Kiz-1, a Protein with LIM Zinc Finger and Kinase Domains, Is Expressed Mainly in Neurons

Ora Bernard, Soula Ganiatsas, George Kannourakis, and Ralf Dringen

The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050 [O. B., S. G., R. D.], and Leukaemia Auxiliary Royal Children’s Hospital Cancer Research Unit, The Royal Childrens Hospital, Parkville, Victoria 3050 [G. K.], Australia

Abstract

The olfactory epithelium is the only neuronal tissue capable of generating new neurons during adult life and hence must express genes responsible for this phenomenon. Therefore, we have used mRNA from immortalized olfactory epithelial cells to search for novel protein tyrosine kinases by polymerase chain reaction, using as primers conserved sequences from the catalytic domain of known kinase genes. A full-length complementary DNA clone corresponding to one such polymerase chain reaction product was isolated and sequenced. This complementary DNA, designated Kiz-1, encodes a protein containing two prominent domains; the NH₂-terminal region contains a cysteine/histidine-rich moiety previously identified as a zinc-finger domain in proteins of the LIM family, while the COOH-terminal contains a kinase domain. Kiz-1 is expressed mainly in the brain of adult mice but also in a range of cultured cell lines, regardless of their tissue of origin. Immunohistochemical studies on adult mouse brain demonstrated that Kiz-1 is expressed exclusively in neurons, not in astrocytes or oligodendrocytes. In the developing embryo, however, Kiz-1 is expressed in all tissues. In COS cells transfected with Kiz-1 complementary DNA and in the immortalized olfactory epithelial cells, Kiz-1 was found mainly in the cytoplasm, but in neurons of the adult brain, it resided also in the nucleus. Two Kiz-1 mRNA species are expressed in cell lines as well as in the murine and human brain. One transcript lacks a region of 60 nucleotides, which lies within the catalytic domain of the kinase and is encoded by a separate exon. Our results suggest that Kiz-1 may play distinct roles in dividing cells and in differentiated neurons.

Introduction

In the central and peripheral nervous systems of the mouse, cellular differentiation into the different types of neurons and glial cells is completed within the first month after birth. Although neural precursors can be found in the central nervous system during adult life, these cells do not differentiate in vivo and cannot give rise to new neurons or glial cells. The only neuronal tissue that has the ability to generate neurons during adult life is the olfactory epithelium (1). The olfactory epithelium is a simple neuronal tissue containing only one type of neuron, olfactory receptor neurons. This tissue, which lines the nasal cavity, is responsible for the sense of smell in vertebrates. The olfactory receptor neurons are derived from basal epithelial cells that have self-renewal capacity as well as the ability to differentiate into olfactory receptor neurons (2). Because the olfactory epithelium can constantly give rise to new neurons, it provides an ideal tissue in which to study the genes that are responsible for this process.

PTK4 growth factor receptors are comprised of at least six different classes, but all have a transmembrane region and an intracellular tyrosine kinase domain (3). Many tyrosine kinase receptors and their ligands are believed to play important roles during embryonal development, and some are also important in lineage determination in the hematopoietic system. The c-kit receptor mutants known as W (4, 5), the PDGFα receptor mutant patch (6), and the colony-stimulating factor-1 mutant op (7) are all developmental mutants, indicating the importance of these receptors in embryonal development. The c-kit and colony-stimulating factor-1 receptors are also important for the development of hematopoietic stem cells and macrophages. Because of the importance of tyrosine kinase receptors in embryonal development and lineage commitment, we attempted to isolate novel tyrosine kinases from olfactory epithelial cells. Using a recombinant retrovirus harboring the N-myc proto-oncogene, we have previously generated immortalized olfactory epithelial cell lines (8). Some of these cell lines represent immortalized olfactory neuronal precursors which express the neuronal marker neurofilament protein (8). These immortalized cells represent cycling olfactory epithelial cells at the penultimate stage of differentiation, and they may correspond to the immature neurons described by Calof and Chikarashii (2). We have used one such olfactory epithelial cell line to isolate cDNA clones corresponding to newly identified tyrosine kinases. Previously, we have isolated new members of the PTK family from mouse neuroepithelial cells by applying the PCR, in combination with degenerate oligonucleotide primers based upon two highly conserved elements in the catalytic domains of PTKs (9). In this paper, we describe a cDNA with a very unusual structure which encodes a protein containing three domains; the NH₂-terminus has a cysteine/histidine-rich region, previously described as a zinc finger domain belonging to the LIM family (10), separated by a

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3 To whom requests for reprints should be addressed.

