Journal of Neurochemistry
Raven Press, Ltd., New York

Rapid Communication

Identification of Cortexin: A Novel, Neuron-Specific, 82-Residue Membrane Protein Enriched in Rodent Cerebral Cortex

Phillip M. Coulter II, *Erwin A. Bautista, Jody E. Margulies, and Joseph B. Watson

*Mental Retardation Research Center, Department of Psychiatry and Biobehavioral Sciences, UCLA School of Medicine, Los Angeles; and *Department of Animal Physiology, University of California, Davis, California, U.S.A.*

Abstract: Nucleotide sequence analysis of a cDNA clone of a rat cortex-enriched mRNA identifies a novel integral membrane protein of 82 amino acids. The encoded protein is named cortexin to reflect its enriched expression in cortex. The amino acid sequence of rat cortexin and its mouse homologue are nearly identical (98% similarity), and both contain a conserved single membrane-spanning region in the middle of each sequence. Northern blot analysis shows that cortexin mRNA is brain-specific, cortex-enriched, and present at significant levels in fetal brain, with peak expression in postnatal rodent brain. In situ hybridization studies detect cortexin mRNA primarily in neurons of rodent cerebral cortex, but not in cells of the hindbrain or white matter regions. The function of cortexin may be particularly important to neurons of both the developing and adult cerebral cortex. **Key Words:** cDNA—Nucleotide sequence—Membrane-spanning—Cortex-enriched—Neuron-specific.
J. Neurochem. **61,** 756–759 (1993).

It is often difficult to isolate integral membrane proteins by biochemical means due to their presence in low amounts or loss of specific binding to a ligand when solubilized away from their normal membrane environment. In recent years, many of these problems have been overcome by expression cloning in either *Xenopus* oocytes (Masu et al., 1987) or mammalian cell lines (Arrufo and Seed, 1987). An alternative approach is to isolate cDNA clones of membrane proteins by subtraction-based approaches (Hedrick et al., 1984; Travis et al., 1989). We previously reported the cloning of a collection of nine novel brain mRNAs (CPu1–9) by screening a rat caudate putamen cDNA library with a caudate putamen-minus-cerebellum single-stranded cDNA probe, generated by subtractive hybridization (Watson et al., 1992). The CPu9 clone detected a rare 1.25-kb mRNA that was highly enriched in cerebral cortex, but was reduced in expression in caudate putamen and hippocampus and absent from cerebellum. Here cDNA sequence analysis shows that CPu9 mRNA encodes a novel membrane-spanning protein of 82 residues highly conserved in both rat and mouse brain. Based on northern blot and in situ hybridization studies, we have named the encoded molecule cortexin to reflect its enriched expression in neurons of rodent cerebral cortex.

EXPERIMENTAL PROCEDURES

Construction of cDNA libraries

A λGT11 cDNA library was prepared from cerebral cortex poly(A)$^+$ RNA isolated from male Sprague–Dawley rat brains (Aviv and Leder, 1972; Chirgwin et al., 1979). The library was constructed using a Superscript kit (GIBCO-BRL) employing oligo(dT) primers for reverse transcription and *Eco*RI adapters for ligation. The library contained approximately 3.8×10^5 pfu prior to amplification. A mouse brain plasmid cDNA library, which was used to isolate the mouse cortexin cDNA clone (pMCTX), has been described previously (Watson et al., 1990).

DNA sequencing

The nucleotide sequence of the full-length rat cortexin cDNA subcloned in pBluescript KS (pRCTX) was determined by double-stranded sequencing using the dideoxy chain termination method (Sanger et al., 1977). A combination of exonuclease III digestions (Henikoff, 1984) and synthetic deoxyoligonucleotides (Watson et al., 1990) was used to generate contiguous sequences for both strands of the rat cortexin clone. A similar strategy was used to determine the sequence of the mouse cortexin homologue (pMCTX).

