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CLONING OF A NOVEL PUTATIVE PROTEIN KINASE HAVING A LEUCINE ZIPPER DOMAIN FROM HUMAN BRAIN[1]

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We report a novel putative serine/threonine protein kinase containing a leucine-zipper domain, isolated from an human neuronal cell line. The teratocarcinoma cell line NT2 was differentiated to postmitotic NT2-N neurons by treatment with retinoic acid, and degenerate oligonucleotide primers to the catalytic domains of protein kinases were employed to PCR amplify subtractive cDNAs. We identified a clone, represented at higher abundance in NT2-N neurons than in the parental cell line, which encodes a putative serine/threonine kinase of 668 aminoacids, the leucine-zipper protein kinase (zpk). Zpk protein contains a leucine-zipper domain, found in many DNA-binding proteins, but few protein kinases. Steady-state mRNA levels for zpk are high in human brain and kidney. Further studies are required to evaluate the role of zpk in neuronal differentiation.

Protein kinases fall into two general classes: those that transfer phosphate to serine or threonine and those that transfer phosphate to tyrosine (1). A few protein kinases, such as wee1+, are capable of phosphorylating both serine/threonine and tyrosine residues (2). Protein phosphorylation regulates many aspects of cellular metabolism, but is of particular significance in controlling mitogenesis and cellular differentiation (3). Receptors for a number of polypeptide growth factors are transmembrane tyrosine kinases (4). Phosphorylation of intracellular tyrosine residues in these receptors in turn stimulates serine/theronine kinases such as protein kinase C, MAP kinase and $p74^{raf}$, thus initiating a cascade of events that transduce extracellular signals to the nucleus (5-7).

The human teratocarcinoma cell line, NT2, has phenotypic properties of primitive neuroectoderm (8), and can be differentiated into post-mitotic NT2-N

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Abbreviations: PCR, polymerase chain reaction; zpk, leucine-zipper protein kinase; NTera2/D1 (NT2), teratocarcinoma cells.

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neurons by treatment with retinoic acid (9,10). We decided to use NT2 cells and NT2-N neurons to identify kinases differentially expressed during human neuronal development. Our approach was to employ the polymerase chain reaction (PCR) with degenerate protein kinase specific oligonucleotide primers (11) to amplify consensus protein kinase catalytic domains (12), and to isolate mRNA's expressed differentially by NT2 cells or NT2-N neurons. Here we report the molecular cloning and DNA sequence analysis of a leucine-zipper putative protein kinase (zpk) cDNA. Furthermore, we describe steady-state zpk mRNA levels in various tissues and compare this unique cDNA with other protein kinases.

MATERIALS AND METHODS

Cells

Human teratocarcinoma cell line NT2 was differentiated into postmitotic NT2-N neurons with retinoic acid as previously described (9,10). Poly(A)$^+$ RNA was isolated from both NT2 cells and NT2-N neurons using the Invitrogen mRNA kit (San Diego, CA).

Subtractive hybridization and DNA amplification

Invitrogen's Subtractor probe kit was used according to manufacturer's instructions to isolate two different subtracted cDNA populations, UND and DIFF. UND is enriched in transcripts expressed in the undifferentiated NT2 cells, whereas DIFF is enriched in transcripts present in NT2-N neurons. One microgram portions of UND and DIFF cDNAs were used for PCR amplification with degenerate primers (PTK1 5' CGGATCCACAGNGACCT 3' and PTK11 5'GGAATTCCAAAGGACCA-GACGTC 3') having restriction enzyme sites Bam H1 for PTK1 and Eco R1 for PTK11. PCR was performed using a Geneamp kit (Perkin Elmer, Norwalk, CT) with 1 µg of degenerate primers and the final concentration of magnesium in the reaction was 2.1 mM. PCR cycling was performed on a Perkin-Elmer 480 thermal cycler for 39 cycles with a profile of 1.3 min at 95°C (denaturation), 2 min at 45°C (annealing), and 2 min at 64°C (elongation).

