Cellular Localization of the Diazepam Binding Inhibitor in Glial Cells with Special Reference to Its Coexistence with Brain-type Fatty Acid Binding Protein

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Summary. The diazepam binding inhibitor (DBI) was originally isolated from the brain as an intrinsic ligand of the benzodiazepine binding site on the type-Á γ-aminobutyric acid receptor (GABAₐ receptor). Its widespread distribution in non-neural tissues outside the brain suggests that DBI has various functions other than GABA-mediated neurotransmission. Since DBI is identical with the acyl-CoA binding protein, which has the ability to bind long chain acyl-CoA esters, the major function of DBI may possibly be related to lipid metabolism. This idea was supported by our previous study showing the consistent coexpression of DBI and fatty acid binding proteins (FABPs) in epithelia throughout the gastrointestinal tract. The present histochemical study focused on the distribution of DBI in neural tissues, and revealed a definite existence of DBI in non-neural supporting cells in both the central and peripheral nervous systems. In the brain, intense immunoreactivity for DBI was detected in the cerebellar Bergmann glia, olfactory ensheathing glia, subgranular layer of the dentate gyrus, and retinal Müller cells. In the peripheral nervous system, satellite cells in sensory/autonomic ganglia, Schwann cells, and sustentacular cells in the adrenal medulla were immunoreactive to a DBI antibody. Moreover, the colocalization of DBI and brain-type FABP (B-FABP) was observed in most of the non-neuronal supporting cells mentioned above, indicating that DBI and B-FABP are cooperatively involved in the energy metabolism of astrocytes and related cells, which are thought to support neuronal development and functions.

The diazepam binding inhibitor (DBI) is a highly conserved 10 kDa cytosolic protein, that was initially isolated from the rat brain as an endogenous ligand of the type α γ-aminobutyric acid receptor (GABAₐ receptor) (GUIDOTTI et al., 1983). It has been found in a large variety of living forms, including yeasts, plants, and invertebrates (KRAGELUND et al., 1999). In the mammalian brain, the localization of DBI has been reported in pyramidal neurons of the cerebral cortex, hippocampal pyramidal cells, and neurons of the reticular thalamic nucleus (COSTA and GUIDOTTI, 1991). DBI has also been demonstrated in astrocytes inhabiting distinct areas of the brain, for example, the Bergmann glia in the cerebellar cortex, astrocytes in the area postrema, and ependymal cells lining the ventricles (COSTA and GUIDOTTI, 1991). DBI has been reported to be an allosteric modulator of GABAₐ receptors in neurons (BORMANN et al., 1985) and a regulator of neurosteroid synthesis in glial cells (COSTA and GUIDOTTI, 1991). As for the peripheral nervous system, BOVOLIN et al. (1990) and ALHO et al. (1991) briefly described the existence of DBI immunoreactivity only in glia-like sustentacular cells of the adrenal medulla. On the other hand, DBI is widely distributed in non-neural tissues such as the liver, intestine, adrenal cortex, testis, ovary, lung, heart, kidney, and spleen, suggesting multiple functions for this peptide (BOVOLIN et al., 1990). KNUDSEN et al. (1989) have demonstrated that DBI is identical with the acyl-CoA binding protein (ACBP), which preferentially binds medium and long chain acyl-CoA esters (ROSENDAL et al., 1993); hence, DBI is believed to play an important role in the intracellular transfer and pool formation of acyl-CoAs (KNUDSEN et al., 2000). Thus, both in neural and non-neural tissues, it is possible to postulate that the major function of DBI may not be GABA transmission.

Fatty acid binding proteins (FABPs) comprise a multigene family of intracellular proteins that participate in the metabolic processing of long chain fatty acids (GLATZ and VAN DER VUSSE, 1996). Nine members of the FABP family have been identified with a
specific tissue distribution (Hertzel and Bernlohr, 2000). Our previous study revealed the coexistence of DBI and FABPs throughout the gastrointestinal tract and discussed the functional relationship between them (Yanase et al., 2001). Here we report on the cellular localization of DBI in the central and peripheral nervous systems mainly by the use of immunohistochemistry, with reference to its colocalization with FABP. Among the nine subtypes of FABP, the present study focused on the brain type (B-FABP), whose expression has been reported to be glia-specific (Kurtz et al., 1994). The distribution and colocalization of DBI and B-FABP in neural tissues indicates a functional cooperation between them, especially in non-neuronal supporting cells.

