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Distribution of Protein Inhibitor of Neuronal Nitric Oxide Synthase in Rat Brain

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In neuronal cells, nitric oxide (NO) is synthesized by neuronal nitric oxide synthase (nNOS) and performs various functions including neurotransmission, modulation of nociception and long-term potentiation and memory. Recently, a novel 89-amino acid protein, designated PIN, has been shown to interact specifically with nNOS and inhibit nNOS dimerization. In this report, we investigated the distribution and the correlation of PIN with that of nNOS in various brain regions of rats. Amplified PIN cDNA from brain tissues revealed an open reading frame which is identical to that of human PIN. Northern blotting of brain RNA with PIN cDNA produced two transcripts, a major 0.9 kb and a minor 2.5 kb. Expression of PIN cDNA as a fusion protein in *E. coli* produced a 10 kDa protein which interacted specifically with pure nNOS in an overlay assay. Immunoblotting of rat brain regions with nNOS antibody demonstrated strong expression in the cerebellum, moderate expression in the cerebral cortex, midbrain, medulla and hippocampus with only weak expression in the spinal cord. By comparison, PIN expression was stronger in the cerebral cortex, midbrain, hippocampus and medulla compared with that of cerebellum and spinal cord. We conclude that PIN interacts strongly with nNOS and is constitutively expressed in various brain regions. The dissimilarity between nNOS and PIN expressions in various brain regions may explain the well known differences in NOS activity between these regions. Our results also suggest that PIN may serve other functions other than nNOS inhibition.

Key Words: **nitric oxide; nervous system; nitric oxide synthases.**

Nitric oxide (NO), a second messenger with a wide biological function such as regulation of vascular tone, synaptic transmission, and immunological responses, is synthesized from L-arginine by a group of hemoproteins known as nitric oxide synthases (NOS)(1). Three isoforms of NOS have been identified so far, the neuronal NOS (nNOS) which was first identified in neuronal cells, the endothelial isoform which was cloned initially from the endothelial cells and the inducible isoform which is expressed in various cells in response to inflammatory cytokines and endotoxin (2).

Early reports on brain NOS expression revealed that nNOS is the main NOS isoform expressed in various regions of the brain (3,4). Bredt *et al.* (5), using NOS activity assay and western blotting, reported that the distribution of nNOS expression is not homogenous in rat and primate brains. The highest NOS activity and nNOS expression occur in the cerebellum and the lowest levels are in the brain stem and cerebral cortex. Subsequent studies indicated that nNOS expression in certain brain regions is involved in numerous physiological processes such as regulation of neurotransmitter release, modulation of nociception and long-term potentiation and memory (6). Moreover, excessive release of NO may play a pathophysiological role in Huntington's chorea, Alzheimer's disease and cerebral ischemia (7).

Recently a novel protein inhibitor of nNOS (PIN) has been identified by interactive cloning (8). Further genetic and biochemical analysis suggests that PIN interacts with a short stretch of amino acids which are unique in the N-terminus of nNOS. Moreover, it has been proposed that PIN functions by interfering with essential dimerization of nNOS monomers and it may actually dissociate previously formed nNOS homodimers (8). PIN has also been cloned from *Drosophila* cells and identified as a highly conserved 89 amino acid-polypeptide with sequences that are similar to the light chain of cytoplasmic dynein (9). Very little is currently known regarding the expression of PIN in various mammalian cells. In fact, the interaction of nNOS and PIN has been described in a single report (8).

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The main aim of this study was to study the distribution and the correlation between PIN and nNOS expressions in various brain regions. We have also cloned and expressed PIN in a bacterial system in order to confirm the nature of the interaction between PIN and nNOS.

METHODS

Animal preparation. Adult male Sprague Dawley rats (250-300 g) were sacrificed by decapitation and tissues were dissected, quickly frozen in liquid nitrogen and stored at −80 °C for later use. Total RNA was isolated from tissues using standard thioguanocyonate phenol/chloroform method (10). One μg of total RNA was reverse transcribed and used for PCR amplification using the following PIN specific oligonucleotides: forward: 5′-ATGTGCGACCGAAAGGCC-GTGATC-3′ and reverse 5′-TTAACCAGATTTGAACAGAAGAAT-GGCC-3′. PCR was carried out at 94° C for 30 sec, 50 °C for 30 sec and 72° C for 30 sec for a total of 30 cycles. The resultant PIN cDNA was cloned into the pCRII.1 vector (TA cloning, Invitrogen).

