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Differential expression of dihydropyrimidinase-related protein genes in developing and adult enteric nervous system

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Abstract Dihydropyrimidinase-related proteins (DRPs) are involved in axonal outgrowth and pathfinding. However, little is known about their significance in the enteric nervous system (ENS), the largest and most complex division of the peripheral nervous system. Using in situ hybridization (ISH) and northern blotting, we examined mRNA expression of DRP-1–4 transcripts in the developing and adult mouse digestive tract and in the adult human colon. ISH detected the mouse DRP-3 transcript in the developing ENS on embryonic day (E)12 and at the later stages as well as in the adult intestine. Mouse DRP-1 and -2 transcripts appeared at E14. DRP-2 transcript was also detected in the adult intestine although DRP-1 expression was lower in the adult. DRP-4 gene was not expressed in the ENS during development or adulthood whereas the signal was apparent in the developing and adult central nervous system (CNS). The DRP expression pattern in the human colon was similar to that of the mouse large intestine. Northern blot analysis showed that DRPs were differentially expressed in the mouse and human intestines, supporting the results of ISH. These data suggest that DRPs play a role not only in the CNS but also in the ENS.

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Introduction

We have recently isolated four members of the dihydropyrimidinase-related protein family (DRP-1, -2, -3, and -4) which were originally identified in human by their homology to dihydropyrimidinase (Hamajima et al. 1996; Wang and Strittmatter 1997; Kato et al. 1998). Non-human counterparts of the human DRPs have been identified and named in relation to the history of their discovery; chicken collapsin response mediator protein (CRMP)-62 (Goshima et al. 1995), rat turned on after division (TOAD)-64 (64 kDa; Minturn et al. 1995), and mouse unc-33-like phosphoprotein (Ulip; Li et al. 1992; Byk et al. 1996, 1998). Based on the comparison of primary structures, these genes were classified into four groups; DRP-1/CRMP-1, DRP-2/CRMP-2/TOAD-64, DRP-3/CRMP-4/Ulip, and DRP-4/CRMP-3 (Hamajima et al. 1996). Amino acid sequences of DRP proteins show 64–79% identity to each other (94–99% interspecies conservation including chicken). CRMP-2 protein was recently reported to be involved in neural development in *Xenopus* (Kamata et al. 1998a) and bovine (Kamata et al. 1998b) nervous systems.

Members of the DRP family, which are intracellular proteins, are involved in the axonal outgrowth and pathfinding through the transmission and modulation of extracellular signals (Goshima et al. 1995; Minturn et al. 1995; Byk et al. 1996). One of the identified extracellular signals is mediated by proteins of the collapsin/semaphorin family which are necessary to create the complex patterns of neuronal connectivity (Minturn et al. 1995; Kolodkin 1996) in collaboration with their receptor, neuropilin (He and Tessier-Lavigne 1997; Kolodkin et al. 1997). Recently, it was shown that neuropilin is expressed in coordinate fashion to DRP-2/CRMP-2 in axonal injury (Pasterkamp et al. 1998).

So far, DRP genes have been studied mainly in the central nervous system (CNS) of the developing embryo (Wang and Strittmatter 1996). Little is known about DRP involvement in the enteric nervous system (ENS) during development and adulthood. Enteric neurons receive a

minority of their innervation from the CNS, are supported by glia instead of Schwann cells, and collagen is excluded from the interior of enteric ganglia. In fact, the structure of the ENS resembles that of the brain more than that of the peripheral nervous system of other regions. Probably the most striking difference between the enteric ganglia and other peripheral ganglia is that only the former are capable of mediating reflex activity in the absence of input from the brain or spinal cord. The independence of the ENS is reflected in both a large size and a complex organization, which includes intrinsic primary afferent neurons, many interneurons, and motor neurons that excite or relax smooth muscle or stimulate epithelial cell secretion (Costa and Brookes 1994; Goyal and Hirano 1996; Kunze and Furness 1999).

We hypothesized that DRPs play a role in the development and functions of the ENS as well as the CNS. In this study, mRNA expression of four proteins of the DRP family was investigated in the ENS of developing and adult mouse using in situ hybridization and northern blot analysis. In addition, the expression of DRPs in the human adult colon was examined.

