Developmental regulation and neuronal expression of the mRNA of rat *n*-chimaerin, a p21^{rac} GAP:cDNA sequence

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Human *n*-chimaerin is a GTPase-activating protein (GAP) for p21^{rac} and a phorbol ester receptor. We have isolated rat *n*-chimaerin cDNA and investigated the cellular and developmental pattern of mRNA expression in the brain. There is extensive sequence conservation with human *n*-chimaerin in the coding region and the first 400 nucleotides of the 3'-untranslated region (UTR) (90% and 83% identity respectively). The rat cDNA encodes an additional 35 N-terminal amino acids compared with the reported human cDNA, which has a 5'-UTR sequence inversion and a 41-nucleotide deletion including the putative initiator methionine. The rat cDNA encodes a 334-amino acid protein (38 200 *M_r*, pI 8.04) with 97% amino acid sequence identity with the human protein, after correction of the human 5'-DNA sequence. *n*-Chimaerin mRNA was detectable in embryonic rat brain at day 15 and increased in amount postnatally from birth to 20 days, coincident with cellular differentiation and synaptogenesis. *n*-Chimaerin mRNA is restricted to neurons, with highest concentrations in hippocampal pyramidal cells, granule cells of the dentate gyrus and cortical neurons. In the cerebellum the mRNA was detected only in Purkinje neurons. The pattern and specificity of mRNA expression suggests an important role for *n*-chimaerin in neuronal signal-transduction mechanisms.

INTRODUCTION

Human *n*-chimaerin cDNA encodes a brain p21 GTPase-activating protein (GAP) (Hall *et al.*, 1990; Diekmann *et al.*, 1991). It has sequence similarity in separate domains to the regulatory region of protein kinase C (PKC) and to the C-terminal of the product of the breakpoint-cluster-region gene (BCR). The BCR gene on chromosome 22 is involved in reciprocal (22;9)(q11;q34) translocation associated with chronic myelogenous leukaemia, which generates an expressed oncogenic BCR/*abl* hybrid (Heisterkamp *et al.*, 1985, 1990). With the use of recombinant *n*-chimaerin proteins expressed in *Escherichia coli*, the C-terminal domain has been shown to function as a p21^{rac} GAP, as does the C-terminal of BCR (Diekmann *et al.*, 1991). p21^{rac}, p21^{rho} and p21^{cdc42H} are members of a subgroup of the p21^{ras} oncogene superfamily implicated in cytoskeletal organization (Hall, 1990). *n*-Chimaerin and BCR are members of a new GAP family; both are capable of activating p21^{rac} GTPase, although they are likely to have different effector or regulatory roles. The PKC-like cysteine-rich domain of *n*-chimaerin binds the diacylglycerol (DAG) analogues, phorbol esters (Ahmed *et al.*, 1990), in a zinc-dependent manner, as does PKC (Ahmed *et al.*, 1991). PKC, which is activated by receptor-mediated release of DAG, is a central component of signal-transduction pathways (Nishizuka, 1988). Similarly, p21^{ras} GAP is involved in receptor-mediated activity and has been shown to inhibit *in vitro* the opening of muscarinic receptor-coupled K⁺ channels (Yatani *et al.*, 1990). The existence of *n*-chimaerin as a DAG (phorbol ester) receptor with GAP activity has important implications for signal transduction in the brain. Here we show that *n*-chimaerin mRNA is selectively expressed in neurons and increases in amount during rat brain development, coincident with synaptogenesis. Rat and human sequences are highly conserved.