Northern blot analysis. Thirty μg of total cerebellar RNA was separated by electrophoresis on a formaldehyde-agarose gel, transferred to nylon membrane and challenged with the random primed ^{32}P-labelled PIN cDNA according to the manufacturer's protocol (Promega Inc.). The blot was washed in 0.2 × SSC, 0.2 % sodium dodecyl sulfate (SDS) for 10 minutes at room temperature then at 65 °C for one hour. The membrane was then exposed to X-ray film using an intensifying screen at −70 °C.

Production of glutathione-s-transferase (GST)-PIN fusion protein. The PIN cDNA Sma I- Sma I fragment was subcloned from pCRII.1 vector in both orientations into the unique Sma I site in the polylinker of the bacterial expression vector pGEX-3. DH10B (Pharmacia Inc.) *E. coli* cells were transformed with the pGEX-3-sense PIN, pGEX-3-anti-sense PIN and pGEX-3. The culture conditions to induce the production of GST proteins utilizing IPTG, as well as the purification of the polypeptides was essentially as described by the manufacturer (Pharmacia Inc.). GST was removed from the GST-PIN fusion protein using Factor Xa protease (Pharmacia Inc).

Overproduction of PIN in C2C12 cells. To clone PIN cDNA in mammalian expression vector pTEJ-8 (11), PIN cDNA was reamplified from the cloned PIN cDNA to introduce a unique HindIII site 5′ to the translational start site and a unique BamHI site 3′ to the translational stop site. In addition, a kozak consensus sequence was also introduced. PCR was carried out with 50 ng of pCRII.1-PIN plasmid DNA at 94 ° C for 45 sec, 50° C for 45 sec and 72 °C for 45 sec for a total of 25 cycles with following olignucleotides, forward: 5′-ACGGATCCCCAAGCT-TGCCGCCACCATGTGCGACCGAAAGGCCGT-3′ and reverse: 5′-ACGTGGATCCTTAACCAGATTTGAACAGAAGAATG-3′. The PCR product was directly subcloned into the unique HindIII and BglII sites of pTEJ-8 to create pTEJ-8-PIN. Mouse myoblast C2C12 (ATCC) cells were maintained in a high glucose DMEM media supplemented with 10% fetal calf serum. Cells were transfected to G418 resistance using pTEJ-8-PIN and maintained with 80 μg/ml of G418. For western blotting, cells were washed once with phosphate buffer saline (PBS), scraped using PBS, collected by centrifugation and quickly frozen and stored at −80 °C for further analysis. Proteins were extracted in extraction buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 μg/ml phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin and 1% Triton X-100. The crude homogenates were centrifuged at 4°C for 15 min at 9,000 xg. The supernatant (30 μg) were loaded on 18% Tris-Glycine SDS-polyacrylamide gel and probed with anti-PIN antibody (see below).

Western blot analysis. Frozen brain tissues were homogenized in 6 volumes (w/v) of homogenization buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithioreitol, 1 mg/ml PMSF, 0.32 mM sucrose, 10 μg/ml leuopeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). The crude homogenate was centrifuged at 4°C for 15 min at 3,000 × g. The supernatants (80 μg) were heated for 5 min at 90 °C and then loaded on either 18% (for PIN) or 4-12% (for nNOS) Tris-Glycine SDS-polyacrylamide gels (NOVEX Inc.). Proteins were separated and then electrophoretically transferred onto polyvinylidene difuoride (PVDF) membranes and blocked with 5% non-fat dry milk and subsequently incubated overnight with either polyclonal affinity purified anti-PIN antibody (1:1000)(donated kindly by Dr. S. Snyder)(8) or monoclonal anti-nNOS antibody (1:750)(Transduction Laboratories Inc.). Recombinant PIN expressed in *E. coli* and lysate of pituitary glands were used as positive controls, respectively. Specific proteins were detected using Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence reagents provided with a chemiluminescence kit (ECL, Amersham Canada, Oakville, Ontario). The blots were scanned with an imaging densitometer (Model GS700, Bio-Rad Inc., 12-bit precision and 42 μm resolution) and optical densities of protein bands were quantified with a software (SigmaGel, Jandel Scientific, San Rafael, CA). Predetermined MW standards (Novex Inc.) were used as markers.