4 The abbreviations used are: PTK, protein tyrosine kinase; cDNA, complementary DNA; PCR, polymerase chain reaction; bp, base pairs; kb, kilobase(s); PMA, phorbol myristate acetate; kD, kilodaltons; NP-40, Nonidet P-40; RIPα, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; poly(A)⁺, polyadenyllic acid, polyadenylated; PBS, phosphate-buffered saline; FCS, fetal calf serum.
Neuron-specific Protein with LIM and Kinase Domains
region rich in proline and serine residues, and a COOH-
terminal located kinase domain. We have designated this
protein with kinase and zinc finger domains Kiz-1 in order
to reflect the unusual combination of these two domains in
a single protein. We report the isolation and full sequence
of this cDNA clone, show its subcellular localization, its
pattern of expression, and describe some characteristics of
this unusual protein. Recently, a human cDNA was isolated
from a human hepatoma HepG2 cell cDNA library and
named LIMP. This cDNA is the human homologue of
mouse Kiz-1 (11).

Results
Isolation of Full-length cDNA Clone Encoding KIZ-1.

mRNA isolated from the olfactory epithelial cell line
olf.4.4.1 was used for the synthesis of cDNA (8), which was
used in a PCR reaction to amplify PTK-related sequences
with degenerate oligonucleotides to conserved regions of
the kinase domain (12). The amplified DNA fragment of
approximately 200–240 bp was cloned into M13 mp19,
and approximately 150 independent clones were screened
by a single base (G) sequencing reaction. These clones were
divided into 12 groups, according to their sequence pattern,
and a single clone from each group was fully sequenced.
Three newly identified clones, which contained the puta-
itive amino acid sequence WMAPE, were selected for fur-
ther analysis. This amino acid sequence previously was
demonstrated mainly in PTK receptors (13) and were, there-
fore, chosen to be fully characterized. A cDNA library from
olf.4.4.1 was constructed in the λZapII vector, but the
screening of this library yielded only partial clones. Since
Northern blot analysis indicated that Kiz-1 was expressed in
the brain (Fig. 4A), a mouse brain cDNA library was used
for the isolation of full-length cDNA clones. Subsequently,
a human brain cDNA library was probed with the mouse
Kiz-1 cDNA to isolate the human Kiz-1 cDNA.

Sequence Analysis of Mouse Kiz-1 cDNA. The nucleo-
tide and predicted amino acid sequence of the murine
Kiz-1 is presented in Fig. 1A. The longest cDNA clone
harbored an insert of 3.2 kb. This clone contained a single,
long open reading frame that may start with either the
methionine codon at nucleotide 204 or that at position 246
(amino acid 1; Fig. 1A). We consider the second methio-
nine codon as the putative translation initiation site because
it conforms most closely to the Kozak consensus sequence
(14). Translation of the open reading frame of the full size
cDNA predicts 633 amino acids with a molecular mass of
about 70 kd. Although the sequence of the PCR fragment
amplified with PTK1 and PTK2 contained predicted amino
acids specific to tyrosine kinase receptors, the NH2-termi-
nal region contained neither a hydrophobic leader se-
quence nor long stretches of hydrophobic residues, suggest-
ing that the protein is not secreted and does not possess a
transmembrane domain.

Analysis of the predicted amino acid sequence of the
Kiz-1 protein revealed that it has three major domains (Fig.
1B). The NH2-terminal domain of 139 amino acids is rich
in cysteine and histidine residues (boxed in Fig. 1A). A
search of available data bases revealed homology of this
domain with proteins belonging to the LIM family repre-
sented by lin-11 (10) and mec-3 (15), both isolated from the
nematode Caenorhabditis elegans, Isl-1, the murine insulin
enhancer binding protein (16), and Xlim-1 from Xenopus
(Ref. 17; Fig. 2A). Kiz-1, like the other members of the LIM
family, contains a tandem repeat of the consensus sequence
CX9CX11–19HX1CX12–19CX12–19CX12–19CX12–19CX12–19
LIM domain; (LIM domain; Fig. 2A). The homology is not restricted to these conserved
amino acids because an overall identity of about 32% exists
between these five proteins (Fig. 2A). The LIM domains of
Kiz-1 are more closely related to Isl-1 (38%) and lin-11
(35%) than to mec-3 (25%) and Xlim-1 (28%). The LIM
domains were found to bind zinc ions tetrahedrally coor-
dinated with a stoichiometry of two zinc ions per domain
(18–20). Therefore, we predict that the structure of the LIM
domains of Kiz-1 is as shown in Fig. 2B, where each LIM
domain contains two zinc binding fingers. Each finger is
generated by binding one zinc ion to two pairs of ligand
amino acids (C, H, or D; Ref. 21).

The middle region of Kiz-1, which extends from amino
acid residue 140 to 309, is rich in serine (15%) and proline
(10%) residues. Regions rich in proline, glutamate, serine,
and threonine are known as PEST regions and can result in
rapid intracellular degradation of the proteins which con-
tain them (22).