MATERIALS AND METHODS

Isolation and sequencing of rat *n*-chimaerin cDNA

A cDNA library was constructed from rat brain free polyribosomal mRNA (Hall *et al.*, 1987) by using Pharmacia cDNA synthesis and Stratagene (Cambridge, U.K.) λgt10 cloning kits. It was screened with human *n*-chimaerin cDNA (2.2 kb), ³²P-labelled using Klenow polymerase with random primers (Sambrook *et al.*, 1989). Hybridization was at 60 °C in 6 × SSC/5 × Denhardt's solution/10 mM-EDTA/0.5% SDS/salmon sperm DNA (100 µg/ml)/poly(A) (10 µg/µl). [1 × SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7.0; 1 × Denhardt's solution is 0.02% Ficoll 400/0.02% poly(vinylpyrrolidone)/0.2% BSA.] Filters were washed at 60 °C in 2 × SSC/0.1% SDS (30 min) and at 50 °C in 0.1 × SSC/0.1% SDS (30 min). The cDNA was excised from λgt10 with *Eco*RI as two fragments (nucleotides 1–935 and 936–2309) which were subcloned into Bluescript BSSK[−] (Stratagene) in both orientations. Nested deletions were generated by using exonuclease III/mung bean nuclease according to manufacturers' protocols and single-stranded DNA fully sequenced in both orientations (Sanger *et al.*, 1977).

Hybridization *in situ*

Oligonucleotide probes complementary to nucleotides 854–907 and 1601–1644 of rat *n*-chimaerin were labelled to a specific radioactivity of 1–2 d.p.m./µg with [³⁵S]thio[dATP by 3' end labelling with terminal transferase and purified on NENSORB columns [oligonucleotide labelling system; NEN–du Pont (U.K.)]. Coronal brain sections (12 µm) were cut on a cryostat and thaw-mounted on to poly(L-lysine)-coated slides. Sections were fixed in fresh 4% paraformaldehyde/phosphate-buffered saline (PBS), rinsed in PBS and further pretreated as described by Brandt *et al.* (1987). Sections were prehybridized (2 h) in 4 × SSC/5 ×

Abbreviations used: GAP, GTPase-activating protein; DAG, diacylglycerol; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; BCR, product of the breakpoint-cluster-region gene; 1 × SSC, 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7.0; PBS, phosphate-buffered saline.

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The rat cDNA sequence data in Fig. 1 will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession number X67250.

Fig. 1. For legend see facing page.

Denhardt's solution/50 mM-sodium phosphate (pH 7.0)/10% dextran sulphate/1 µg of yeast tRNA/ml/10 µg of poly(A)/ml/100 µg of sheared single-stranded salmon sperm DNA/ml/20 µM-α-thio-dATP. Hybridization was at 42 °C for 16 h in the same solution with the omission of α-thio-dATP, but containing 50 mM-dithiothreitol and ³⁵S-labelled oligonucleotide (0.5 × 10⁶–1 × 10⁶ c.p.m. per section). Sections were washed in 1 × SSC/10 mM-dithiothreitol at 20 °C for 15 min, 1 × SSC at 50 °C for 45 min, 0.2 × SSC at 55 °C for 30 min, followed by 1 × SSC at 20 °C for 2 h and exposed to β-max Hyperfilm (Amersham) for 1 week. Subsequently they were dipped in LM-1 nuclear emulsion (Amersham), developed after 4–6 weeks and counterstained with Methyl Green/pyronine stain (Sigma). Control sense-strand oligonucleotides showed an absence of hybridization in Northern-blot analyses and histochemistry *in situ*. The hybridization was also effectively inhibited by a 20-fold excess of unlabelled oligonucleotide.

Northern-blot analysis

Preparation of rat forebrain polyribosomal RNA and Northern-blot analysis were as previously described (Hall *et al.*, 1987). A 3' *Bst*XI fragment (667 nucleotides; which excluded the CA-rich repeat section) of rat *n*-chimaerin cDNA, ³²P-labelled using Klenow polymerase with random primers, was hybridized to RNA on Amersham Hybond N filters. Filters were subsequently rehybridized to a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control cDNA. Film autoradiographs were scanned using a Joyce-Loebl Centriscan 3 densitometer to determine the relative amounts of GAPDH and *n*-chimaerin hybridization.

