HPC-1/Syntaxin-1A Activity in the Enteric Nervous System of Developing Rat Gastrointestinal Tract

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Abstract

The HPC-1/syntaxin-1A antigen was originally identified as a neuron-specific membrane protein in the central nervous system. The presence of HPC-1 antigen in the nervous system of the fetal rat gastrointestinal tract was immunohistochemically demonstrated using the antibody against HPC-1 to clarify the role of this protein in the development of the enteric nervous system. Rat gastrointestinal tract from 14-, 16-, 18-, and 20-day fetuses and adults were immunohistochemically examined for HPC-1 antigen by light microscopy. Acetylcholinesterase (AchE) activity was also examined as a comparison. HPC-1 activity was first detected on 18th day of gestation. AchE activity was first detected at the Auerbach’s plexus of the esophagus on the 16th day of gestation. The presence of HPC-1 in the developing rat intestine revealed that the HPC-1 antigen may be a good indicator for expressing the maturation of enteric nervous system in the development of the enteric nervous system.

Key words: Enteric nervous system, HPC-1 antigen, Immunohistochemistry, rat gastrointestinal tract, development

Introduction

The HPC-1 antigen was originally found to be a neuron-specific protein in the central nervous system (Inoue et al., 1992). This antigen is thought to play important roles in intracellular membrane transport, the exocytotic process of neurotransmitters and also the regulation of neuronal sprouting (Yamaguchi et al., 1996). Immunohistological studies have shown that HPC-1 antigen was widely distributed in rat central nervous system (Akagawa et al., 1990; Barnstable et al., 1983; Inoue et al., 1993), but a detailed histo-cytochemical study of the fetal enteric nervous system has not yet been reported. To clarify the role of HPC-1 antigen in the development of the enteric nervous system, we investigated the presence and cytological distribution of HPC-1 antigen in the enteric nervous system of the fetal rat gastrointestinal tract by immunohistochemical methods. The difference between the central nervous system...
and the enteric nervous system was examined by Western blotting.

**Materials and methods**

**Immunohistochemistry:** Timed pregnant Wistar rats were obtained from Clea Japan Inc. (Tokyo, Japan). Pregnant rats were sacrificed by exposure to ether vapors and the embryos were taken at 14, 16, 18, or 20 days of gestation. The embryo specimens were then fixed in 4% paraformaldehyde for 3 hours and immersed in 20% sucrose for 24 hours at 4°C. The tissue blocks were embedded in OCT compound (Tissue Tek II, Miles, Elkhart, IN, USA) and cryostat sections (4 µm) were collected on slide glasses. The specimens were rinsed in 0.3% Triton ×100 in phosphate-buffered saline (PBS) (0.01 M phosphate buffer (PH 7.3) containing 0.88% NaCl). Endogenous peroxidases were blocked by 0.3% H₂O₂ for 10 minutes. The samples were incubated with rabbit polyclonal antibody against HPC-1 (1: 1,000 dilution) in PBS with 1% normal goat serum. The HPC-1 antibody has been previously characterized (Inoue et al., 1992). After washing in PBS, the samples were stained by the ABC method (Hau et al., 1981). Acetyl-cholinesterase (AchE) was stained by the Karnovsky-Roots method (Karnovsky et al., 1964). Three specimens were prepared for each gestational age studied.

**Immunoblotting:** Tissues from adult rat brain and colon were homogenated in 10 volumes (brain) or 2–3 volumes (colon) of Ca²⁺, Mg²⁺-free PBS with 10 mM phenyl-methyl-sulfonyl-fluoride (PMSF) at 0°C. Nuclear pellets were removed by centrifugation at 3,000 × g for 10 min at 4°C. Membrane fractions were collected by ultracentrifugation at 40,000 × g for 1 hr at 4°C. Protein concentrations were determined by a protein assay kit (Bio-Rad, Hercules, CA, USA). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and visualized by an enhanced chemiluminescence (ECL) system (Amersham International plc., Bucks., U.K.). Antisera were raised in Japanese White rabbits against the fusion protein expressed in E. coli with the exception of the membrane-bound region, as previously reported (Kushima et al., 1995).