The carboxy-terminal region of Kiz-1 extends from resi-
due 310 to 633 and contains most of the highly conserved
motifs typical of PTK domains. This kinase domain contains
the typical ATP-binding motif GXGXXG (amino acids 332–
337) together with the invariant lysine at position 354 (13).
The IHRDL (amino acids 443–447) of subdomain VI is
typical of the PTK family, and subdomain VIII, represented
by WMAPE (amino acids 501–505), is mainly found in
growth factor receptor tyrosine kinases. An important fea-
ture of all tyrosine kinases is the presence of a tyrosine
residue between subdomains VII and VIII. Its presence at
this position is considered diagnostic of PTK family mem-
bership (3). Kiz-1 contains a tyrosine residue (amino acid
493) at this position.

The kinase domain of Kiz-1 is of great interest because it
contains an insert of eight amino acids, KKPDRKKR (resi-
dues 485–492), in the middle of the catalytic domain. This
sequence of basic residues is homologous to nuclear local-
ization signals present in all proteins transported to the
nucleus (23). We have previously observed that the region
between subdomains VI and IX amplified with the oligonu-
cleotides PTK1 and PTK2 is about 200–220 nucleotides in
all PCR products analyzed (9). Because of the extra eight
amino acids, the PCR product of Kiz-1 amplified with PTK1
and PTK2 contains 240 nucleotides.

While Kiz-1 bears greater homology to tyrosine kinases
than to serine/threonine kinases, it contains several amino acid
residues characteristic of the latter. For example, while amino
acids GMAY (433-436) are present only in tyrosine kinases, the absence of AAR or RAA at position 448-450 is a feature of serine/threonine kinases. It is interesting to note that the COOH-terminal 100 amino acids of Kiz-1 contains three tyrosine residues (53B, 556, 618) and five serine residues which might serve as putative phosphorylation sites. Overall, the kinase domain of Kiz-1 has homology with several PTKs; the human c-raf-1 and Abelson murine leukemia virus have 62% nucleotide identity in the kinase domain, but the amino acid identity was only 30%. The highest homology was to Rous sarcoma virus and human c-src, where nucleotide identity was 65% and amino acid identity 35%.

Identification of Two Distinct Kiz-1 Transcripts. The human and murine brain cDNA libraries consistently yielded clones of a single Kiz-1 species. The sequence of the human Kiz-1 cDNA is shown in Fig. 1A. However, the cDNA library constructed from the olfactory epithelial cell line of 8.1 yielded an additional cDNA clone which differed from the sequence depicted in Fig. 1A. It lacked a region of 60 nucleotides encoding a stretch of 20 amino acids (shaded in Fig. 1A) present within the murine and human cDNAs isolated from the brain (Kiz-1). This region lies within the catalytic site of the kinase domain between regions V and VI and is conserved among many PTKs, including those belonging to the Src family. The short form of Kiz-1 (Kiz-1-S), was generated by splicing of an exon bearing these 60 nucleotides (Fig. 3A). Interestingly, we were able to demonstrate by PCR that the Kiz-1-S mRNA was expressed not only in neural cell lines and Rat-2 cells but also in mouse brain (Fig. 3B). No correlation could be found between the expression of Kiz-1-S and the cell cycle phase of the cells. Both cycling cells and nondividing neurons expressed similar levels of the two transcripts (off 8.1, off 4.4.1, and brain; Fig. 3B).

Expression of Kiz-1 in the Adult Mouse Is Restricted to the Central Nervous System. Northern blot analysis of poly(A)+ RNA isolated from different mouse tissues and cell lines indicated that Kiz-1 transcripts of 3.5 kb was expressed in the brain of adult mice but not in other tissues such as heart, muscle, liver (Fig. 4A), spleen, thymus, kidney, lung, testis, ovaries, lymph nodes, or bone marrow (data not shown). The level of expression in the brain was comparable with that in the different neural cell lines and the three olfactory epithelial cell lines examined (Fig. 4A). When mRNA was prepared from different regions of the brain, it was observed that the level of Kiz-1 expression in the forebrain and the hindbrain was higher than that in the midbrain. The highest level of expression was observed in the olfactory cell lines off 4.4.1 and off 4.4.2 (8), while in the neuroepithelial cell lines NZen17 and NZen37 (24), the level was somewhat lower (Fig. 4B). It was interesting to find that all of the cell lines examined expressed Kiz-1, regardless of whether they were derived from neural, fibroblastic, early embryonic, or hemopoietic origin (data not shown). Because the Northern blot analysis did not reveal expression in tissues other than brain, we used the more sensitive RNase-protection technique to detect the presence of Kiz-1 mRNA in different murine tissues and in the developing brain (Fig. 4B). The expected RNase-protected RNA species of 274 bases was detected only in the adult and developing brain (Fig. 4B) but not in any other tissue (data not shown). Interestingly, the level of Kiz-1 mRNA was twice as high in E13-E15 brain as in E16 brain.