Subcloning of amplified DNAs and DNA sequence analysis

PCR products were electrophoresed on a 4% (w/v) Nusieve agarose gel. The amplified band in the size range of ~220 bp was excised and purified using a Magic PCR Kit (Promega, Madison, WI). Amplified DNA was digested with the restriction enzymes Bam H1 and Eco R1, and the digest was subcloned into Bam H1/Eco R1 digested Bluescript plasmid (Stratagene). Two hundred clones (100 UND and 100 DIFF) were examined by DNA sequence analysis using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc, Foster City, CA).

Plasmid DNA was isolated using a Qiagen column 20 (Chatsworth, CA). Cycle sequencing reactions were performed in a Perkin-Elmer 480 thermal cycler for 25 cycles with a profile of 96°C for 30 sec, 40°C for 15 sec and 60°C for 4 min. Following separation of the extension products on a Select-D G-50 column (5-Prime/3-Prime, Boulder, CO), the reaction mixtures were dried, resuspended in 4 µl of 5:1 (v/v) formamide/50mM EDTA, loaded on a 6% (w/v) sequencing gel,

electrophoresed and analysed using an Applied Biosystems 373 laser-activated fluorescence-emission sequencer.

cDNA library screening

The 210 bp 10.2 PCR clone was radiolabeled with a-[^{32}P]dCTP by random priming using a kit from Promega (Madison, Wisconsin), and used to probe ~10^6 plaques from an amplified human fetal brain library (Stratagene, La Jolla, CA) to obtain larger cDNA clones. Hybridization was done overnight at 42°C in 50% (v/v) formamide, 5xSSPE, 5xDenhardt's, 1% (w/v) SDS, 100 μg/ml sheared salmon sperm DNA and 1x10^6 cpm/ml of probe. Filters were washed at 60°C twice in 2xSSC containing 0.1% (w/v) SDS, and exposed overnight to Kodak XAR-5 film at -70°C.

Sequence determination

The cDNAs were subcloned into the plasmid vector BluescriptSk. For complete sequence determination, unidirectional nested deletions were made using the Exo111/Mung Bean nuclease kit from Stratagene, and the resultant clones were subjected to DNA sequence analysis as described earlier using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Sequence comparisons

All sequence comparisons were done on a VAX using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package. Complete sequence was determined after assembly of nested deletions into contigs using the Program Sequencher 2.0 (Gene Codes Corp; Ann Arbor, Michigan). Each contig was the result of the assembly of at least 8 individual clones. The DNA sequence was verified by resequencing. Protein homologies were established using MacVector (IBI).

RNA analysis

Multiple human tissue Northern blots (Clontech Laboratories, Palo Alto, CA). were hybridized to radiolabeled zpk cDNA probe following conditions previously described for library screening. Filters were washed to a final stringency of 0.1xSSC/0.1% (w/v) SDS at 65°C before exposure to XAR-5 X-ray film.

RESULTS AND DISCUSSION

We used subtractive hybridization to identify cDNAs differentially expressed in human NT2 neuroectodermal cells or in NT2-N neurons, followed by PCR amplification of kinase consensus sequences (11,12). PCR products were cloned, and 200 clones were isolated and characterized by DNA sequence analysis. From these, we selected one novel clone represented more frequently in the NT2-N neurons, and used it to obtain full length cDNA clones from a fetal human brain cDNA library. Three independent clones were isolated, and DNA sequence determined for one of these clones. We used 5'-RACE and primer extension methods to obtain the complete 3387 nucleotide cDNA sequence as shown in figure 1, and validated this