Materials and methods

Pregnant mice were killed by exposure to ether vapor at 10–16 days of gestation. In each mouse, whole embryos and the digestive tract (small and large intestines) were rapidly removed and immediately frozen on dry ice. Samples of normal adult human colon were obtained in the course of routine resections for cancer and were snap frozen within 30 min of surgical removal. The brain tissues from newborn and adult mice and from adult humans were also obtained as controls. Frozen materials were stored in sealed containers at –80°C. No differences in hybridization were noted between specimens used immediately and those stored. Total RNAs were extracted using the acid guanidium thiocyanate/phenol/chloroform method.

To perform in situ hybridization, 10-μm-thick frozen sections (mouse embryos, adult mouse brain, adult mouse large intestine, and adult human colon) were cut, thaw-mounted onto glass slides, and air dried. Hybridization was carried out as previously described (Kato et al. 1998). Briefly, after being fixed in 4% paraformaldehyde/phosphate buffer for 15 min, sections were predigested with proteinase K solution (10 μg/ml proteinase K, 10 mM TRIS, pH 8.0, and 1 mM EDTA), followed by refixation in 4% paraformaldehyde/phosphate buffer. Sections were incubated in 0.2 N HCl and 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride, then dehydrated and air dried. Eight single-strand RNA probes (each against four DRPs, mouse or human) were labeled with digoxigenin (DIG RNA labeling kit; Boehringer Mannheim, Mannheim, Germany). The probes were diluted in hybridization solution (50% formamide, 10 mM TRIS-HCl, pH 7.6, 200 mg/ml yeast tRNA, 1×Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA, pH 8.0), and the optimal probe concentration was determined by serial dilution. The probes were applied onto the sections, and then clean coverslips were overlaid to spread the hybridization solution evenly. Sections were incubated for 16 h at 62°C in a moisture chamber containing 50% formamide. After hybridization, sections were washed in solution containing 50% formamide and 2×SSC at 62°C for 30 min, and were placed in TNE buffer (500 mM NaCl, 10 mM TRIS-HCl, pH 7.6, 1 mM EDTA) for 10 min at 37°C. Sections were treated with 10 μg/ml ribonuclease A in TNE buffer at 37°C for 30 min and were washed in 2×SSC at 50°C for 20 min, then 0.2×SSC at 50°C for 20 min twice. Hybridized digoxigenin-labeled probes were detected using the anti-digoxigenin–alkaline phosphatase conjugate system (DIG nucleic acid detection kit; Boehringer Mannheim). The sections were counterstained with contrast red (Kirkeguard and Perry Laboratories, Gaithersburg, Md., USA) and mounted in crystal mount (Biomeda, Foster City, Calif., USA). Negative controls consisted of no probe inclusion, prehybridization ribonuclease treatment, and sense probes instead of antisense probes.

In northern blot analysis, total RNAs from adult mouse small and large intestines and adult human colon were denatured using glyoxal, separated on a 1% agarose gel, and blotted onto nylon membranes (Hybond-N; Amersham, Aylesbury, UK). Membranes were prehybridized for 10 min at 65°C in 3×SSC, 20 min at 65°C in 3×SSC and 10×Denhardt's solution, and finally for 30 min at 65°C in 10×Denhardt's solution, 1 M sodium chloride, 50 mM TRIS, 10 mM EDTA, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. The membranes were then hybridized with ^{32}P-labeled mouse or human DRP DNA probes at 65°C for 16–20 h, washed twice for 30 min in 0.1×SSC and 0.1% SDS at 65°C, and exposed to X-ray films at –80°C for 1–5 days with a lightening plus intensifying screen. Negative controls consisted of hybridization with no probes.