Subcellular Localization of Kiz-1 Protein. To determine the subcellular localization of Kiz-1 protein, we generated an epitope-tagged derivative of Kiz-1 (Kiz-1-HA; see Fig. 1B) which contained a 9-amino acid insert derived from the influenza virus hemagglutinin; this epitope is detectable by
binding to the monoclonal antibody 12CA5. The cDNA encoding Kiz-1 was cloned into the mammalian vector PSTC011 containing the influenza hemagglutinin epitope and transfected into COS cells. The cellular localization of Kiz-1 was then determined by immunocytochemistry. High levels of immunoreactivity were found in the cytoplasm of transfected COS cells, and very little staining was detected in the nuclei (Fig. 5).

We have also attempted to raise rabbit polyclonal antibodies corresponding to sequences at the NH2-terminus (amino acids 9–24) and the COOH-terminus (amino acids 618–633) of Kiz-1. Only rabbits injected with the COOH-terminal peptide gave rise to anti-Kiz-1 antibodies. When these antibodies were used to stain COS cells transfected with the expression vector bearing Kiz-1 cDNA, the immunoreactivity was confined mainly to the cytoplasm. This immunoreactivity was inhibited by the presence of the peptide used to immunize the rabbits. The staining pattern did not change when transfected COS cells were incubated with the differentiating agents PMA or ionomycin for 1, 2, or 6 h prior to the immunohistochemistry (data not shown).

Staining of off 4.4.1, one of the cell lines that expressed the highest levels of Kiz-1 mRNA (Fig. 4A), with anti-Kiz-1 antibodies indicated that Kiz-1 was expressed only in the cytoplasm of these cells (Fig. 6). As in the case of transfected COS cells, Kiz-1 did not translocate to the nucleus when the cells were incubated with either PMA or ionomycin (data not shown).

The Kiz-1 Protein. COS cells transfected with Kiz-1-HA and Kiz-1 cDNA in the pEF-Bos vector (EF-Bos-Kiz-1) were labeled with [35S]methionine for 4–5 h prior to immunoprecipitation of cellular proteins with anti-HA and anti-Kiz-1 antibodies. The immunoprecipitates of cells transfected with the Kiz-HA construct revealed a single band of about 70 kD when precipitated with anti-Kiz antiserum, anti-Kiz antibody affinity purified on a CNBr-Kiz- peptide column, or anti-HA antibody (Fig. 7A, Lanes 2, 4, and 6, respectively). This band was absent from immunoprecipitates with preimmune serum (Fig. 7A, Lane 1), anti-Kiz-1 antibodies in the presence of the COOH-terminal peptide (Fig. 7A, Lanes 3 and 5), or anti-HA in the presence of the HA peptide (Fig. 7A, Lane 7).

When COS cells transfected with EF-Bos-Kiz-1 DNA were metabolically labeled with [35S]methionine and lysed and solubilized with 0.1% digitonin, a protein of about 40 kD coprecipitated with Kiz-1 (Fig. 7B). This protein was not observed when the cells were lysed with 2% NP-40 or when the immunoprecipitates were washed with RIPA buffer containing 0.2% SDS and 0.5% deoxycholate (Fig. 7A), suggesting that the 40 kD protein was a noncovalently associated protein and not a Kiz-1 degradation product.

Western blot analysis of COS cells transfected with either Kiz-HA or EF-Bos-Kiz-1 constructs probed with anti-Kiz-1 antibodies also revealed a single band of 73 or 70 kD, respectively (data not shown). Kiz-1 protein was also detectable mainly in the cytoplasmic fraction of off 4.4.1, Rat-2, and 2.3D cells (data not shown). We were unable to demonstrate Kiz-1 in adult brain extracts (data not shown), probably due to the low levels of Kiz-1 protein.

In the Adult Mouse, Kiz-1 Expression Is Restricted to Neurons. Northern blot analysis indicated that, in the adult mouse, the brain is the only organ in which Kiz-1 is present at a detectable level (Fig. 4A). In order to determine which cells within the brain expressed Kiz-1, we immunostained midsagittal and coronal frozen sections of adult brain. Expression of Kiz-1 was observed only in neurons and the epithelial cells of the choroid plexus. Examination of stained sections at low magnification revealed that the cells in the white matter, which contain mainly oligodendrocytes and astrocytes, did not contain Kiz-1 protein. By contrast,
most of the neurons in the cerebral cortex, but not the astrocytes or the oligodendrocytes, stained with anti-Kiz-1 antibodies (Fig. 8F). In the deep gray matter, the neurons of the basal ganglia stained for Kiz-1, and in the cerebellum, Purkinje cells and their axons were also clearly stained; however, it was unclear whether other neurons in the cerebellum stained (Fig. 8A). In the hippocampus, only the neurons of the pyramidal layer stained, whereas the neurons of the dentate gyrus did not express Kiz-1 (Fig. 8C-E).