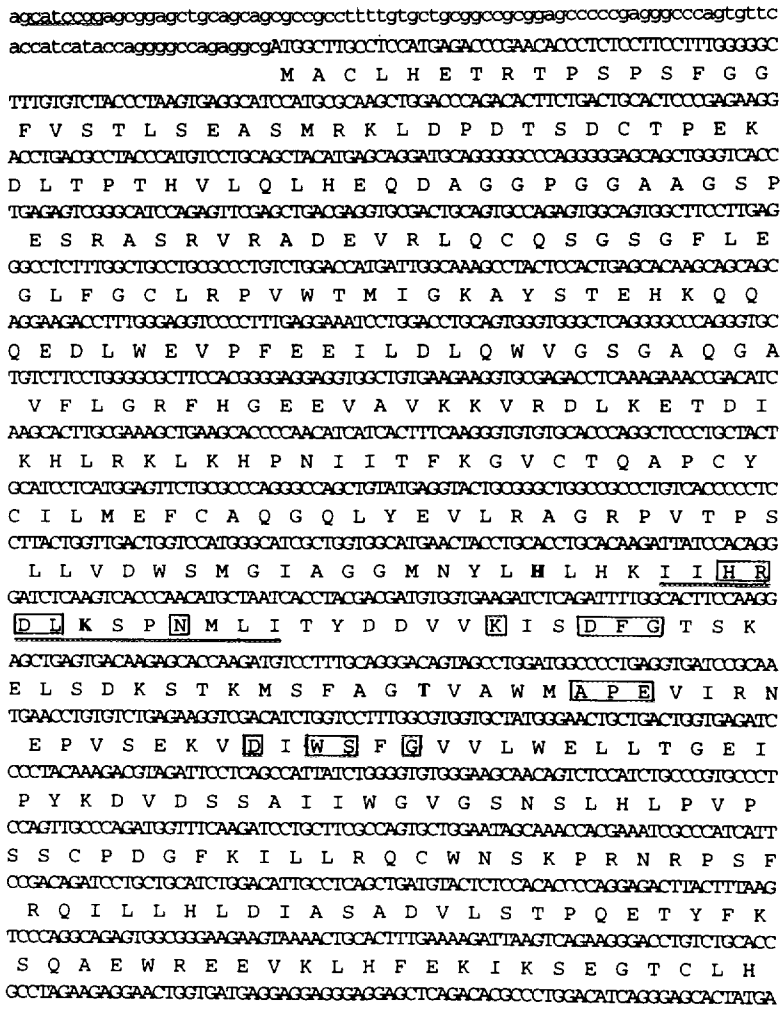

Fig. 1. Nucleotide sequence of zpk cDNA (numbered on left) and the deduced amino acid sequence (single letter code and numbered on right) are shown. Putative locations for the 5' cap site and the polyadenylation signal sequence are underlined. Protein kinase conserved residues are boxed, while those conserved in protein-serine/threonine kinases are indicated in bold, the protein- kinase site is underlined, while the leucines in the leucine-zipper are circled, and the ATP-binding site is indicated by arrows. A second ATP binding site may also be present at residues 132-139.

sequence by partial characterization of the other two fetal human brain cDNA clones we had obtained.

The cDNA has a long open reading frame encoding 668 aminoacids, with a Kozak consensus sequence for translation initiation at position 99-101 (13) and a polyadenylation signal sequence (AATAAC) at nucleotides 3347-3352 (14). A 5'-AmpliFINDER RACE kit (Clontech) was used to obtain the 5' end of the cDNA.