Results

In situ hybridization

In developing mouse, no DRP mRNA was detected in the gut on embryonic day (E)10 whereas transcripts of all four DRP members (DRP-1, -2, -3, and -4) were apparent in the CNS, spinal cord, and dorsal root ganglia. The DRP expression in these organs continued from E10 through E16. On E12, DRP-3 transcript first appeared in the enteric ganglia of the immature gut (Fig. 1C). On E14, expression of DRP-1 and -2, as well as DRP-3, was detected in the enteric ganglia of the developing esophagus, stomach, and intestines (Fig. 1A,B,D). DRP-4, still negative at this stage, was not detected at further stages. On E16, a mRNA expression pattern of DRPs similar to that of E14 was observed in each portion of the digestive tract. The DRP transcripts were localized in the ganglia of the myenteric plexuses and not apparent in those of the submucosal plexuses. No signals were detected in the muscular or mucosal layer at any stage. In addition to the ENS and CNS, DRP-1, -2, and -3 were exhibited in the retroperitoneal and mediastinal ganglia on E12 and at later stages, but not in the lungs, cardiac muscle, liver, kidneys, or urinary bladder throughout the stages examined. DRP-4 mRNA was observed only in the CNS, spinal cord and dorsal root ganglia, but not in any other parts of the peripheral nervous system or visceral organs.

In the adult mouse large intestine, expression of DRP-2 and -3 was detected in most of the myenteric neuronal cells and not in the submucosal ganglia (Fig. 2A,B). The mRNA signals were found in the cytoplasm of the neuronal cells with unlabeled areas corresponding to the neuronal cell nuclei. Expression of DRP-1 was very weak and that of DRP-4 was totally negative. No signals were detected in muscular or mucosal layers. In the adult human colon, the DRP mRNA expression pattern was similar to that of adult mouse (Fig. 2C,D), and neither DRP-1 nor -4 mRNA was detected. Muscular or mucosal layers were not labeled. In all experiments, negative con-