RESULTS

Rat brain *n*-chimaerin cDNA sequence

Rat *n*-chimaerin cDNA (2.3 kb) isolated from a brain cDNA library (see the Materials and methods section) contained an open reading frame extending from nucleotides 339 to 1419, with the first methionine at nucleotide 420 (Fig. 1). When compared with the sequence of the rat cDNA, the reported human cDNA was found to have a sequence inversion of the entire 5'-UTR and

a 41-nucleotide deletion, possibly as a result of a cDNA cloning artefact (Hall *et al.*, 1990). The rat cDNA encoded a 334-amino acid protein, 35 amino acids longer at the *N*-terminus than the reported human protein. That the rat cDNA 5'-sequence was in the correct orientation was established by both PCR amplification of cDNA ends (Frohman & Martin, 1989) derived from rat brain mRNA and comparison with the human genomic DNA sequence (H. H. Lim, P. Smith & L. Lim, unpublished work). The 5'-UTR of the rat cDNA had 77% sequence identity with the corresponding human genomic sequence and with the reversed 5'-UTR of the human cDNA (Fig. 1). This region in both rat and human cDNAs is capable of forming stable hairpin/loop structures (–502 kJ for rat *n*-chimaerin). The rat and human cDNA sequences were highly similar over the coding region (90%) and the first 400 nucleotides of the 3'-UTR (83.7% overall similarity), suggesting a regulatory function for this region of the mRNA (Fig. 1). The rat cDNA contains a CA-rich repeat sequence inserted in the 3' region, which is not present in the human cDNA. A core sequence, CACACCACAAACAC, is repeated six times in a 200-nucleotide CA-rich section which includes a 38-nucleotide direct repeat. A second cDNA had a 50-nucleotide deletion eliminating one of the 38-mer repeats (cf. Fig. 1). This region may be variably expressed in the mRNA or subject to deletion during cloning. The 3'-terminal 200 nucleotides of rat and human cDNAs were 71% similar. There are two polyadenylation signals in rat cDNA; these are conserved with the human sequence where both are utilized (Hall *et al.*, 1990).

n-Chimaerin protein sequence

With the human 5' sequence supplied by genomic DNA (cf. Fig. 1), the predicted human and rat *n*-chimaerin protein sequences share 97% identity over 334 amino acids (Fig. 2). In both the rat and human sequences, A and C residues at positions –3 and –4, respectively, preceding the first ATG of the long open reading frame, conform to the Kozak (1987) consensus. In both species, ATG codons and short open reading frames are present upstream of the presumptive initiation site. The additional 35 *N*-terminal amino acid sequence is capable of forming an amphipathic helix, exposing a hydrophobic face with

Fig. 2. Comparison of rat and human *n*-chimaerin amino acid sequences

Amino acid substitutions are boxed. The first 35 amino acids (not in the published human sequence; Hall *et al.*, 1990) (—), PKC-like (——) and BCR-like regions (====) are underlined as indicated. Potential phosphorylation sites for PKC (*) and cyclic AMP-dependent protein kinase (o) are indicated.

Fig. 1. Sequence conservation of rat *n*-chimaerin

The DNA sequence of rat *n*-chimaerin cDNA (numbered) is aligned with the human sequence. The first 450 nucleotides of the rat sequence are aligned with human genomic DNA sequence (and correspond to the reverse complement of the human cDNA 5'-UTR; P. Smith, H. H. Lim & L. Lim, unpublished work) and the remainder is aligned with the human cDNA sequence described previously (Hall *et al.*, 1990). The rat amino acid sequence is shown below. The rat CA-rich repeat core sequence is underlined; a 38-nucleotide direct repeat was present in a 50-nucleotide partially overlapping repeat (↔); a second cDNA clone contained a deletion of this sequence (□). Two potential polyadenylation signals are double underlined.