MATERIALS AND METHODS

Antibody

To produce polyclonal antisera against DBI, mouse DBI was expressed in Escherichia coli, and an emulsion of the purified proteins with Freund's complete adjuvant (Difco, Detroit, USA) was injected intradermally into rabbits followed by booster doses in incomplete Freund's adjuvant at two-week intervals, as described elsewhere (Yanase et al., 2001).

To raise polyclonal antisera against B-FABP, the full coding sequence of rat B-FABP cDNA was expressed as a glutathione S-transferase (GST) fusion protein, using the pGEX-4T-2 plasmid vector (Pharmacia Biotech AB, Uppsala, Sweden). The production and purification of polyclonal guinea pig antisera were done as reported previously (Yamada et al., 2000).

Immunohistochemistry

Under deep pentobarbital anesthesia, adult male ddY mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain, retina with optic nerve, olfactory mucosa, dorsal root ganglia, sciatic nerve, vagal nerve, adrenal gland, and small intestine were dissected out and immersed for an additional 6 h in the same fixative solution. After dipping in 30% sucrose solution overnight at 4°C, tissues were embedded in Tissue-Tec O. C.T. compound (Sakura Finetechical, Tokyo, Japan) and quickly frozen in liquid nitrogen. Tissue sections were cut at a 10 μm thickness on a cryostat, and collected onto slides coated with poly-L-lysine. The sections were pretreated with 0.3% Triton X-100-containing phosphate-buffered saline (PBS, pH 7.4) for 1 h, and with 0.03% H2O2 in methanol for the inhibition of endogenous peroxidase activities.

Immunoreactivity for DBI was detected by the avidin-biotin complex (ABC) method. After treatment with normal goat serum for 30 min, the sections were incubated overnight at room temperature with an affinity-purified rabbit polyclonal antibody for DBI (0.78 μg/ml). The antigen-antibody reactions were detected by incubation with biotin-labeled goat anti-rabbit IgG (Histofine kit; Nichirei, Tokyo), followed by streptavidin-peroxidase complex (VECTASTAIN; Vector Labs., Burlingame, USA). The peroxidase activities were visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine (DAB) and 0.001% H2O2.

Immunostaining for B-FABP was conducted by the indirect immunoperoxidase method. After pretreatment with normal goat serum, the sections were incubated with a guinea pig antibody raised against B-FABP (0.97 μg/ml) overnight at room temperature. They were then incubated with peroxidase-labeled porcine anti-guinea pig IgG (Dako Japan, Kyoto) for 1 h, and the peroxidase activities were visualized by incubation in the Tris-HCl buffer containing DAB and H2O2.

Double immunostaining

Frozen sections, 10 μm in thickness, were prepared from the paraformaldehyde-fixed brain and small intestine as described above. After treatment with normal goat serum, the sections were incubated with the rabbit anti-DBI antibody (3.9 μg/ml), followed by Cy3-labeled donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, USA), and then incubated with the guinea pig anti-B-FABP antibody (1.46 μg/ml), followed by FITC-labeled donkey anti-guinea pig IgG (1:200, Jackson ImmunoResearch). The immunostained sections were observed under a confocal laser scanning microscope (Fluoview, Olympus, Tokyo).

In situ hybridization

Two non-overlapping antisense oligonucleotides were used for each procedure of in situ hybridization. They were complementary to nucleotide residues 81–125 (GACAAGGCGGCTGAGGAGTGAGCCGCT CAAGACTCAAGCCA) and 206–250 (GGACCT CAAGGCGGCAAGCCAAGTGGACCTCGT GGAA ACAAGCTGAA) of mouse DBI cDNA (DBB accession No. X61431), 4110–4115 (TGGGCTTTGCCACT AGGCCAGTGAGGAGAAGTTGACCACAAACAACTG) and 4875–4920 (CGTTTGAGTGGAGACAGACGC TCATTCTGTGAAAGTGGAGATG) of mouse B-FABP cDNA (U04827). These oligonucleotides were labeled with 32P-dATP, using terminal deoxynucleotidyl transferase (Promega, Madison,
USA) at a specific activity of $0.5 \times 10^6$ dpm/µg DNA.