**Results**

**Immunohistochemistry (Table 1):** HPC-1 and AchE activities were both absent at 14 days of gestation. The enteric nervous system was first detected by the AchE staining method in the esophagus at 16 days of gestation, but no reaction was observed in the rectum at this stage (Fig. 1A, B). HPC-1 was not detected at 16 days of gestation. HPC-1 was first detected in the esophagus and small intestine of fetal rats at 18 days of gestation, but not in the rectum at that time (Fig. 2A, B). The HPC-1 reactivity became stronger in the rectum compared to that in the small intestine and esophagus at 20 days of gestation (Fig 3). The distribution pattern of the HPC-1 was similar to that of the adult rat enteric nervous system. The surface of the ganglion cells were prominently stained compared to that of the cytoplasm. The two ganglionated plexuses are situated in the submucosa (Meissner’s plexus) and between the layers of the muscle coat (Auerbach’s plexus). But only a dense network of strongly HPC-1-positive
Table 1. HPC-1 Immunoreactivity in the Rat Fetal Intestine

| Gestational age (day) | HPC/AchE   | Esophagus | Small Intestine | Rectum |
|-----------------------|-------------|-----------|-----------------|--------|
| 14                    | HPC-1       | –         | –               | –      |
|                       | Ach-E       | –         | –               | –      |
| 16                    | HPC-1       | –         | –               | –      |
|                       | Ach-E       | +         | ±               | –      |
| 18                    | HPC-1       | +         | +               | –      |
|                       | Ach-E       | +         | +               | –      |
| 20                    | HPC-1       | ±         | +               | +      |
|                       | Ach-E       | +         | +               | –      |

(Abbreviations: –, absent; ±, weak; +, moderate; †, strong; AchE, acetylcholinesterase)

Fig. 1. Light micrograph of AchE staining in the esophagus (A) and rectum (B) at 16 days of gestation. Auerbach’s plexus (arrows) is at the outside of the circular muscle layer at the esophagus but yet of the rectum (brown in original section, black in this illustration). Magnification: A, 400×; B, 200×

Fig. 2. HPC-1 reactivity in the small intestine (A) and rectum (B) at 18 days of gestation. HPC-1-positive nerve fibers are recognized in the Auerbach’s plexus in the small intestine (arrows), but not in the rectum. Magnification: A, 400×; B, 100×
nerve fibers were seen in Auerbach’s plexus. Nerve fibers in Meissner’s plexus formed a less dense network with a similar distribution; the immunohistochemical staining there was weaker.

*Western blot analysis:* Since the HPC-1 antigen was immunohistochemically positive in the

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**Fig. 3.** HPC-1 reactivity in the rectum at 20 days of gestation. Strongly HPC-1-positive stainings are recognized in Auerbach’s plexus (arrowheads). Magnification: 400×.

**Fig. 4.** Western blotting of membrane fraction from adult rat brain and colon. Membrane fractions from the colon and brain were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Two main bands were detected with polyclonal anti-HPC-1 antiserum. With a monoclonal antibody, one band was detected in the brain membrane fraction but two bands were detected in the membrane fraction from the colon.
enteric nervous system in developing and adult rats, further immunoblots were performed (Fig. 4) to confirm the expression of the HPC-1 antigen in the enteric nervous system. Membrane fractions of the cells were immunoblotted and examined with polyclonal and monoclonal antibodies against HPC-1. HPC-1 antigen in the membrane fractions of rat brain tissues was detected as two bands, with the upper band being syntaxin-1B and the lower being HPC-1/syntaxin-1A. HPC-1 antigen in the colon was also visualized as two bands, identical to those of the brain tissue. With the monoclonal antibody 14D8, which is specific for HPC-1/syntaxin-1A (Akagawa et al., 1997), only one band was seen in the membrane fraction of the brain tissue, whereas two bands were detected in the colon.