Overlay assay. To investigate whether r PIN interacts with nNOS, we used an overlay assay similar to that described by Jaffrey and Snyder (8). Pure nNOS (7.5 μg)(Sigma Chemicals) was dissolved in homogenization buffer (see above), heated for 5 min at 90 °C and then loaded on 4-12% Tris-Glycine SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked with 5% non-fat dry milk and subsequently incubated with 30 μg/ml of either pure PIN protein or pure PIN-GST fusion protein. After washing, the membrane was probed with monoclonal anti-GST antibody (Santa Cruz Biotechnology) and specific proteins were detected with secondary antibody and ECL kit as mentioned above.

RESULTS AND DISCUSSION

Cloning of rat PIN cDNA. RT-PCR on rat tissue RNA with oligonucleotides corresponding to the 5′ and 3′ ends of the coding sequence of rat PIN sequence (8) produced a single band (273 bp). Cloning and sequence of that band revealed an open reading frame of 89 amino acid protein with 100% homology with human homologue of dynein light chain (9). Searching the data bank revealed that a high degree of sequence similarity exists between the coding sequence of rat PIN and that of Mr 8,000 dynein light chain of *D. melanogaster* (8), *Chlamydomonas* (12) and *C. elegans* (13) (figure 1). Probing cerebellar RNA with rat PIN cDNA revealed a major 0.9 kb transcript (empty arrow in figure 2) and a minor transcript of 2.5 kb (filled arrow in figure 2). Although not previously seen in rat tissues, a minor 2.5 kb transcript has been reported to be present in a number of human tissues (9). The relationship of the larger PIN-related transcript to PIN itself is not yet known.

Expression of PIN protein in E. coli. Subcloning of rat PIN cDNA into the bacterial expression vector pGEX-3 in the sense orientation and transforming DH10B *E. coli* strain with that vector produced a prominent 36 kDa polypeptide. *E. coli* transformed with pGEX-3 alone produced a 26 kDa polypeptide representing the known molecular weight of the GST pro-

FIG. 1. Sequence analysis of closely related dynein light chain homolgues. Sequence comparison between human dynein light chain (9,17), rat PIN (8) and dynein light chain homologues from *Drosophila* (9), *C. elegans* (13) and *Chlamydomonas* (12). The alignment was generated by the software DNAMAN (Lynnon BioSoft, Vaudreuil, Quebec). Residues conserved across species are shaded.

tein. No prominent polypeptide was present in the extracts of cells containing the GST-antisense PIN, probably due to the lack of an inframe translational stop site. Western blot analysis of *E. coli* extracts showed that the 36 kDa polypeptide was recognized by the anti-PIN antibody, thereby, indicating that the polypeptide is indeed a GST-PIN fusion protein (figure 3). Anti-PIN antibody did not recognize any polypeptide in extracts prepared from cells expressing the GST alone or the GST-antisense PIN (figure 3).

Figure 4 illustrates the result of overlay assay in which immobilized pure nNOS protein was probed with GST-sense PIN fusion protein or GST alone followed by anti-GST antibody. A prominent band of about 160 kDa was detected when GST-sense PIN fusion protein was used, no band was detected when GST alone was used. These results indicate that GST-PIN fusion protein interacts with nNOS *in-vitro,* whereas no such interaction was detected between pure nNOS and GST alone (figure 4).

Although the anti-PIN antibody detected PIN when fused to GST, it remained to be determined if the antibody could detect mammalian PIN expression. We therefore, established a stable cell line which over express PIN. Western blot analysis detected weak PIN expression in C2C12 cells and more than 5-fold increase in PIN expression in C2C12 cells transfected with PTEJ-8-PIN (figure 5). In addition, pure bacterially produced PIN (obtained by cleaving purified GST-PIN fusion protein at the GST-PIN junction with Factor Xa protease) migrated on SDS-PAGE with the same apparent molecular mass as the mammalian PIN (compare lane 1 with lanes 2 and 3 in figure 5). We, therefore, conclude that the 10 kDa PIN is specifically recognized by the anti-PIN antibody. In addition, these results indicate that PIN does not undergo major post-translational modification in mammalian cells.