In order to determine the subcellular localization of Kiz-1 in neurons, alternate sections were not counterstained with hematoxylin after staining with anti-Kiz antibodies (data not shown). These studies indicated that Kiz-1 staining in the neurons was found in both the cytoplasm and the nucleus.
In particular, strong nuclear staining was observed in hippocampal neurons (Fig. 8E). In contrast, the cells of the choroid plexus were stained only in the cytoplasm (data not shown).

The Human Kiz-1 cDNA. We isolated human Kiz-1 cDNA clones from a human brain (medulla) cDNA library using a full-length mouse Kiz-1 cDNA clone as a probe. The screening of this library yielded a large number of clones (~100), 20 of which were analyzed further. Comparison of the nucleotide sequence of the human and mouse cDNA indicated 87% identity. The differences in the nucleotide sequence were restricted mainly to the third base and different amino acid codon usage. Amino acid identity between the mouse and the human was 95%. Most of the differences were in the proline/serine-rich region (amino acids 140–309) where amino acid identity was 91%. In the cysteine/histidine-rich region, the amino acid identity was 95%, and in the kinase domain, 96%. Interestingly, within the kinase domain, the nonidentical amino acids were replaced mainly by homologous amino acids; thus, the structure of the protein was conserved. For example, arginine 473 in the mouse was replaced by lysine in the human Kiz-1, and glutamic acid 625 was replaced by aspartic acid in the human protein. The 20 amino acids present only in Kiz-1-L are identical in the mouse and the human, and the nucleotide sequence of this region differed only in five nucleotides (data not shown). The identity in the amino acid sequence between the mouse and the human in this region suggests that these amino acids play an important role in the function of Kiz-1.

In situ hybridization of sections of human brain with Kiz-1 anti-sense oligonucleotides revealed that the expression pattern of Kiz-1 mRNA was similar to that demonstrated by immunohistochemistry in mouse brain (Fig. 9C). The antisense oligonucleotides hybridized mainly to neurons and not to glial cells, as indicated by the large number of grains on cells in regions rich in neurons such as the cerebral cortex, hippocampus, brain stem, and cerebellum, and by the absence of signal in the white matter (Fig. 9, C and D, and data not shown). In rare cases where grains were detected over oligodendrocytes, they appeared to form close contact with neurons (data not shown). The in situ hybridization experiments on human brain sections confirmed that expression of Kiz-1 in the adult brain, as in the mouse, is mainly restricted to neurons.

Discussion

In this study, we have identified and characterized a new gene encoding a protein with three distinct domains, namely zinc finger and kinase domains separated by a 170-amino acid domain rich in proline and serine. This gene was recently isolated also by Mizuno et al. (11). Kiz-1 is highly conserved between the human and the mouse, and its expression is restricted mainly to the brain in adults. In contrast, in the mouse embryo, Kiz-1 is expressed in all tissues (data not shown). It is also expressed in cell lines derived from various mouse tissues including embryonic stem cells. We have analyzed mouse brain by immunohistochemistry and determined that the protein is present mainly in neurons and not in oligodendrocytes or astrocytes. However, not all neurons expressed Kiz-1. In the hippocampus, for example, the neurons of the pyramidal layer, but not those of the dentate gyrus, expressed Kiz-1. Similar results were obtained by in situ hybridization to sections of mouse and human brain. The observation that Kiz-1 was not expressed in all neurons suggests that it may be important for the function of specific types of neuron.

The observation that, during embryonal development, Kiz-1 is expressed in all tissues is in agreement with our finding that all cell lines examined expressed Kiz-1 mRNA and that Kiz-1 is expressed in dividing cells from all cell lineages. However, the subcellular localization of Kiz-1 appeared to be different in neurons and in rapidly dividing cells. In COS cells transfected with Kiz-1 cDNA, in an olfactory epithelial cell line, and in the developing embryo, the protein was confined to the cytoplasm. By contrast, it was localized in both cytoplasm and nuclei of neurons in

Fig. 5. Cellular localization of Kiz-1 protein in transfected COS cells. COS cells transfected with Kiz-1-HA cDNA were stained with anti-HA antibodies 48 h after transfection. × 500.
the adult brain. The amount present in the nuclei varied between the different types of neurons. In the hippocampus, Kiz-1 was mainly in the nucleus, whereas in cortical neurons, it was found equally in the nucleus and the cytoplasm. These findings suggest that Kiz-1 may play a different role in rapidly dividing cells from that in neurons.