After sacrifice with a lethal dose of pentobarbital anesthesia, the brain, dorsal root ganglia, and small intestine of the male ddY mice were quickly removed. Fresh-frozen sections, 10–14 µm in thickness, were prepared and mounted on glass slides precoated with 3-amino-propyltriethoxysilane. They were fixed with 4% paraformaldehyde for 10 min and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0).

The sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4 × SSC (1 × SSC; 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 200 µg/ml tRNA, 1 mM EDTA and 10% dextran sulfate. Hybridization was performed at 42°C for 10 h in the hybridization buffer supplemented with 10,000 cpm/µl of 35S-labeled oligonucleotide probes. The slides were washed at room temperature for 20 min in 2 × SSC containing 0.1% sarkosyl and twice at 55°C for 40 min in 0.1 × SSC containing 0.1% sarkosyl. The sections were either exposed to Hyperfilm-β max (Amersham, Buckinghamshire, England) for two weeks, or dipped in Kodak NTB2 nuclear track emulsion and exposed for two months.

RESULTS

Immunohistochemical staining revealed that the immunoreactivity to the DBI antibody was preferentially localized in the cytoplasm of astroglial cells in several areas of the brain. In the cerebellum, Bergmann glia showed the most intense immunoreactivity for DBI over the entire length, making their characteristic processes (Bergmann fibers) extending towards the brain surface clearly visible (Fig. 1a). The DBI immunoreactivity was also found in the astrocytes dispersed in the granular layer of the cerebellar cortex, cerebellar medulla, cerebral cortex, and olfactory bulb. The olfactory ensheathing glia in the first two layers of the olfactory bulb, i.e.,
the olfactory nerve layer and glomerular layer, displayed an intense immunoreactivity to the DBI antibody (Fig. 2a). In the hippocampus, the cellular lining at the bottom of the granular layer in the dentate gyrus was immunoreactive to the DBI antibody (Fig. 2c). The immunoreactivity was highly concentrated in the ependymal cells of the ventricles and moderately so in the choroid plexus (Fig. 2e). In the retina, Müller's supporting cells and pigment epithelial cells showed the immunoreactivity (Fig. 2g).

In the peripheral nervous system, an intense immunoreactivity to the DBI antibody was detected in satellite cells of the dorsal root ganglia (Fig. 3a) and of submucosal/myenteric nerve plexuses in the small intestine (Fig. 1d). In the adrenal medulla, DBI immunoreactivity was detected in stellate-shaped sustentacular cells of glial origin (Fig. 3c). Endocrine cells in the adrenal cortex stained positively, while medullary chromaffin cells were free of the DBI immunoreactivity, as reported previously (BOVOLIN et al. 1990) (Fig. 3c). Schwann cells were positively immunostained in the dorsal root (Fig. 3a), sciatic nerve (Fig. 3e), vagal nerve, optic nerve, olfactory nerve (Fig. 3g), and intramural nerve plexuses in the small intestine.

Double immunostaining of single sections with antibodies against DBI and B-FABP as well as alternative staining of serial sections with either of the two antibodies revealed that both immunoreactivities were frequently but not invariably colocalized in the central and peripheral nervous systems. In the cerebellar cortex, Bergmann glia showed intense immunoreactivities to DBI and B-FABP antibodies (Fig. 1a–c). Both immunoreactivities were also localized in the olfactory ensheathing glia (Fig. 2a, b) and cellular lining in the subgranular layer of the dentate gyrus (Fig. 2c, d), while no immunoreactivity for B-FABP was detectable in glial cells of the cerebellar medulla, ependyma/choroid plexus (Fig. 2f) or retinal Müller cells/pigment epithelial cells (Fig. 2h). In the peripheral nervous system, satellite cells in the dorsal root ganglia (Fig. 3a, b) and submucosal/myenteric nerve plexuses of the small intestine (Fig. 1d–f) showed both DBI and B-FABP immunoreactivities. In the adrenal medulla, sustentacular cells were stained equally with both antibodies (Fig. 3c, d). On the other hand, Schwann cells in the vagal nerve, sciatic nerve (Fig. 3e, f), optic nerve, and dorsal root (Fig. 3a, b) showed only DBI immunoreactivity, and were devoid of immunoreaction for B-FABP. Schwann cells in the olfactory nerve (olfactory ensheathing glia) were an exceptional case, being doubly positive for DBI and B-FABP (Fig. 3g, h).

