnrk also shares somewhat lower levels of similarity with those of the Trk-related Drosophila RTK, Dtrk, and the mammalian muscle-specific kinases (MuSKs) (35, 36).

Like the previously reported Trk and Ror family RTKs, Dnrk contains the YXXDYY sequence motif (amino acids 590–595; Figs. 1 and 2A), corresponding to the autophosphorylation site of insulin receptor(s) (18, 37). Interestingly, Dnrk possesses the two putative ATP-binding motifs (G354/G359/K380 and G437/G442/K459) within its TK domains, which is a characteristic feature found in the Dtrk protein (19). Furthermore, there are several putative tyrosine-containing motifs that may interact with SH2 regions of cellular signaling molecules upon tyrosine phosphorylation (see “Discussion”).

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. 2B). 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. 1). As shown in Fig. 2B, the Dnrk extracellular domain contains two cysteine-rich domains and a membrane-proximal kringle domain, which are characteristic features of the Ror family RTKs (Fig. 2B). The kringle domain is a highly folded structure, rich in cysteines, and is found in certain blood coagulation proteins, apolipoprotein, and hepatocyte growth factor (38–41). Although the functional role of the kringle domain in these RTKs remains unclear, it is believed to be involved in mediating protein-protein interactions (20, 38). 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).

Chromosomal Mapping and Expression of Dnrk—To determine the cytological location of the 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 chromosome (Fig. 3A; Ref. 42).

To characterize the temporal expression pattern of the Dnrk gene, we performed Northern blot analysis with RNA samples from embryos (0–4 h, 4–10 h, and 10–22 h), larva, pupa, and adult flies. Radiolabeled cDNA, covering the entire open reading frame, was used as a probe. The Dnrk probe detected a major band about 3.5 kb in size (Fig. 3B). Dnrk is expressed at high levels in 4–22-h embryos, larva, and pupa, with maximal expression in pupa where the restructuring of the nervous system occurs (Fig. 3B). It is noteworthy that during embryogenesis the level of Dnrk expression accumulated a maximum during 4–10 h and gradually declined thereafter (Fig. 3B). A decreased level of expression was observed in adult flies.

We next performed in situ hybridization experiments to whole-mount embryos to determine the tissue specificity of Dnrk transcripts during embryogenesis (see “Experimental Procedures”). The 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. 4A). 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. 4B). The distribution of transcripts after germ band shortening (stage 13, Fig. 4C) matched the profile of developing commissures and connectives. Expression of Dnrk was also observed in cells in positions corresponding to those of the peripheral nervous system at stage 17, when larval sensory cells have differentiated (Fig. 4D). No mutations having defects in neural development are known to be located at the 49f chromosomal region to which Dnrk mapped.

Protein Kinase Activity of Dnrk—To test the catalytic activity of the putative TK domain of Dnrk, we constructed expression vectors encoding the HA-tagged cytoplasmic kinase domains of Dnrk (see “Experimental Procedures”). Because the TK domain of Dnrk possesses two putative ATP-binding motifs (distal to the following kinase subdomains and proximal to the following kinase subdomains), the two different HA-tagged kinase domains of Dnrk, HA-DnrkS (amino acids 404–714 of Dnrk) and HA-DnrkL (amino acids 337–714 of Dnrk) were expressed transiently in COS7 cells (see “Experimental Procedures”). The HA-DnrkL protein possesses the two ATP-binding motifs, whereas the HA-DnrkS protein lacks the region (amino acids 337–403) containing the distal ATP-binding motif (Fig. 5A).

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. 5B, anti-HA antibody clearly detected HA-DnrkS and HA-DnrkL with expected molecular masses (38 and 45 kDa, 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 with 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 \([\gamma-\text{P}]\text{ATP}\). Specific phosphorylation of DnrkL with an expected molecular mass was observed (Fig. 5C). DnrkS lacking the distal ATP-binding motif was also specifically phosphorylated \textit{in vitro}, yet to a lesser extent (about 1/3 compared with DnrkL) (Fig. 5C). Because 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 with DnrkS. Phosphoamino acid analysis revealed that phosphorylations of DnrkL and DnrkS occurred on tyrosine as well as serine/threonine residues (Fig. 5D). The extents of tyrosine \textit{versus} serine/threonine phosphorylation on DnrkL and DnrkS were comparable (data not shown). It is likely that phosphorylation of DnrkL and DnrkS on serine residues was due to a contaminating serine/threonine kinase; however, we cannot entirely rule out the possibility that phosphorylation of DnrkL and DnrkS on tyrosine residues is mediated by a contaminating tyrosine kinase.