Kiz-1 represents a new member of the LIM-domain-containing family of proteins. Before the discovery of Kiz-1, the LIM family could be divided into two groups; the first group contains a homeodomain at the carboxy terminus, and the second group has only the LIM domain. To the first group belong the three proteins from which the acronym LIM was derived, i.e., lin-11, Isl-1, and mec-3 (10, 15, 16) as well as the Drosophila protein ap (25, 26), the Xenopus protein Xlim-1 (17), and its mouse homologue Lim-1 (27), together with Lmx-1 (28) and LH-2 (29). The members of the second group are the rhombotins (30), the cystein-rich protein CRP (21, 31), the cysteine-rich intestinal protein CRIP (32), and zyxin (33).

Kiz-1 does not contain a homeodomain but instead has a putative kinase domain at the carboxy terminus of the protein. Therefore, Kiz-1 founds a new subgroup of the LIM protein family.

Although the function of the LIM domain is not yet understood, LIM domains were shown to contain two zinc atoms (18–20). The presence of four zinc atoms per molecule in proteins containing two LIM domains indicates that the LIM motif forms four zinc fingers; the spacing of paired cysteine and histidine residues separated by two amino acids suggests a possible function in DNA binding because
this motif has been found in a variety of DNA-binding proteins (34–36). Alternatively, it has been suggested that the LIM motif plays a role in protein-protein interactions via the metal-binding domain (33, 37). There are precedents for the combination of protein-binding and kinase domains in a single polypeptide chain. For example, the Src protein tyrosine kinase contains the protein association domains SH2 and SH3. The formation of a complex with the SH2 domain is important for the activation of the kinase domain (38). It is possible that heterodimer formation may activate the kinase domain of Kiz-1 and be responsible for its phosphorylation; indeed, we have demonstrated that Kiz-1 coprecipitated with a 40 kD protein.

The structure of the kinase domain of Kiz-1 is most intriguing. Although it contains most of the features of tyrosine kinases, it also has some amino acids indicative of serine/threonine protein kinases. For example, subdomain VIII has some of the features of serine/threonine kinases rather than tyrosine kinase. Since we have not as yet been able to demonstrate autophosphorylation of Kiz-1, we have not yet elucidated the amino acid specificity of the kinase domain of this unusual protein.

Two distinct Kiz-1 mRNA species are expressed in adult mouse and human brain and in cell lines. These two species reflect alternative splicing; the region of 60 nucleotides absent from Kiz-1-S cDNA lies on a separate exon which
can be spliced out, resulting in a shorter mRNA. This exon lies in the middle of the kinase domain, and its encoded amino acid sequence is identical in the human and the mouse. In addition, it bears high homology to the corresponding region of several tyrosine kinases belonging to the Src family, indicating its importance. Although we as yet do

Fig. 8. Immunohistochemistry of mouse brain with anti Kiz-1 antibodies. Frozen sagittal sections of adult mouse brain were stained with anti-Kiz-1 antibodies and detected with horseradish peroxidase, except control section (B) where the antibody was omitted. A, staining of mouse cerebellum with anti-Kiz antibodies indicated high level of Kiz-1 expression in the cytoplasm and nuclei of Purkinje cells. × 200. g, granule layer; p, Purkinje cell layer; m, molecular layer of the cerebellum. C, pyramidal cells from the CA1 region of the hippocampus (ca) and not the dentate gyrus cells (dg) express Kiz-1 (× 100). D, higher power view (× 400) of cells from CA1 region expressing Kiz-1 in the cytoplasm and the nuclei. E, pyramidal cells of the CA3 region of the hippocampus express Kiz-1 mainly in the nucleus (× 300). F, neurons (n) of the cerebral cortex and not glial cell (g) express high levels of Kiz-1 in both cytoplasm and nuclei (× 200).

Fig. 9. In situ hybridization of sections of human cerebral cortex to Kiz-1 oligonucleotides. A and B, negative controls indicate no hybridization to sense Kiz-1 oligonucleotides; C and D, hybridization to antisense Kiz-1 oligonucleotides resulted in large numbers of grain on cortical neurons. C, dark field; D, bright field. × 200.
not know the function of the short Kiz-1-S protein, it is tempting to speculate that it acts as an inhibitor of the kinase activity of Kiz-1-L by forming heterodimers with it. One precedent is that Bcl-x (the short form of Bcl-x) can inhibit the function of the long form of this protein (39). Similarly, short splice variants of the transcription factor mTFE3 can inhibit the function of full size protein (40). We believe that this is the first time that a splice variant missing a part of the catalytic domain of a protein kinase has been isolated and characterized. It is possible that the role of the truncated protein is to regulate the level of Kiz-1 in the cell, since high levels of Kiz-1 expression in stably transfected COS cells and Rat-2 cells are toxic to the cells (data not shown).