The specificity of immunoreactions for DBI and FABP were confirmed according to a conventional procedure, including an absorption test. Moreover, in situ hybridization using antisense probes could detect the selective expression of DBI and FABP mRNAs in the brain (Fig. 4a, b) and peripheral nervous system. When sections from the dorsal root ganglia and small intestine were hybridized with antisense probes specific for either DBI or FABP, both signals were localized in small cells surrounding the neuronal somata (Fig. 4c, d).

**DISCUSSION**

DBI was first isolated from the rat brain as an intrinsic modulator of the GABA_A receptor (GUIDOTTI et al., 1983), but its broad existence in various nonneural tissues has suggested multiple biological actions of DBI apart from GABA_A-mediated neurotransmission (BOVOLIN et al., 1990). One of the major organs for DBI production outside the brain is the gastrointestinal tract. Our previous study demonstrated its consistent distribution in epithelia from the oral cavity to the anus (YANASE et al., 2001), in contrast to the reported specialized DBI regulation of secretion of cholecystokinin and insulin from gastrointestinal endocrine cells (CHEN et al., 1988; LI et al., 1999). Another representative organ for DBI synthesis is steroidogenic tissue, including fat tissue and steroid hormone-secreting cells (GRAY et al., 1986; TRANZO et al., 1994). *In vitro* studies using these tissues have shown that DBI regulates steroid biosynthesis through peripheral-type benzodiazepine receptors on the mitochondrial membrane (YANAGIBASHI et al., 1988; BESSMAN et al., 1989; PAPADOPOULOS et al., 1991). On the other hand, KNUDSEN et al. (1989) have demonstrated that DBI is identical with the acyl-CoA binding protein, which binds medium and long chain acyl-CoA esters with high affinity (KNUDSEN, 1991), and DBI is believed to play a significant role in intracellular acyl-CoA transfer and pool formation (KNUDSEN et al., 1999). Acyl-CoA esters, which are substrates and intermediates in the lipid metabolism, also regulate various cellular functions, including enzyme activity, cell signaling, and gene expression (FÆRGEN and KNUDSEN, 1997). DBI may thus be a key substance for the precise understanding of numerous acyl-CoA-mediated intracellular phenomena.

Previous histochemical studies on DBI in the brain have reported its localization in the pyramidal cells of the cerebral cortex, hippocampal pyramidal cells, and neurons of the reticular thalamic nucleus, as well as in the glial cells of the cerebellar cortex, including the Bergmann glia, and those in the area postrema.
Fig. 2. Immunostaining of DBI and B-FABP in the olfactory bulb (a, b), dentate gyrus of the hippocampus (c, d), ependyma/choroid plexus (e, f), and retina (g, h). The ensheathing glia of the olfactory bulb are stained with both antibodies (a, b). Double-positive cells for DBI and B-FABP line the subgranular layer of the dentate gyrus (c, d). DBI immunoreactivity is intense in the ependyma, and moderate in the choroid plexus (e), whereas B-FABP immunoreactivity is almost negative in both structures (f). Retinal Müller cells and pigment epithelium also show a single immunoreactivity for DBI (g, h). Bar: 20 μm (a, b, e, f), 50 μm (c, d, g, h)
Fig. 3. Legend on the opposite page.
The present study showed the selective expression of DBI in particular glial cells. The immunoreactivity was especially intense in cerebellar Bergmann glia, olfactory ensheathing glia, and retinal Müller cells, whereas none of the neurons throughout the brain contained the immunoreactivity. DBI was immunostained also in the cellular lining of the subgranular layer of the dentate gyrus, which is thought to comprise neural stem cells (PALMER et al., 1997). In the peripheral nervous system, satellite cells in sensory/autonomic ganglia, Schwann cells, and sustentacular cells in the adrenal medulla were immunolabeled with the DBI antibody. BOVOLIN et al. (1990) and ALHO et al. (1991), who immunohistochemically examined the distribution of DBI in peripheral tissues, recognized DBI immunoreactivity only in adrenomedullary sustentacular cells. It was concluded that the distribution of DBI was confined predominantly to glial and related cells in neural tissues, indicating its importance in regulating the function and metabolism of glial cells or glia-mediated neuronal activities.