Distribution of PIN in rat brain. Figure 6 illustrates a western blot of different rat brain regions probed with anti-PIN antibody. Weak expression was detected in the spinal cord and the cerebellum. By comparison, relatively strong PIN expression was observed in the cerebral cortex, midbrain, medulla and hippocampus (209%, 206%, 200 and 150% of that of the cerebellum, respectively). Probing these areas with anti-nNOS antibody revealed relatively high expression in the cerebellum and weaker expression in the spinal

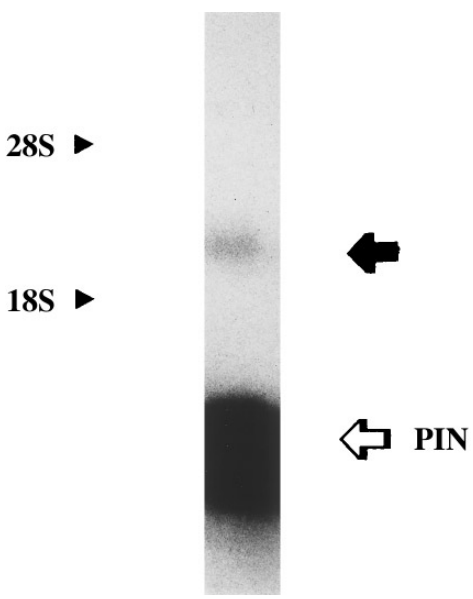

FIG. 2. Northern blot analysis of total cerebellar RNA. Hybridization with PIN cDNA revealed a major PIN transcript (empty arrow) of about 0.9 kb in size. A minor transcript (filled arrow) of about 2.5 kb was also detected.

FIG. 3. Detection of GST-PIN fusion protein with anti-PIN antibody. Extracts of E. coli transformed with pGEX-3 vector (GST), pGEX-3 vector containing full coding sequence of PIN in the sense direction (GST-Sense PIN) or antisense direction (GST-Antisense PIN) were loaded on 4–12% Tris–Glycine SDS–PAGE, transferred to PVDF membrane and probed with anti-PIN antibody.

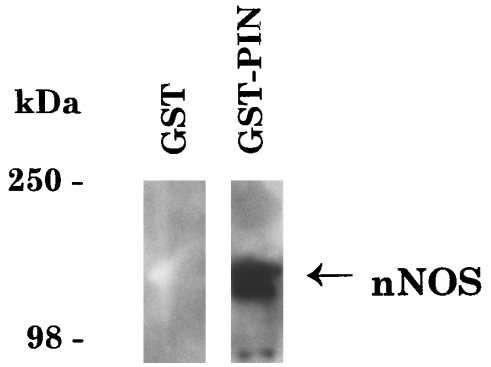

FIG. 4. Overlay assay in which immobilized pure nNOS was probed with GST alone or GST-Sense PIN fusion protein. Notice that nNOS protein was detected when GST-Sense PIN fusion protein was used a probe, whereas no band was detected when GST alone was used.

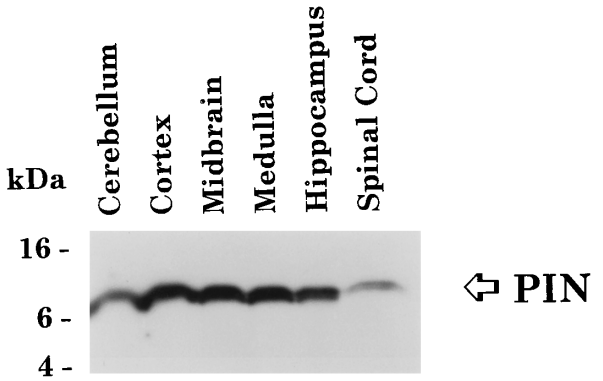

FIG. 6. Distribution of PIN in various rat brain regions. Western blotting with anti-PIN antibody detected weaker expression in the cerebellum and spinal cord compared with other brain regions.

cord (figure 7). Unlike PIN, expression of nNOS in the cerebral cortex, midbrain, medulla and hippocampus was lesser than that of the cerebellum (50%, 52%, 42% and 32% of cerebellum nNOS expression, respectively)(figure 7).

The existence of an endogenous protein which selectively interacts and inhibits nNOS activity was first reported by Jaffrey and Snyder (8). These authors used a yeast two-hybrid screening of rat hippocampal cDNA library to identify a 10 kDa protein, designated PIN which selectively associates with residue 163 to 245 of nNOS. No such interaction was found between PIN and the other two NOS isoforms. *In-vitro* transfection with PIN cDNA revealed that PIN interactions with nNOS results in the destabilisation of nNOS homodimer thereby inhibiting nNOS activity. Little is known about the expression of PIN in mammalian cells. Jaffrey and Snyder (8) cloned PIN from rat hippocampal cDNA library and reported strong mRNA expression in the testes and intermediate levels in the brain, whereas only weak expression in peripheral tissues. Similarly, Dick and colleagues (9) cloned a human homologue of PIN and showed ubiquitous mRNA expression in various human tissues.