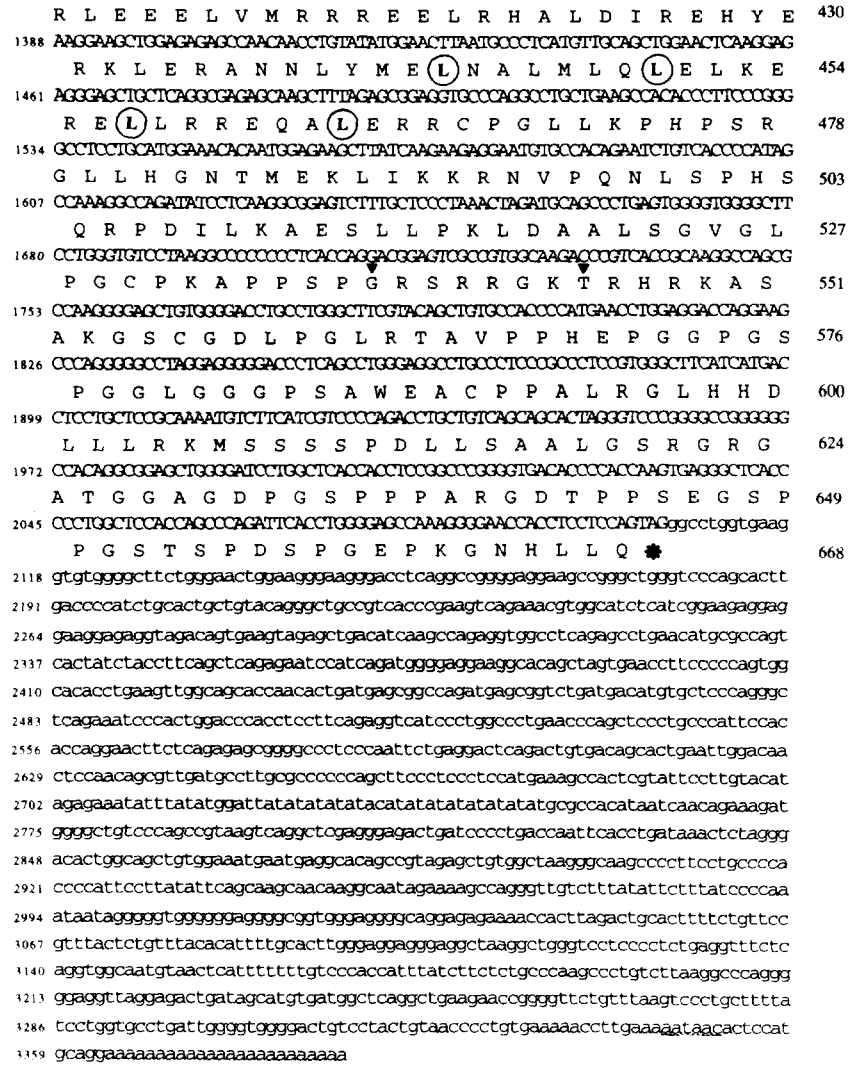

Fig. 1. - Continued

Results suggested the 5' cap site to be CATCCG, 90 bp from the initiation start site (data not shown).

Northern blot analysis of various human tissues using radiolabeled zpk probe showed a single transcript of 3.4 kb in brain (Fig. 2; upper panel), with comparable steady-state levels in adult (panel to the right) and fetal brain (panel to the left). A smaller transcript, ~3.0 Kb, was detected in kidney and skeletal muscle, while both transcripts were seen in adult lung at very low levels. In fetal tissues, we detected the zpk transcript in brain but not in other tissues. After initial probing with zpk cDNA, the blots were hybridized with beta-actin to show uniformity of RNA loading (lower panel). Zpk mRNA steady-state levels are higher in