Fig. 3. Proteins with BCR-like domains

The amino acid sequence of rat *n*-chimaerin (n-chim; 140–334), bovine phosphatidylinositol kinase-associated p85 α (amino acids 113–305) and p85 β (amino acids 109–283; Otsu *et al.*, 1991), human BCR (amino acids 1007–1200; Lifshitz *et al.*, 1988) and *rho*GAP peptide (Diekmann *et al.*, 1991) are compared; amino acids identical with *n*-chimaerin (■) or with BCR (□).

membrane-binding potential. There are two consensus sites for cyclic AMP-dependent phosphorylation and eight for PKC phosphorylation in both rat and human proteins (Fig. 2). *n*-Chimaerin sequence relationships to PKC and BCR have been described (Hall *et al.*, 1990; Ahmed *et al.*, 1991). The PKC-like cysteine-rich region (amino acids 79–135) is identical in rat and human sequences except for a valine to isoleucine substitution (Fig. 2). The phosphatidylinositol kinase-associated 85 kDa proteins have sequence similarity to the BCR C-terminal domain (Otsu *et al.*, 1991) and to *n*-chimaerin (Fig. 3). The regions most conserved between BCR and *n*-chimaerin are also those of greatest similarity to the p85 proteins, although the latter have not so far been shown to have GAP activity. One of these regions is also present in a peptide fragment of purified *rho*GAP (Diekmann *et al.*, 1991; Fig. 3). The rat *n*-chimaerin, expressed in *E. coli* as a glutathione *S*-transferase fusion protein, has p21^{rac} GAP activity, as does the human counterpart (Diekmann *et al.*, 1991; M. Teo, J. Lee, S. Ahmed & L. Lim, unpublished work).

Developmental expression of *n*-chimaerin mRNA

On Northern-blot analysis of brain RNA, *n*-chimaerin cDNA detected a 2.3 kb mRNA (Fig. 4). The slightly larger size than the human counterpart may be due to the CA-rich insertion in the 3'-UTR region. In addition, a 1.9 kb mRNA was detected by both the cDNA and oligonucleotide probes. *n*-Chimaerin mRNA was present in embryonic rat brain at day E15 (Fig. 4). The amounts of mRNA increased postnatally in the forebrain from postnatal day 1 to maximal levels at day 20, coincident with the period of cellular differentiation and synaptogenesis.

n-Chimaerin mRNA is expressed in central nervous system neurons

The cellular distribution of *n*-chimaerin mRNA in the rat

Fig. 4. Developmental increase in *n*-chimaerin mRNA

Total RNA from free polyribosomes (10 μ g) from rats aged embryonic day 15 (E15) to postnatal day 40 was hybridized with *n*-chimaerin cDNA and subsequently to GAPDH. The graph shows developmental changes in the amount of *n*-chimaerin mRNA hybridization (2.3 kb band) determined by densitometric scanning, corrected for equal RNA loading on the basis of the GAPDH signal for the same blot.

Fig. 5. Expression of *n*-chimaerin in rat brain

Coronal sections from various regions of adult rat brain (*a-d*) were hybridized with an ^{35}S -labelled *n*-chimaerin antisense oligonucleotide and apposed to autoradiographic film (see the Materials and methods section); c, cerebral cortex; aon, anterior olfactory nucleus; pir, piriform cortex; neocortex corresponding to layer V indicated by an arrow; s, striatum; a, basolateral nucleus of the amygdala; h, hippocampus; dg, dentate gyrus; p, pons; cbl, cerebellum. Bar, 2.5 mm.

Fig. 6. Localization of *n*-chimaerin mRNA in the cortex, hippocampal formation and cerebellum

Hybridized sections were emulsion-dipped, developed after 4 weeks and counterstained (black silver grains can be distinguished from lighter counterstain of cells). (*a*) Layers I, II and III of the cerebral cortex. (*b*) Hippocampal formation: py, pyramidal cell layer CA1; ep, ependymal cells; rad, stratum radiatum; or, stratum oriens. (*c*) Dentate gyrus: g, granule cell layer; p, polymorphic neurons of the hilar region. (*d*) Cerebellar cortex: Pc, Purkinje cells; m, molecular layer; gc, granule cell layer; wm, white matter. Bar, 50 μm .