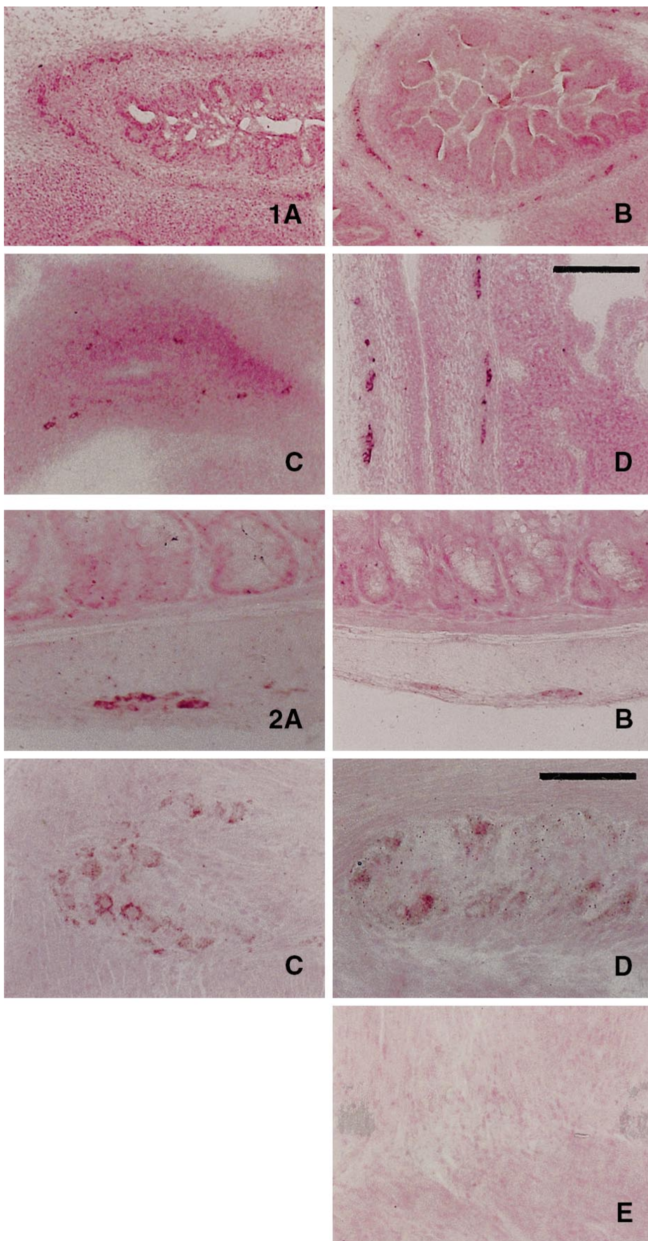

Fig. 1A–D Developmental expression of dihydropyrimidinase-related protein (DRP) transcripts in fetal mouse myenteric plexuses of the digestive tract using non-isotopic in situ hybridization. **A** DRP-1 expression in the myenteric plexuses of the small intestine; embryonic day (E)16. **B** DRP-2 is expressed in the myenteric plexus of the small intestine; E16. **C** DRP-3 signal is detected in the immature gut on E12. **D** DRP-3 expression is displayed in the myenteric plexus of the esophagus; E16. The pulmonary tissue is seen at the *right* of the figure. *Scale bars* 50 μm in **A–D**

Fig. 2A–E Expression of DRP-2 and -3 in the adult mouse large intestine and human colon detected by non-isotopic in situ hybridization. The transcripts are visualized in the neuronal cells of the myenteric plexus with some unlabeled areas occurring over the center of the sectioned cell nuclei. **A** DRP-2 in the mouse large intestine. **B** DRP-3 in the mouse large intestine. **C** DRP-2 in the human colon. **D** DRP-3 in the human colon. **E** Negative control, human colon, DRP-3, sense probe. *Scale bars* 100 μm in **A–E**

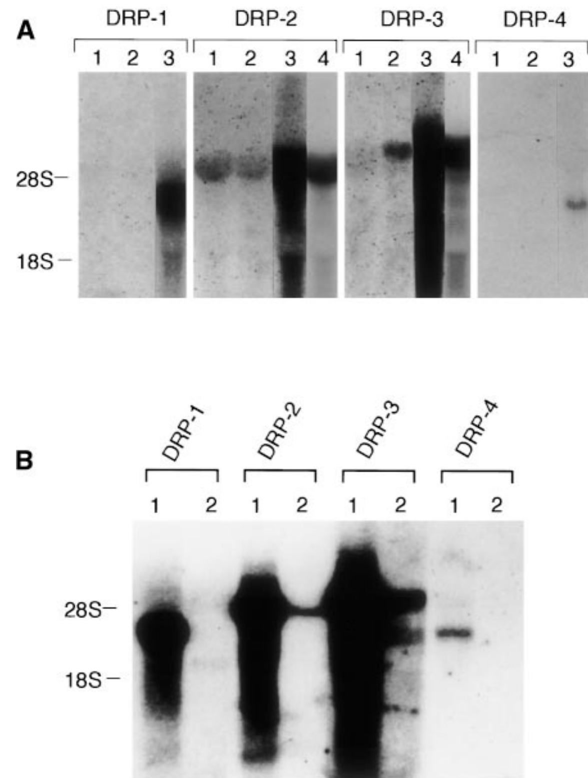

Fig. 3 Northern blot analysis of four DRP genes in the adult mouse intestines (**A**) and adult human colon (**B**). Hybridizations are carried out with ^{32}P-labeled probes specific for each DRP mRNA. **A** Adult mouse: *lane 1* small intestine, *lane 2* large intestine, *lane 3* fetal mouse brain (control), *lane 4* short exposure of lane 3. **B** Adult human: *lane 1* adult human brain (control), *lane 2* human colon. The position of 28 S and 18 S ribosome RNA is shown. The amount of sample RNA is equilibrated by visualizing with ethidium bromide. Note that DRP genes are differentially expressed in the mouse small and large intestines and that the DRP expression in the human colon is similar to that in the mouse large intestine. Estimated lengths of DRP-1–4 transcripts are 3.0, 4.5, 5.8, and 3.0 kb, respectively, and each transcript is similar in size between the mouse and human. The size of human DRP transcripts is previously shown (Hamajima et al. 1996)

trols (no probe inclusion, prehybridization ribonuclease treatment, and sense probe instead of antisense probe) produced no specific signals (Fig. 2E).