**Fig. 5.** 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 (amino acids 404–714 of Dnrk) (pEF-HA-DnrkS), and HA-DnrkL (amino acids 337–714 of Dnrk) (pEF-HA-DnrkL) are shown. The whole-cell lysates (equivalent cell numbers) were analyzed by anti-HA immunoblotting as described under “Experimental Procedures.” Expression of HA-DnrkS detected by immunoblotting was constantly higher (1.5–2-fold) than that of HA-Dnrk (see text). C, \textit{in vitro} kinase activities of HA-DnrkS and HA-DnrkL. Cell lysates (equivalent cell numbers, see B) 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”). \textit{In vitro} kinase assay was performed as described under “Experimental Procedures.” About 3-fold higher autophosphorylation of HA-DnrkL was observed when compared with HA-DnrkS, although amounts of HA-DnrkL immunoprecipitated with anti-HA antibody was lower (about two-thirds) than that of HA-DnrkS (data not shown, see B). D, phosphoamino acid analysis of HA-DnrkS and HA-DnrkL phosphorylated \textit{in vitro}. Phosphoamino acid analysis was performed as described under “Experimental Procedures.” PS, PT, and PY, phosphoserine, phosphothreonine, and phosphotyrosine, respectively.
kinase. In this respect, it should be noted that bacterially expressed fusion proteins, DnrkS and DnrkL, respectively, fused to glutathione S-transferase, as well as HA-tagged DnrkS and DnrkL, were phosphorylated on tyrosine residues in vivo as assessed by anti-phosphotyrosine immunoblot (data not shown). Because DnrkS exhibited autophosphorylation activity, yet to a lesser extent compared with 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.

**DISCUSSION**

Using a PCR-based approach, we have cloned a cDNA of a novel Drosophila RTK, Dnrk, that is expressed exclusively in the nervous system during embryogenesis. Dnrk possesses typical structural features that have been reported for the Trk and Ror family RTKs, in particular for Ror family RTKs. The cytoplasmic TK domain of Dnrk exhibits a high degree of homology with those of the Trk and Ror family RTKs (8–10, 18, 20) (Fig. 2A). Interestingly, like Dtrk, the Trk-related Drosophila RTK (18), the TK domain of Dnrk contains two tandemly repeated putative ATP-binding sites at its N-terminal portion (Fig. 1). Within the extracellular domain of Dnrk, there are two cysteine-rich domains and a membrane-proximal kringle domain, the unique domain(s) shared with the Ror family RTKs (mammalian Rors and Drosophila Dror) and muscle-specific RTKs (mammalian MuSKs and Torpedo RTK) (18, 20, 35, 36) (Fig. 2B and 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. Because these RTKs, including Dnrk, share particular similarity within their extracellular domains, it is likely that their cognate ligands (soluble or cell surface molecules) are also structurally related.

Similar to Dror, distinct expression of Dnrk was not detected before the extended germ-band stage (Fig. 4). In addition, like Dror, Dnrk is exclusively expressed in the nervous system. The determination and differentiation of neuroepithelial cells in both the central nervous system and peripheral nervous system 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 central nervous system and peripheral nervous system.

In addition to the structural features of Dnrk, our in vitro kinase analysis of Dnrk revealed that Dnrk indeed possesses tyrosine kinase activity (Fig. 5, C and D). As described, Dnrk contains two putative ATP-binding motifs within its TK domain. Because antibody against the Dnrk protein is not presently available, we constructed and expressed the HA-tagged cytoplasmic TK domains of Dnrk in COS cells. The HA-Dnrk-Lacking the distal ATP-binding motif still exhibits autophosphorylation activity in vitro, albeit to a lesser extent when compared with the HA-DnrkL that possesses both ATP-binding motifs (Fig. 5C). 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.