The broad colocalization of DBI and B-FABP in the present study further implies their intimate functional relationship in neural tissues. In the brain, astrocytes in several regions, represented by cerebel-

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**Fig. 4.** Detection of DBI and B-FABP mRNAs in the sagittal sections of the brain (a, b, X-ray film) and in the dorsal root ganglia (c, d, bright-field micrograph). a and b. The olfactory bulb and cerebellum show the intense expression of DBI (a) and B-FABP (b) mRNAs. Less intense but significant signals for both mRNAs are also seen in the hippocampus including the dentate gyrus (arrows). c and d. In the dorsal root ganglia, intense signals for DBI (c) and B-FABP (d) mRNAs are found to be localized in the satellite cells around neuronal soma, displaying a ring structure. Bar: 1 mm (a, b), 50 μm (c, d)
lar Bergmann glia, possessed both DBI and B-FABP. In the peripheral nervous system, their coexpression was observed in non-neuronal supporting cells, including satellite cells of sensory ganglia and enteric nerve plexuses, and sustentacular cells of the adrenal medulla. Many previous studies have suggested the function of B-FABP during the development of the central nervous system (FENG et al., 1994; KURTZ et al., 1994; OWADA et al., 1996). In the adult mouse brain, B-FABP is distributed in the olfactory bulb and in the subgranular layer of the dentate gyrus (KURTZ et al., 1994), as confirmed by the present study, where neurons are featured in their permanent turnover throughout life (KUIN et al., 1996; KATO et al., 2001). In such germinal zones, the coexistence of DBI and B-FABP has been recognized specifically in non-neuronal supporting cells and not in the differentiated neurons themselves. Recent studies have raised the possibility that a metabolic neuron-glial relationship is fundamental in both the developing and adult brain, through the trophic or regulatory guidance of glial cells for neuronal activities. One good example is the supply of the non-essential amino acid L-serine, which plays an essential role in neuronal development and function. The initial enzyme for de novo biosynthesis of L-serine, 3-phosphoglycerate dehydrogenase, is preferentially expressed in glial cells associated with active neurons, for example, Bergmann glia and olfactory ensheathing glia (FURUYA et al., 2000; YAMASAKI et al., 2001). Taking the definite DBI/B-FABP colocalization in the non-neuronal supporting cells into consideration, it is assumed that DBI and B-FABP are cooperatively involved in the energy metabolism in astrocytes or related cells, which are thought to support neuronal development and function under physiological and pathological conditions.

Our previous study demonstrated the colocalization of DBI with other members of the FABP family in various non-neuronal tissues (YANASE et al., 2001). DBI in gastric epithelial cells, intestinal epithelial cells, hepatocytes and adipocytes was seen to coexist with epidermal (E), intestinal (I), liver (L), and adipocyte (A)-FABP, respectively. The present study failed to show the colocalization of DBI and B-FABP in Schwann cells, ependyma, and retinal Müller cells/pigment epithelium. It is believed that Schwann cells in myelinated nerve fibers contain a myelin-type FABP as a substitute for B-FABP. Other DBI-immunoreactive cell elements, which lacked B-FABP, may contain other known or unknown subtypes of FABP. Long chain fatty acids and their acyl-CoA derivatives are required for membrane synthesis and energy production through highly regulated metabolic pathways. The widespread and constant colocalization of DBI and FABPs suggests that they cooperate in various intracellular fundamental functions, and that the specialization of their cooperative functions might be decided by the tissue-specific expression of FABP subtypes.

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