Another interesting feature of the kinase domain is the insertion of eight amino acids containing four lysine and two arginine residues. This stretch of basic residues is similar to the consensus sequence of nuclear localization signals (23). It seems likely that this motif accounts for our observation that, in the brain, Kiz-1 resides in the nuclei of neuronal cells.

The function of Kiz-1 is not yet known. However, from the available data, we can predict that it has at least two different functions, as a protein binding protein which co-precipitates with 40 kD cytoplasmic protein as observed in transfected COS cells, and probably as a DNA-binding protein in mature neurons. It is possible that one function of Kiz-1 is similar to that of c-Raf-1. c-Raf-1, like Kiz-1, has a cysteine-rich domain (known as a cysteine finger) and a serine/threonine kinase domain. Recently, it was demonstrated that c-Raf-1 can bind directly to the Ras protein via the cysteine finger and that it is an indispensable downstream effector of the mitogenic response to Ras (41, 42). Similarly, Kiz-1 may be an effector of a Ras-like protein in neurons.

Materials and Methods

Analysis of RNA Expression. Poly(A)· was prepared as described (43). Samples of 2 µg were fractionated on 1% agarose/formaldehyde gel and transferred onto nitrocellulose Hybond C Extra (Amersham) as described by Thomas (44). Probes were labeled by random priming and hybridized under standard conditions.

RNase Protection Analysis. RNase protection analysis was performed as described (45), except that 0.4 µg of RNase T1 per ml (Sigma Chemical Co.) and 8 µg of RNase A per ml (Sigma) were used. All samples analyzed contained 1 µg of poly(A)· RNA or 20–40 µg total RNA and 50 µg of yeast tRNA. Hybridization, following denaturation (80°C for 5 min), was carried out overnight at 45°C in buffer containing 50% formamide, 200 mM 1,4-piperazinediethanesulfonic acid (pH 6.7), 2 mM NaCl, and 5 mM EDTA, followed by RNase digestion. Protected products were resolved by electrophoresis in 5% acrylamide/7 M urea gels.

DNA Amplification. DNA amplification was essentially as described (9). cDNA was prepared from poly(A)· RNA isolated from the olfactory epithelial cell line off 4.4.1 (5 µg). PCR was performed on 1/10th of the cDNA sample by using Taq DNA polymerase (Cetus) and PTK1 and PTK2 oligonucleotides (12). Conditions for amplification were as follows: 35 cycles of 1 min at 95°C, 1 min at 45°C, and 2 min at 72°C. The amplified DNA of 220–240 bp was purified on low melting agarose before digestion with BamHI and EcoRI and ligation into M13 mp19. Sequencing was performed by the dideoxy chain termination method using a Sequenase kit (USB). For analysis of the two forms of Kiz-1, cDNA was prepared from 1 µg of poly(A)· RNA and amplified using oligonucleotides corresponding to nucleotides 1350–1370 and 1733–1753 (Fig. 1A). The amplified DNA fragments were resolved in 2% agarose gel.

cDNA Cloning. A cDNA library was prepared from 5 µg of poly(A)· RNA isolated from the olfactory epithelial cell line off 8.1. This library was constructed using the oligo dT-primed cDNA kit (Amersham) with λ-ZapI vector (Stratagen). Initially, 6 × 105 clones were screened with the 240-bp PCR generated fragment designated off 8.1 #114 clone. To obtain a full-length cDNA clone, a mouse brain cDNA library in the Uni-Zap XR vector (Stratagen) was screened with a 5’ terminal end 300-bp EcoRI fragment of that clone. To isolate human Kiz-1 cDNA, a human brain (medulla) cDNA library (Clontech) was probed with a full-length mouse cDNA clone.

Construction of Expression Vectors. Mouse Kiz-1 cDNA was ligated into the XbaI site of PE-BOSS vector (46) after removal of the stuffer. Kiz-1-HA was constructed by ligation of Kiz-1 cDNA to the Clal site of PSTC011 vector containing the hemagglutinin epitope YPYDVPDYA.

COS Cell Transfection. COS cells were transfected by a DEAE-dextran method. Forty % confluent overnight cultures were transfected with 5 µg plasmid per 150-mm dish in 7.5 ml of 400 µg/ml DEAE-dextran (Sigma)-0.1 mM chlo-roquine (Sigma) in RPMI 1640 supplemented with 10% Nu-serum (Collaborative Research). After 2–3 h, when the cells started to appear abnormal, the transfection medium was replaced by 10 ml of 10% dimethyl sulfoxide in PBS for 2 min. The dimethyl sulphoxide was removed, and the cells were washed twice with 10 ml RPMI10% FCS and incubated in the same medium for 48–72 h. The concentration of ionomycin and PMA were 1 µg/ml and 2–5 ng/ml, respectively.