Northern blot analysis

RNA blot analysis was performed as described previously (Watson et al., 1990) using tissue and brain poly(A)$^+$ RNA isolated from adult male Sprague–Dawley rats or brain poly(A)$^+$ RNA prepared from C57BL/6CR mice at different developmental times.

Resubmitted manuscript received April 20, 1993; accepted April 21, 1993.

Address correspondence and reprint requests to Dr. J. B. Watson at 48-241 NPI Mental Retardation Research Center, Department of Psychiatry and Behavioral Sciences, UCLA School of Medicine, 760 Westwood Plaza, Los Angeles, CA 90024, U.S.A.

Abbreviations used: SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate buffer.

In situ hybridization

^{35}S-Radiolabeled full-length cRNA was synthesized by in vitro transcription of linearized pRCTX plasmid cDNA with either T7 (antisense) or T3 (sense) RNA polymerases as described previously (Watson et al., 1990). Conditions for in situ hybridization were a modification of previous methods (Angerer et al., 1987; Popper and Micevych, 1991). Frozen coronal brain sections (20 μm) from male Sprague–Dawley rats (~250 g) were mounted on slides, fixed in 4% paraformaldehyde, dehydrated in 100% ethanol, digested with proteinase K, acetylated, and incubated at 42°C in prehybridization solution [50% formamide, 0.3 *M* NaCl, 50 m*M* PIPES, 5 × Denhardt's solution, 0.05% sodium dodecyl sulfate (SDS), 150 μg/ml salmon sperm DNA, 20 m*M* dithiothreitol]. Sections were hybridized overnight at 55°C under glass coverslips with 40 μl of prehybridization solution containing 5% dextran sulfate and 5 × 10^5 cpm (~5 ng) of ^{35}S-labeled sense or antisense cortexin cRNA. Sections were RNased and washed at a final stringency of 0.1 × saline–sodium citrate buffer (SSC)/1 m*M* dithiothreitol at 55°C. For cellular resolution, sections were dipped in Kodak NTB-3 emulsion and exposed at 4°C for 10 days. After development, sections were counterstained with cresyl violet.

RESULTS AND DISCUSSION

To obtain full-length cDNA clones of rat cortexin mRNA, an unamplified λGT11 cDNA library prepared from rat cerebral cortex poly(A)$^+$ RNA was plated out at high density, transferred to nylon filters (Grunstein and Wallis, 1979), and hybridized to a 1.1-kb CPu9 cDNA insert (see Watson et al., 1992) radiolabeled with [α-^{32}P]dCTP using the random oligonucleotide priming method (Feinberg and Vogelstein, 1983). Filters were washed at high stringency (0.1 × SSC/0.5% SDS at 68°C) and exposed to x-ray film with intensifying screens. Of 14 plaque isolates obtained from the library, two clones (pRCTX) were identical and contained cDNA inserts of about 1.25 kb in length, which matched the size of cortexin mRNA detected previously on northern blots (Watson et al., 1992). A mouse cortexin cDNA clone (pMCTX) of approximately 1.1 kb was also isolated by screening a mouse brain plasmid cDNA library with the rat clone. This was a nearly full-length clone of the mouse 1.25-kb mRNA seen on northern blots (data not shown) and contained the likely open reading frame (see sequence analysis below).

As shown in Fig. 1, the cDNA sequence of clone pRCTX consists of 1,210 nucleotides. The longest open reading frame generated by conceptual translation is 246 nucleotides encoding a protein of 82 amino acids with a calculated molecular mass of 9.1 kDa. There are 231 nucleotides of 5′ untranslated sequence and 733 nucleotides of 3′ untranslated sequence not including the $(A)_n$ tail. Based on the sequence of the shorter 1.1-kb rat clone, there are at least 52 adenosine residues added by polyadenylation, which predicts a mature rat cortexin mRNA of 1,262 nucleotides in length. As expected, the nucleotide sequences encoding the rat and mouse open reading frames are highly similar (97%), although there is an increased frequency of nucleotide substitutions in the mouse 5′ flanking region and an abrupt deletion of 14 nucleotides in its 3′ untranslated region, very close to the termination codon.