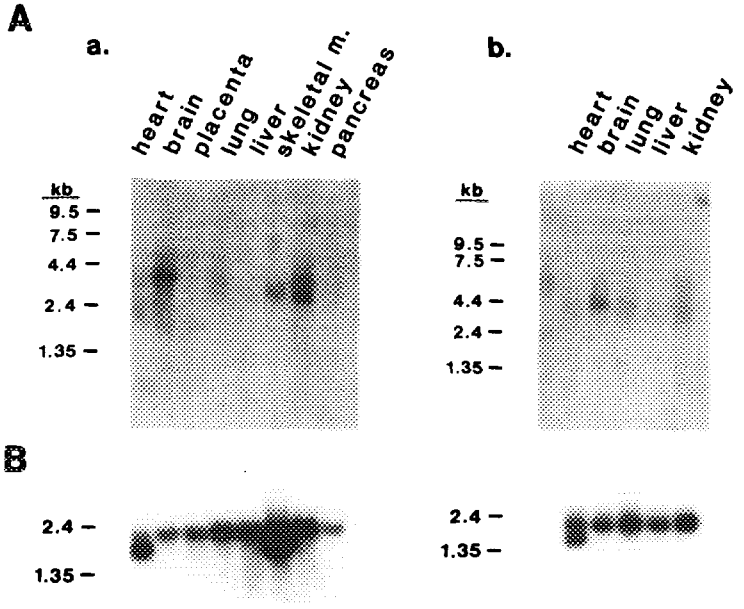

Fig. 2. Steady-state zpk mRNA levels in various human tissues. Northern blots were purchased from Clontech laboratories. 2 μg of poly$^+$A RNA from different human tissues was loaded in each lane. Panel A represents Northern blots hybridized to α-[^{32}P] labeled zpk cDNA (a) from human adult tissues, (b) from human fetal tissues. These blots were hybridized to α-[^{32}P] labelled β-actin cDNA as represented in the lower panel B.

neuronally differentiated NT2-N cultures than in undifferentiated NT2 neuroectodermal cells (data not shown).

Protein and DNA database searches showed no homology to any known protein kinase. Partial amino acid sequence alignment of zpk with the catalytic domains of several serine/threonine specific protein kinases shows conserved regions with the Raf sub-family in subdomains V1b and V111, and with the Kin2 subfamily in subdomain 1X (Fig. 3). A consensus sequence for two potential ATP-binding sites, Gly-Xaa-Gly-Xaa-Xaa-Gly are located in zpk (residues 132-139 and 538-545), while a putative protein kinase domain is located at position 232-244 (3). In addition, there are two overlapping sites of leucine zipper motif (LXXXXXXLXXXXXXLXXXXXXL) between amino acids 443-471(15), and a putative endoplasmic reticulum-targeting sequence between amino acids 416-419 (16).

Endoplasmic reticulum targeting sequences have been identified in both soluble (16) and transmembrane (17) ER proteins. A lysine-rich motif at the cytoplasmically-exposed C-terminus of some transmembrane proteins confers ER localization, although a more complex retention signal at the C-terminus has been

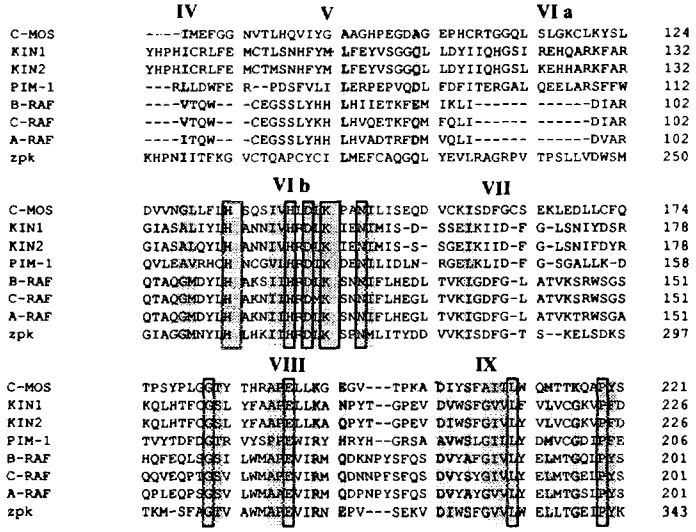

Fig. 3. Alignment of the amino acids in the catalytic domains IV-IX encoded by zpk with other members of the protein serine/threonine kinase family. The catalytic subdomains are indicated above the alignment in Roman numerals (3). Amino acids identical in all these kinases are boxed and amino acids identical in at least 3 kinases are represented without a box.

also postulated (18). The ER-targeting motif is not present at the extreme C-terminus in zpk, thus the significance of this motif is not known.