Northern blot analysis

DRP-2 and -3 genes were differentially expressed in the mouse intestines (Fig. 3A). In the small intestine, DRP-2 signal was more intense than DRP-3 signal whereas DRP-3 was more intense than DRP-2 in the large intestine. DRP-1 and -4 signals were not detected. In adult human colon, a DRP expression similar to that of the mouse large intestine was obtained; DRP-3 signal was more intense than DRP-2 signal, and DRP-1 and -4 signals were negative (Fig. 3B). The major band of each DRP transcript was identical in size with that of the brain. No probes or hybridization with labeled sense probe yielded any bands.

Discussion

Most features of DRPs characterized to date imply that they have neuron-specific functions: CRMP-62 involvement in collapsin-induced growth cone collapse is evidenced by inhibition of such collapse by introducing anti-CRMP-62 antibodies into neuronal cells (Goshima et al. 1995); expression of TOAD-64 gene and protein is coincident with initial neuronal differentiation and decreases when the majority of axon growth is complete (Minturn et al. 1995); Ulip phosphorylation is regulated by nerve growth factor (Byk et al. 1996); there is differential but nervous system-restricted expression of four CRMP family members (Wang and Strittmatter 1996); and *Caenorhabditis elegans* unc-33, considered to be the nematode homolog of DRP, is involved in the guidance and outgrowth of neurons (Li et al. 1992).

In the present study, DRP family genes were differentially expressed not only in the CNS but also in the ENS. In the mouse ENS, DRP-1 was expressed at the late embryonic stages and reduced in the adult, suggesting a preferential association of DRP-1 during development. The expression of DRP-2 and -3 was detected in adult as well as embryonic ENS. This observation may indicate that these two DRP members are also involved in extra-developmental functions. We speculate that in the adult ENS, DRPs could be associated with control of the motility, exocrine and endocrine secretions, intestinal microcirculation, and immune or inflammatory processes, although the biologic functions of DRPs in the enteric neuronal cells still need to be clarified. DRP-4 was absent from the ENS at all stages whereas it is well expressed in the CNS, indicating that DRP-4 is more associated with the CNS than the ENS. The northern analysis showed that DRP signals were differentially expressed in the adult mouse and human intestinal tissues, supporting the results of the DRP mRNA expression detected by in situ hybridization. DRP-3 signal was more intense than that of DRP-2 in the mouse and human large intestine. Interestingly, the opposite expression pattern was observed in the mouse small intestine, suggesting a tissue-specific expression of DRP family. Expression of DRP genes in the intestines was previously examined by northern blots in chick (Goshima et al. 1995), mouse (Byk et al. 1996), and rat (Wang and Strittmatter 1996). However, no DRP signals were detected. This is probably because the amount of DRP gene transcripts is very small in comparison with those of other tissues, especially the fetal or newborn brain.

A study of the rat CNS (Wang and Strittmatter 1996) using in situ hybridization suggests that DRP-2/CRMP-2 and DRP-4/CRMP-3 are expressed in the adult brain as well as during brain development and that DRP-1/CRMP-1 and DRP-3/CRMP-4 are more stringently regulated during development. DRP/CRMP expression pattern in the mouse ENS analyzed in the present study was somewhat different from that of the rat CNS This discrepancy may be explained by the differences either of the tissues or of the species analyzed. As regards the tissue specificity, we have shown that DRPs are differentially expressed between the mouse small and large intestines. The species is also important in examining the DRP expression; in the rat brain, DRPs are mainly expressed at the late embryonic stage (Wang and Strittmatter 1996) whereas they are detected at the middle stage in the mouse CNS in the present study.

In conclusion, we have shown that DRPs are differentially expressed in the ENS, the largest and most complex division of the peripheral nervous system. It is almost certain from the previous and present studies that DRPs play a role in both central and peripheral nervous systems. However, their involvement is not restricted to the developing and adult nervous systems. We most recently showed that DRP-3 gene was expressed in the postmeiotic spermatids of the adult mouse testis (Kato et al. 1998). This observation suggests that DRPs are also important in extraneuronal cells. Further studies are needed to clarify various DRP functions in the nervous and other systems.

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