Fig. 7. Neuronal specificity of *n*-chimaerin mRNA

The Figure shows a higher-magnification analysis of emulsion-dipped sections. (a) Cerebral cortex: $\blacktriangleright$, cells with a neuronal morphology, $\blacktriangleleft$, small glial cells; (b) hilar region of the dentate gyrus: gc, labelled granule cells; $\blacktriangleright$, unlabelled glial cells; $\blacktriangleleft$, unlabelled large neurons; (c) cerebellar cortex: $\blacktriangleright$, Purkinje cells; gc, granule-cell layer; m, molecular layer. The bar represents 10 μ m.

brain was investigated by hybridization *in situ*. Specific hybridization was exclusively localized to cells of neuronal morphology. High concentrations of mRNA were present in the hippocampus and cerebral cortex (Figs. 5a–5c). The anterior olfactory nucleus was labelled to the same extent as the adjacent frontal cortex (Fig. 5a). The densely packed neurons of the piriform cortex were clearly delineated by hybridization (Fig. 5b). At the higher resolution afforded by emulsion-dipped sections (Figs. 6 and 7) silver grains were concentrated over cortical neurons in layers II–VI, whereas layer I, consisting of primarily neuronal processes and glial cells, showed background levels of silver grains (Fig. 6a). The band of lower-intensity labelling in layer V compared with the rest of the cortex on autoradiograms (Figs. 5b and 5c) reflected lower neuronal density rather than decreased labelling of the large pyramidal neurons. In the hippocampus and dentate gyrus, pyramidal cell layers and granule cell layers were strongly labelled (Figs. 6b and 5c), although there were also a minority of neurons that were unlabelled (Fig. 7b). The taenia tectum and induseum griseum were prominently labelled. Neurons in the striatum and the basolateral nucleus of the amygdala were moderately labelled (Fig. 5b). In the olfactory bulb, mitral cells were moderately labelled, whereas the adjacent granule cells were scarcely labelled. Neurons within the thalamus and hypothalamus had similar low concentrations of label. Cells in the brainstem, pontine nuclei (Fig. 5c) and locus coeruleus had low to moderate amounts of label. Most neurons in the stratum radiatum and stratum oriens were unlabelled (Fig. 6b). No labelling was present over the ependyma (Fig. 6b), choroid plexus, blood vessels, oligodendrocytes or other glial cells (Figs. 7a and 7b). Similarly, white matter tracts showed no specific labelling. The cerebellum, which contains relatively low levels of *n*-chimaerin mRNA as determined by Northern-blot analysis (Hall *et al.*, 1990), showed a very restricted cellular distribution of expression. A fine line of label on autoradiograms of the cerebellar cortex (Fig. 5d) represented highly specific labelling of Purkinje cells (Fig. 6d and Fig. 7c). Other neurons of the cerebellar granule cell layer and molecular layer were unlabelled.

DISCUSSION

Conservation between rat and human sequences

Rat and human brain *n*-chimaerin cDNA sequences are highly conserved. The cysteine-rich PKC-like domain is represented in several other proteins, including diacylglycerol kinase (Schaap *et al.*, 1990), the oncogenes *raf* (Beck *et al.*, 1987) and *vav* (Katzav *et al.*, 1989) as well as *Caenorhabditis elegans unc-13* (Maruyama & Brenner, 1991). Sequence comparisons enabled a consensus sequence for phorbol ester binding to be derived (Ahmed *et al.*, 1990, 1991). The complete amino acid identity in this region of rat and human *n*-chimaerin is suggestive of structural/functional constraints which may relate to extreme specificity of ligand binding, since naturally occurring DAGs have diverse acyl groups. Relatively minor sequence changes lead to loss of phorbol ester receptivity (Ahmed *et al.*, 1991). Both rat and correctly orientated human *n*-chimaerin sequences contain upstream ATG codons followed by in-frame terminator codons. This and possible secondary structure of the 5'-UTR may influence translational efficiency. The 5'-UTR of BCR mRNA also contains upstream open reading frames, has the potential to form stable stem loop structures and is inhibitory to translation *in vitro* (Muller & Witte, 1989). Interestingly, the 5'-UTR of the mouse *p21rac1* mRNA has sequence similarity to this region of BCR mRNA and can similarly form stable loop structures (Moll *et al.*, 1991).