Leucine zippers contribute to targeting of various proteins(e.g., glucose transporters (19)), and facilitate dimerization of cytoplasmic hormone receptors and enzymes (20). Leucine zippers are also a common feature of transcription factors, in which they effect homo- or heterodimerization which can result in altered DNA binding. A leucine-isoleucine zipper motif was reported only once previously at the N-terminus of bovine cGMP-dependent protein kinase (15). CD and NMR studies indicated that the active form of this kinase is dimeric (21,22). Protein expression studies will be necessary to establish zpk has kinase activity and whether it depends on dimerization.

Hydrophobicity plots of the predicted zpk protein showed no transmembrane domain (data not shown). Therefore, zpk may belong to the family of non-receptor kinases.

Preliminary DNA sequence analysis of the clones obtained using oligonucleotide primers for consensus kinase domains amplified from NT2 cells and NT2-N neurons suggests that we have detected more than 20 novel kinase-like transcripts in addition to zpk. Thus, the NT2 cell line and the cloning strategy we

used, should provide an useful method for identification and characterization of new members of this important family of proteins.

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REFERENCES

1. Krebs, E. G., & Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923-959.
2. Lindberg, R.A., Quinn, A.M., Hunter, T. (1992) Trends Biochem. Sci. 17, 114-119.
3. Taylor, S.S., Knighton, D.R., Zheng, J., Ten Eyck, L.F., & Sowadski, J.M. (1992) Annu. Rev. Cell Biol. 8, 429-462.
4. Yarden,Y. & Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443-478.
5. Hunter, T., Ling, N. & Cooper, J.A. (1984) Nature 311, 480-483.
6. Morrison, D. K., Kaplan, D.R., Escobedo, J.A., Rapp, U.R., Roberts, T.M., & Williams, L.T. (1989) Cell 58, 649-657.
7. Rossomondo, A.J., Payne, D.M., Weber, M.J. & Sturgill, T. W. (1989) Proc. Natl. Acad. Sci. USA 86, 6940-6943.
8. Lang, E., Mazauric-Stucker M-L., Maelicke A. (1989) J. Cell Biol. 109, 2481-2493.
9. Pleasure, S.J., Page, C. & Lee,V. M.-Y. (1992) J. Neurosci. 12, 802-1815.
10. Younkin, D.P., Tang, C.-M., Hardy, M., Reddy, U.R., Shi, Q.-Y., Pleasure, S.J., Lee,V. M.-Y., & Pleasure, D. (1992) Proc. Natl. Acad. Sci. USA 90, 2174-2178.
11. Wilks, A.F. (1989) Proc. Natl. Acad. Sci. USA 86, 1603-1607.
12. Lai, C and Lemke, G. (1991) Neuron 6, 691-704.
13. Kozak, M. (1981) Nucleic Acid Res 9, 5233-5252.
14. Wickens, M. & Stephenson, P. (1984) Science 226, 1045-1051.
15. Wernet, W., Flockerzi, V. & Hofmann, F. (1989) FEBS 251, 191-96.
16. Pelham, H.R.B. (1989) Annu. Rev. Cell. Biol . 5, 1-23.
17. Jackson, M.R., Nilsson, T. & Peterson, P.A. (1990) EMBO J. 9, 3153-3162.
18. Gabathuler, R. & Kvist, S. (1990) J. Cell Biol. 111, 1803-1810.
19. Asano, T., Takata, K., Katagiri, H., Tsukada, K., Lin J.L., Ishihara, H., Inukai, K., Hirano, H., Yazaki, Y., & Oka, Y. (1992) J. Biol. Chem 267, 19636-19641.
20. Forman, B.M., Yang, C.R., Au, M., Casanova, J., Ghysdael, J., & Samuels, H.H. (1989) Mol Endocrinol 3, 1610-1626.
21. Landgraf, W., Hofmann, F., Pelton, J.T., Huggins, J.P. (1990) Biochemistry 29, 9921-9928.
22. Atkinson, A.R., Saudek, V., Huggins, J.P., Pelton, J.T., (1991) Biochemistry 30, 9389-9395.