A nearly identical amino acid sequence of 82 amino acids

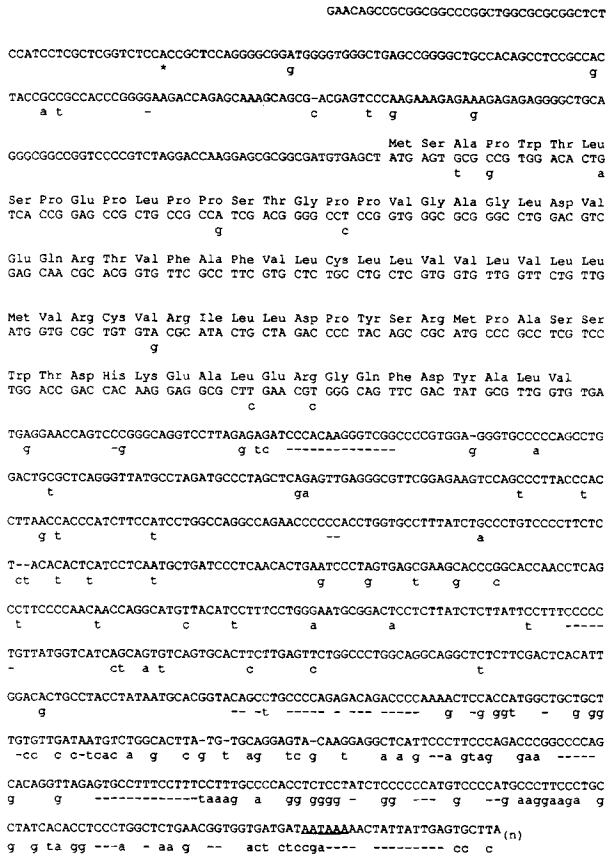

FIG. 1. Cortexin nucleotide sequence. The nucleotide sequences of rat and mouse cortexin cDNA clones pRCTX (1.210 kb) and pMCTX (1.08 kb), respectively, were determined and aligned for comparison. Nucleotide differences detected in the mouse sequence are indicated below the rat sequence by either lowercase letters to denote substitutions or dashes to indicate deletions. The 5′ end of the mouse sequence is indicated by an asterisk. To optimize alignments in the 5′ and 3′ untranslated regions, breaks are made by dashes in the rat sequence. The putative 3′ signal for polyadenylation [$(A)_n$] is underlined. The genetic code was used to translate the longest open reading frame and generate the rat cortexin amino acid sequence (shown above the nucleotide sequence) and the corresponding mouse sequence (see Fig. 2A). Alignments and translations were performed with the GeneWorks Nucleic Acid and Protein Sequence Analysis Software (IntelliGenetics, Inc.). The GenBank accession number for cortexin is L15011.

is predicted from the nucleotide sequences of both the rat and mouse cortexin cDNA clones (Fig. 2A). Changes are in the third and fourth codons, resulting in only two amino acid substitutions in the mouse sequence (Ala^3 → Ser^3; Pro^4 → Ala^4). Previous results showed that the amino acid sequence of RC3, which contains only 78 residues, can be validated by comparing the predicted rat protein sequence with that encoded by a cDNA clone of a homologous mouse mRNA (Watson et al., 1990). The rat and mouse RC3 amino acid sequences were identical, whereas the bovine homologue of RC3 was also highly conserved (75/78 match; Baudier et al., 1991). Similarly, the high conservation of the rat and mouse cortexin amino acid sequences reported here is strong evidence that the correct cortexin open reading frame has been