In support of the findings of Jaffrey and Snyder, our results indicate that PIN is transcribed in rat brain with a major 0.9 kb transcript PIN which translates to a small protein with an apparent mass of 10 kDa (figures 2 and 6). Moreover, we confirmed that PIN interacts selectively with pure nNOS (figure 4). Additionally, we found that PIN is expressed in all regions of rat brain but to variable degrees and in a fashion which is different from that of nNOS. While nNOS expression is concentrated mainly in the cerebellum, PIN is strongly expressed in the cerebral cortex, medulla, hippocampus and medulla.

The main implication of our finding is that NO production in neuronal cells could be determined not only by factors which activate nNOS such as Ca^{++}, L-arginine and tetrahydrobiopterin concentrations but by the presence of endogenous inhibitor such as PIN. Indeed, the relatively high expression of PIN and low nNOS expression in the cerebral cortex, midbrain and medulla could explain the previously reported low relative NOS activity in these regions compared with that of the cerebellum and hippocampus (5).

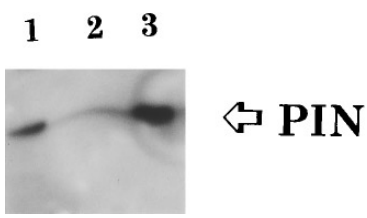

FIG. 5. Overexpression of PIN in C2C12. Western blotting with anti-PIN antibody detected bacterially expressed PIN (lane 1), weak PIN expression in C2C12 (lane 2) and strong PIN expression in transformed C2C12 (lane 3). Notice the similarity in the apparent mass of PIN in the three lanes.

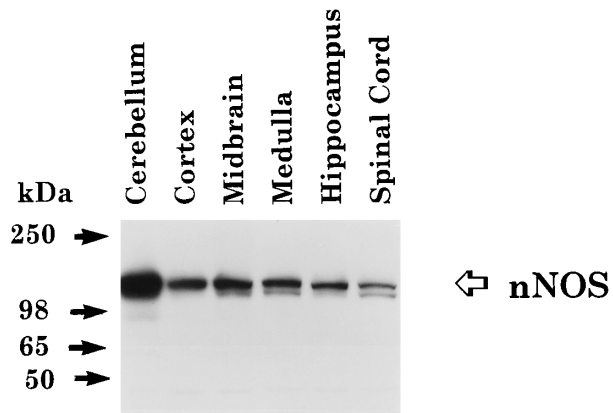

FIG. 7. Western blotting of various brain regions with anti-nNOS antibody. Notice prominent nNOS expression in the cerebellum compared with other brain regions.

Alternative explanation for differences between nNOS and PIN expressions is that PIN may serve other functions in addition to the regulation of nNOS dimerization. The amino acid sequence of rat PIN suggests that it is one of the most conserved proteins in nature and has a high homology with the light chain dynein of *Drosophila* (9), *C. elegans* (13) and *Chlamydomonas* (12). Dyneins are microtubule-based molecule motors which are localized both at the flagellum and cytoplasm (14). In the flagellum, dyneins provide microtubule sliding essential for flagellar bending. Cytoplasmic dyneins are involved in numerous processes such as retrograde transport in axons, subcellular localization of Golgi apparatus, nuclear migration, movement of endosomes and lysosomes and positioning of the mitotic spindle (14).

Unlike the heavy and intermediate chains of cytoplasmic dynein, little is known regarding expression, tissue distribution and functional significance of cytoplasmic light chain dynein. King and Patel-King (12) have recently cloned an M_r 8,000 PIN homologue from the outer arm of *Chlamydomonas* flagella and proposed that this protein along with a closely related protein (M_r 11,000) associate with the intermediate chains at the base of the soluble dynein particle. In *Drosophila*, partial loss of function of PIN homologue (DDLC1) elicited severe pleitropic morphogenetic deficiencies in wing development and female sterility, whereas total loss of function provoked massive apoptosis and embryonic lethality (8). In addition, King and colleagues (15) reported recently that *Chlamydomonas* PIN homologue (dynein light chain M_r 8,000), is expressed in bovine and rat brain and is localized at the cell body and cell processes of mouse oligodendrocytes. These data suggest that PIN may serve both, a structural function as a component of the cytoplasmic dynein and as a regulator of nNOS activity. If true, PIN would be the equivalent of caveolin-1 for endothelial NOS isoform. Recent studies indicate that the structural protein caveolin-1, which is involved in forming cell membrane caveolae, also regulates endothelial NOS isoform activity (16). Clearly more studies are needed to explore the exact nature of PIN function in mammalian cells.

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