Previous studies demonstrate that activation of RTKs result in autophosphorylation of RTKs, thereby creating binding sites for SH2 region-containing cytosolic proteins, including phosphoinositide 3'-kinase, phospholipase Cγ, growth factor receptor binding protein 2, and Shc (43, 44). On the basis of the prediction reported previously, we found several tyrosine-containing motifs within the catalytic TK domain of Dnrk, which are candidate sites for interaction with the SH2 region-containing cytoplasmic signaling molecules. Upon tyrosine phosphorylation, for example, Y507MAP510, Y642FGL645, and Y671ALM674 are assumed to be able to interact with SH2 regions of mammalian proteins (SH2 of Vav and p120-ras-GTPase-activating protein (p120-rasGAP) for PO4-Y507MAP510, SH2 of PTP1C for PO4-Y642FGL645, and SH2 of Shc, Csk, and p85 subunit of phosphoinositide 3'-kinase for PO4-Y671ALM674) (43–46). Thus far, dShc, and Gap1, the Drosophila homologues of PTP1C, Shc, and p120-rasGAP, have been reported (47–49). Such putative tyrosine-containing motifs were not found in the corresponding domain of Dror. These preliminary observations raise an interesting possibility to be tested that Dnrk may interact with cytoplasmic signaling molecules by using such motifs.

Like Dror, expression of Dnrk is restricted to the nervous system during embryogenesis (Fig. 4). Furthermore, the peak expression of Dnrk as well as Dror occurs when early processes of neuronal differentiation, including axonogenesis, occur. Interestingly, it has been reported that mammalian Rors are also expressed relatively early during development and that their expression declines drastically at a later embryonic stage (18). Thus, these Ror family RTKs (Dnrk, Dror, and Rors) may play an important role in early neuronal development. 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 (36). Moreover, it has been shown that a targeted disruption of the MuSK gene results in a failure in the formation of neuromuscular synapses (50, 51). 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, Dror, and mammalian Rors) with MuSK, it is possible that these Ror family RTKs may play an important role(s) in synapse formation in the developing central nervous system. Further studies are required to address this important issue.

**Acknowledgments**—We thank Drs. T. Kono and E. Barsoomian for critical reading of the manuscript. We also thank T. Enomoto (Hitachi Electronics Engineering Co., Ltd.) for technical assistance.

**REFERENCES**

1. Ulrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
2. Petruzelli, L., Herrera, R., Arenas-Garcia, R., Fernandez, R., Birnbau, M. J., and Rosen, O. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4710–4714
3. Nishida, Y., Hata, M., Nishizaka, Y., Rutter, W. J., and Ebina, Y. (1986) Biochem. Biophys. Res. Commun. 141, 474–481
4. Livneh, E., Glazer, L., Segal, D., Schlessinger, J., and Shilo, B.-Z. (1985) Cell 40, 599–607
5. Spranger, F., Stevens, L. M., and Nüsslein-Volhard, C. (1989) Nature 338, 478–479
6. Hafen, E., Basler, K., Edstrom, J.-E., and Rubin, G. M. (1987) Science 236, 55–63
7. Glazer, L., and Shilo, B. Z. (1991) Genes Dev. 5, 697–705
8. Klein, R., Parada, L. F., Coulier, F., and Baracid, M. (1989) EMBO J. 8, 7011–7019
9. Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., and Baracid, M. (1989) Mol. Cell. Biol. 9, 24–33
10. Lamballe, F., Klein, R., and Baracid, M. (1991) Cell 66, 967–979
11. Hempstead, B. L., Martin-Zanca, D., Kaplan, D. R., Parada, L. F., and Chao, M. V. (1991) Nature 350, 678–683
12. Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991) Nature 350, 158–160
13. Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991) Science 252, 554–558
14. Klein, R., Jing, S., Nanduri, O., O'Rourke, E., and Baracid, M. (1991) Cell 65, 189–197