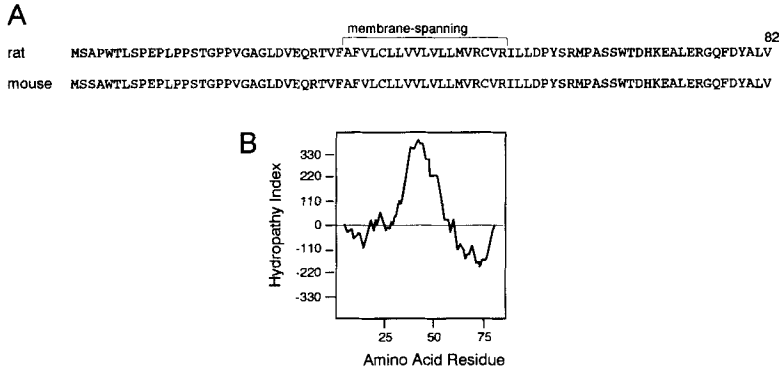

FIG. 2. Comparison of the rat and mouse cortexin amino acid sequences. **A:** Both the predicted rat and mouse cortexin amino acid sequences are 82 residues long and contain a putative membrane-spanning region (bracketed; see also B). **B:** The hydropathy index of the rat cortexin sequence was determined using the Kyte–Doolittle algorithm (Kyte and Doolittle, 1982) with a window setting of 11.

identified. Searches of the current computer protein data bases (NCBI, EMBL) revealed sequence similarity of a portion of the cortexin sequence with hydrophobic-rich, membrane-spanning sequences of numerous, but unrelated, integral membrane proteins (data not shown). Consistent with this similarity, hydropathy analysis predicts that both the rat and mouse cortexin proteins have single membrane-spanning regions, rich in hydrophobic residues, positioned near the midpoint of each protein's sequence (Fig. 2). Otherwise, the cortexin amino acid sequence is completely novel.

Northern blot experiments detected cortexin only in cortex tissue from rat brain (Fig. 3A). Because the heart lane was underloaded two- to threefold as was the cortex lane, we cannot rule out some expression in heart. However, no heart signal was detected after a 2-week exposure of the blot, whereas there was slight expression found in testes. Cortexin mRNA was highly enriched in rat cortex relative to other brain regions, with decreased expression in hippocampus and basal ganglia (Fig. 3B). There was little or no detectable expression in thalamus, brainstem, or cerebellum. During development, cortexin mRNA was detected at moderate levels in fetal mouse brain (embryonic days 14–18) and at high levels beginning on postnatal day 4, with peak expression occurring at approximately postnatal day 18 (Fig. 3C). The northern blot results presented here are in good agreement with results from a previous study (Watson et al., 1992) that used different rat RNA preparations for analysis. The cumulative data indicate that cortexin mRNA is brain-specific, cortex-enriched, and expressed at significant levels early in the developing rodent brain.

To determine cortexin mRNA expression at the cellular level in rat brain, 20-μm tissue sections of rat brain sampled at every 200–400 μm were hybridized in situ to ^{35}S-labeled cortexin cRNA. Robust mRNA hybridization was detected in forebrain regions with the cortexin antisense riboprobe, but not with the sense probe. As expected from northern blot data (Fig. 3), cortexin mRNA was restricted primarily to cell populations of the forebrain, including neocortex, piriform cortex, cingulate cortex, entorhinal cortex, amygdala, CA1/CA3/CA4 fields and dentate gyrus of the hippocampus, basal ganglia (caudate putamen, nucleus accumbens), olfactory tubercle, and selected nuclei in the thalamus and hypothalamus. The most intense hybridization was over pyramidal neurons in laminae II and VI of neocortex (somatosensory, oculomotor, frontal; see Fig. 4A and