Neuronal localization of *n*-chimaerin

n-Chimaerin mRNA is expressed in neurons in brain regions that are involved in learning and memory processes. The mRNA is enriched in the hippocampus, and in the cerebellum it is restricted to Purkinje neurons. There are similarities to the distribution of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase mRNA (Mailleux *et al.*, 1991). $\text{Ins}(1,4,5)\text{P}_3$ (generated together with DAG by receptor-mediated hydrolysis of inositol phospholipids) and $\text{Ins}(1,3,4,5)\text{P}_4$ are involved in the regulation of calcium flux (Berridge & Irvine, 1989). The various PKC subtypes have a variable but partially overlapping distribution in the brain, with high expression in the hippocampus (Nishizuka, 1988). The distribution of the nervous-system-specific γ -PKC, which is expressed in hippocampus, cortex and Purkinje cells of the cerebellum (Brandt *et al.*, 1987), most closely parallels that of *n*-chimaerin mRNA. Like *n*-chimaerin mRNA, γ -PKC rapidly increases in amount during the postnatal period of synaptogenesis in rat brain (Hashimoto *et al.*, 1988; Yoshida *et al.*, 1988). PKC has been implicated in memory-storage processes in studies using radiolabelled phorbol esters, with distributional changes in the hippocampus (and cerebellar deep nuclei) after discrimination learning (Olds *et al.*, 1989, 1990). Since *n*-chimaerin binds phorbol esters in an analogous manner to PKC (Ahmed *et al.*, 1990, 1991), it may be a factor to consider when evaluating PKC involvement in learning on the basis of phorbol ester binding.

Potential function of *n*-chimaerin

n-Chimaerin is a p21 GAP with neuronal specificity. The PKC-like phorbol ester (DAG)-binding region of *n*-chimaerin is a potential regulator of its GAP activity. This region and/or the *N*-terminal amphipathic helical sequence may mediate membrane interactions. Polyclonal antibodies raised against recombinant *n*-chimaerin recognize both membrane-associated and soluble protein species in the brain (C. Monfries & L. Lim, unpublished work). Other types of GAPs are also present in membrane and soluble fractions (Polakis *et al.*, 1991). p21ras GAPs may have an effector function in addition to p21ras GTPase activation (McCormick, 1989). p120 GAP and NF1 GAP both activate p21ras GTPase, but have different affinities and regulation (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990; Bollag & McCormick, 1991). In contrast with the p21ras superfamily which have conserved structure (Bourne *et al.*, 1991), the various GAPs, including p21ras p120 GAP (Vogel *et al.*, 1988), p21rap1 GAP (Rubinfeld *et al.*, 1991) and the BCR/*n*-chimaerin p21rac GAPs, share little sequence similarity. GAPs active on common substrates, such as p120 GAP and NF1 GAP for p21ras and BCR and *n*-chimaerin for p21rac, have sequence similarity which is limited to their respective catalytic domains and are likely to have different effector or regulatory roles. In addition to its p21rac GAP domain, BCR has serine kinase activity and also a region of sequence similarity to *dbl*, a GTP/GDP exchange protein (Hart *et al.*, 1991; Maru & Witte, 1991). p21rac is expressed in a variety of tissues (Didsbury *et al.*, 1989). Whereas in neutrophils it is involved in NADPH oxidase assembly/activation (Abo *et al.*, 1991; Knaus *et al.*, 1991), a uniquely neuronal function may be subserved by its interaction with *n*-chimaerin (alternatively, a neuronal homologue of p21rac may be the natural substrate.) The p21rho/p21rac/p21cdc42H subgroup of the *ras* superfamily plays a role in cytoskeletal organization in both mammalian and yeast cells (Hall, 1990; Drubin, 1991). It is possible that *n*-chimaerin GAP may have an effector function involving the linkage of receptor-stimulated membrane events generating DAG and p21rac-mediated cytoskeletal activities in neurons.

The support of the Glaxo-Singapore Research Fund and the Brain Research Trust are gratefully acknowledged.

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Received 10 February 1992/1 April 1992; accepted 9 April 1992