Immunoprecipitation. Transfected COS cells were preincubated in methionine-free Dulbecco's medium supplemented with 10% dialysed FCS for 2 h prior to the addition of 50 µCi/ml [35S]methionine (NEN) and incubated for an additional 4 h. The cells were then washed with PBS, collected and lysed in 0.2 ml buffer [2% NP-40, 20 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM MgCl2, and 5 mM EDTA containing a cocktail of protease inhibitors. The cytoplasmic fractions were collected and incubated with rabbit anti-Kiz-1 or with anti-HA monoclonal antibody 12CA5 (BAbco) for 1 h. The immune complex was precipitated with protein A-sepharose beads (Pharmacia) for 1 h. The beads were washed five times with RIPA buffer [50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 0.2% SDS, 0.5% deoxycholate, and 0.5% NP-40] before resuspension in 50 µl sample buffer [100 mM Tris-HCl (pH 6.8), 10% glycerol, 0.1% Triton X-100, and 50 mM dithiothreitol]. [35S]-Labeled immunoprecipitated proteins were visualized after electrophoresis on 10% SDS-polyacrylamide gels by autoradiography. For coprecipitations, the cells were lysed in buffer containing 0.1% digitonin instead of 2% NP-40, and the immunoprecipitates were washed in PBS instead of RIPA buffer.

Western Blot Analysis. Lysates were prepared from approximately 1 × 107 cells. Cells were scraped from dishes with a rubber policeman, washed twice with PBS, and lysed in 0.5 ml of lysis buffer. The nuclear and cytoplasmic fractions were separated by centrifugation at 2500 rpm in a mini-centrifuge for 5 min. The nuclei were resuspended in 1.5 ml of lysis buffer and sonicated for 30 s. The cytoplas-
mic and nuclear fractions were fractionated on 10% SDS-polyacrylamide gel and electrophoresed onto nitrocellulose Hybond C-extra. The filters were probed with rabbit anti-Kiz-1 antibodies or anti-HA antibody. After washing, the filters were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse immunoglobulins and visualized by enhanced chemiluminescence (Western blotting detection kit; Amersham).

**Preparation of Antisera.** Two peptides corresponding to the mouse sequence of amino acids 9–24 and 618–633 (5 mg of each) were conjugated to 10 mg keyhole limpet hemocyanin via chloroacetyl group. Rabbirts were first injected s.c. with 250 μg of peptide mixed with Freund’s complete adjuvant (GIIBCO), followed by injections with 250 μg of peptide mixed with Freund’s incomplete adjuvant (GIIBCO). The rabbits were given four injections at 1-month intervals. The anti-Kiz-1 antibodies were affinity purified on CNBr-activated sepharose (Pharmacia) coupled to bovine serum albumin-conjugated peptide. Ten ml of immunized rabbit serum were passed through the column. Elution was according to the manufacturer’s instructions (Pharmacia).

**Immunocytochemistry.** Kiz-1 tagged with the hemagglutinin epitope was detected with either mAb 12CAS or with rabbit anti-Kiz-1 antibodies. Transfected COS cells grown on 3-cm plastic Petri dishes were fixed with methanol for 15 min at −20°C, washed three times with PBS/1% FCS, and incubated for 1 h with the primary antibodies. The cells were then washed three times with PBS/1% FCS and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin (Silenus, Melbourne, Australia) at a dilution of 1:1000. Cultures were washed three times, and the staining was visualized using a fluorescence microscope.

**Immunohistochemistry.** Frozen mouse embryos and adult tissues perfused with 10% sucrose were sectioned on a cryostat. Cryosections (6 μm) were mounted on gelatin-coated slides, fixed at 4°C for 1 min with acetone, air dried, and stored at −70°C. Prior to immunostaining, the sections were fixed again at 4°C with acetone for 5 min, washed with PBS for 5 min, and blocked with 2% nonfat dry milk (Diploma) in PBS for 20 min. The sections were then incubated with anti-Kiz-1 antibodies diluted 1:50 or 1:100 for 2 h. After washing in PBS, the slides were incubated for 1 h with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin antibodies (1:50; Dako P217) and detected using diaminobenzidine. The slides were then stained with hematoxylin for 10–20 s, rinsed, dehydrated, and mounted in di-n-Butylphthalate. Standard negative controls, including preimmune serum and omission of primary antisera, were routinely performed.

**In Situ Hybridization.** In situ hybridization was performed as described previously (47) using oligonucleotide probes labeled with [35S]ATP. Control sense, nonsense, and positive antisense oligonucleotides were used. The sequence of the antisense oligonucleotides corresponded to the sequence of mouse Kiz-1 mRNA in position 722–742.

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**References**