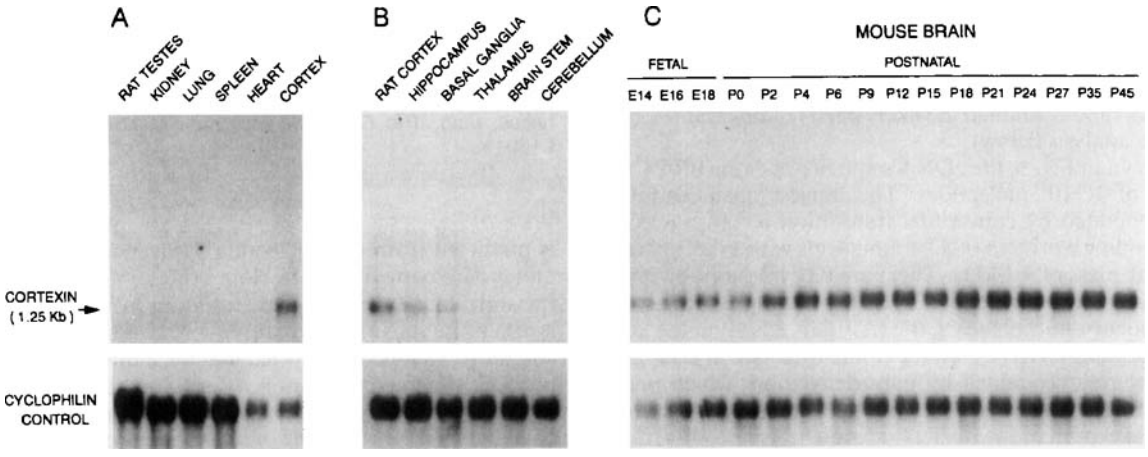

FIG. 3. Northern blot analysis of cortexin mRNA expression. Nitrocellulose filters contained 2 μg of poly(A)$^+$ RNA from either adult rat tissues (**A**), adult rat brain regions (**B**), or mouse brain at different developmental days (**C**). Blots in A and B were hybridized to ^{32}P-labeled rat cortexin cDNA insert (1.210 kb) of pRCTX, and blots in C were hybridized to radiolabeled mouse cortexin cDNA (1.08 kb) of pMCTX. Blots were also hybridized with cyclophilin cDNA (Danielson et al., 1988) as a control. Washing conditions were 0.1 × SSC/0.5% SDS at 68°C. The cortex and heart lanes in A were underloaded based on comparisons to the cyclophilin control hybridization. E, embryonic day; P, postnatal day.

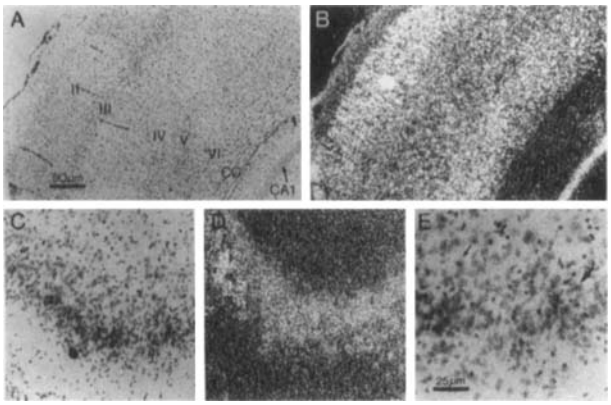

FIG. 4. In situ hybridization of cortexin mRNA in rat cortex. Cortexin mRNA expression was visualized in selected rat cortical regions by hybridization with ^{35}S-labeled rat cortexin antisense cRNA. There was no selective hybridization of sense riboprobes to adjacent sections (data not shown). **A:** Bright-field micrograph of a coronal section of somatosensory neocortex at the level of the hippocampus. Roman numerals (II–VI) indicate laminae of cortex. CC denotes corpus callosum. The CA1 field of hippocampus is also labeled. **B:** Dark-field micrograph of A. **C** and **D:** Bright-field and dark-field micrographs of coronal sections through piriform cortex. **E:** Higher magnification view of selected population of cells from C. Dense grains were detected over likely pyramidal neurons (large arrow) and polymorphic neurons (small arrow). The scale bar (50 μm) in A also applies to B–D.