Discussion

In the present study, the presence of HPC-1 immunoreactivity was demonstrated in almost all components of the enteric nervous system of the fetal rat gastrointestinal tract. It is known that HPC-1 mRNA is present only in neuronal cells and not in glial cells in the rat central nervous system (Inoue et al., 1993). The HPC-1/syntaxin-1A tends to accumulate in synapse-rich regions in the central nervous system (Bennet et al., 1992). In the fetal rat gastrointestinal tract, we observed that the HPC-1-positive nerve fibers were dense in the circular muscle layer of the rectum and stomach (data not shown). The rectum and stomach have thicker muscle layers and more neuromuscular junctions than the other portions of the intestine. This finding suggests that the HPC-1 antigen may play a significant role in synapse formation and/or function in the enteric nervous system. We studied the development of the intramural plexus in rat embryo at different developmental stages using the HPC antibody. For comparison, AchE was studied, since AchE activity is considered to be a marker for identifying premature ganglion cells in Auerbach's plexus. AchE staining is also a good indicator of maturation of the plexus (Ito et al., 1984). Under the same staining conditions as used for adult rat intestine, the first appearance of HPC-1 in the intramural nerve system was observed in the fetal stomach at 18 days of gestation. This is later than the appearance of AchE staining. The HPC-1 appeared later in the small intestine and rectum. Apparent amount of HPC-1 reached maximum at 18 day in the plexus. The distribution pattern was the same as that in the adult rats. These results indicate that the HPC-1 antigen may be a good indicator for expressing the maturation of enteric nervous system in the development of the enteric nervous system. In the immunoblot studies, the polyclonal anti-HPC-1 antibody recognized the two protein bands in the colon, as is the case in the central nervous system. However, the monoclonal antibody revealed only one band in the brain but two bands in the enteric nervous system. At present, we do not know the reason for this discrepancy. The precise physiological roles of the HPC-1 antigen remain to be clarified.

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References

Akagawa K, Takada M, Hayashi H, and Uemura K. (1990) Calcium and voltage-dependent potassium channel in the rat retinal amacrine cells identified in vitro using a cell type-specific monoclonal antibody. Brain Res 518: 1-5.

Akagawa K et al. (1997) J. Molecular Neuroscience: in print.

Barnstable CJ, Akagawa K, Hofstein R, and Horn JP. (1983) Monoclonal antibodies that label discrete cell types in the mammalian nervous system. Cold Spring Harbor Symp. Quant Biol 48: 863-876.

Bennett MK, Calakos N, and Scheller RH. (1992) Syntaxin: A synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257: 255-259.

Inoue A, Obata K, and Akagawa K. (1992) Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. J Biol Chem 267: 10613-10619.

Inoue A and Akagawa K. (1993) Neuron specific expression of a membrane protein, HPC-1: tissue distribution, and cellular and subcellular localization of immunoreactivity and mRNA. Mol Brain Res 19: 121-128.

Ito Y, Sohma S, and Hirano H (1984) Light- and electron-microscopic studies on acetylcholinesterase activity in Auerbach's plexus of developing rat colon. Histochem 81: 209-212.

Hsu SM, Raine L, and Fanger H. (1981) Use of avidin-viotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody(PAP) procedures. J Histochem Cytochem 29: 577-580.

Karnovsky MJ and Roots JL. (1964) A directcoupling triocholine method for cholinesterase. J Histochem Cytochem 12: 219-2218.

Kushima Y, Fujiwara T, Morimoto T, and Akagawa K. (1995) Involvement of HPC1/Syntaxin-1A antigen in transmitter release from PC12h cells. Biochem Biophys Re Comm 212: 97-103.

Yamaguchi K, Nakayama T, Fujiwara T, and Akagawa K. (1996) Enhancement of neurite-sprouting by suppression of HPC-1/syntaxin 1A activity in cultured nerve cells. Brain Research 740: 185-192.

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