B), pyramidal neurons (see CA1 in Fig. 4A and B) and dentate granule cell layer of the hippocampus, and pyramidal and polymorphic neurons of the piriform cortex (Fig. 4C–E). There was little or no detectable mRNA expression in neurons of the midbrain or hindbrain, including cerebellar cortex (data not shown), or in any cells of white matter regions (see corpus callosum in Fig. 4A and B). The cortexin mRNA appears to be neuron-specific and is expected to encode a neuron-specific protein expressed primarily in the cerebral cortex.

The function of cortexin is unknown. Clues to its role, particularly in cortical neurons, may come from studies of its subcellular localization through immunocytochemical and biochemical fractionation studies. For example, it will be important to determine whether cortexin is localized in presynaptic or postsynaptic membranes, or possibly in membranes of synaptic vesicles of neurons in both fetal and postnatal forebrain. Because genomic Southern blot analysis suggests that there is only one cortexin gene (unpublished observations), cortexin's function may be unique to neurons of both the developing and adult forebrain.

Acknowledgment: We wish to thank Kathy Kampf, Vance Handley, and Anthony T. Campagnoni for advice on construction of λ cDNA libraries and for providing mouse brain poly(A)$^+$ RNA. Thanks also to Paul Micevych for helpful suggestions for in situ hybridization studies. Research was supported by NIH grant no. HD25831 (J.B.W.) and an MRRC Postdoctoral Trainee Fellowship (J.E.M.).

REFERENCES

Angerer L. M., Stoler M. H., and Angerer R. C. (1987) In situ hybridization with RNA probes: an annotated recipe, in *In Situ Hybridization: Applications to Neurobiology* (Valentino K. L., Eberwine J. H., and Barchas J. D., eds), pp. 42–70. Oxford University Press, New York.

Arruffo A. and Seed B. (1987) Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA* **84,** 8573–8577.

Aviv H. and Leder P. (1972) Purification of biologically active globin messenger mRNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.

Baudier J., Deloulme J. C., Van Dorsselaer A., Black D., and Matthes H. W. D. (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). *J. Biol. Chem.* **266,** 229–237.

Chirgwin J. M., Przybyla A. E., MacDonald R. J., and Rutter W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18,** 5294–5299.

Danielson P. E., Forss-Petter S., Brow M. A., Calavetta L., Douglass J., Milner R., and Sutcliffe J. G. (1988) P1B15: cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **7,** 261–267.

Feinberg A. B. and Vogelstein B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

Grunstein M. and Wallis J. (1979) Colony hybridization, in *Methods Enzymol.* **68,** 379–389.

Hedrick S. M., Cohen D. I., Nielsen E. A., and Davis M. M. (1984) Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* **308,** 149–153.

Henikoff S. (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28,** 351–359.

Kyte J. and Doolittle R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.

Masu Y., Nakayama K., Tamaki H., Harada Y., Kuno M., and Nakanishi S. (1987) cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature* **329,** 836–838.

Popper P. and Micevych P. E. (1991) Steroid regulation of calcitonin gene related peptide mRNA in motor neurons of the spinal nucleus of the bulbocavernosus. *Mol. Brain Res.* **8,** 159–166.

Sanger F., Nicklen S., and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Travis G. H., Brennan M. B., Danielson P. E., Kozak C. A., and Sutcliffe J. G. (1989) Identification of a photoreceptor-specific mRNA encoded by the gene responsible for retinal degeneration slow (*rds*). *Nature* **338,** 70–73.

Watson J. B., Battenberg E. F., Wong K. K., Bloom F. E., and Sutcliffe J. G. (1990) Subtractive cDNA cloning of RC3, a rodent cortex-enriched mRNA encoding a novel 78 residue protein. *J. Neurosci. Res.* **26,** 397–408.

Watson J. B., Coulter P. M. II, and Sutcliffe J. G. (1992) Identification of genes enriched in expression in mammalian neostriatum and neocortex. *Brain Dysfunct.* **5,** 94–105.