

# Developmentally and Spatially Regulated Expression of HNK-1 Carbohydrate Antigen on a Novel Phosphatidylinositol-anchored Glycoprotein in Rat Brain

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**Abstract.** HNK-1 carbohydrate antigen is an epitope expressed commonly in many cell surface adhesion and recognition molecules in the nervous system. We purified and characterized from rat brain a novel phosphatidylinositol (PI)-anchored 150-kD glycoprotein belonging to the HNK-1 family. The molecule (PI-GP150) was detected by combination of PI-specific phospholipase C treatment of brain membranes and Western blot analysis with mAb HNK-1. HNK-1-positive PI-GP150 was purified from the PI-PLC-released materials with three successive chromatographies (Sephacryl S-300, mAb HNK-1-Sepharose 4B, and Mono Q) and proven to be a novel molecule by immunoblot and structural analyses. Polyclonal antibody was raised against PI-GP150 and used to show that (a) PI-GP150 is expressed on the surface of neuronal cell bodies and their processes in culture, and (b) PI-GP150 appears during embryonic development and is present throughout all postnatal life in all brain regions. However, the expres-

sion of the HNK-1 epitope on PI-GP150 is regulated in both developmental stage-specific and region-specific manners. In newborn rats, the HNK-1 epitope is expressed on PI-GP150 throughout the brain. The level of HNK-1 epitope on PI-GP150 decreases after postnatal day 7 in hindbrain and becomes completely absent in adult myelencephalon and metencephalon. In contrast, HNK-1 epitope on PI-GP150 was constitutively expressed in telencephalon. Thus, while the HNK-1 carbohydrate epitope is strongly coupled to PI-GP150, its expression can be regulated independently of that of PI-GP150. The differential expression of the HNK-1 epitope at different rostro-caudal axial levels was observed also in other HNK-1 family molecules in brain membranes. These results suggest that the HNK-1 epitope plays an important role in adding region-specific and developmental stage-specific modifications on the function of the cell surface molecules.

**D**URING development, construction of the central nervous system is controlled by an intricate program of gene expression, which leads to a precisely timed and spatially localized appearance and disappearance of a vast number of molecules. Such molecules function in a variety of developmental events including neurogenesis, neuronal migration, axonal elongation and fasciculation, and synapse formation. Among them, cell surface adhesion and recognition molecules play key roles in the formation of complicated but highly ordered neural networks. It is, therefore, of fundamental importance to study variations in the expression of cell surface molecules from one brain region or developmental stage to another. Recently, a growing number of neural adhesion molecules have been identified and characterized in the nervous system. Most of them are classified into three groups on the basis of structural homology: Ig superfamily (Williams and Barclay, 1988; Hunkapillar and Hood, 1989;

Yoshihara et al., 1991), cadherin family (Takeichi, 1988), and integrin family (Ruoslahti, 1988).

Carbohydrate moieties expressed on the cell surface molecules may play crucial roles in cell-cell adhesion and recognition processes. Above all, HNK-1, a carbohydrate epitope defined by a mAb HNK-1 (Abo and Balch, 1981), has been found on a large number of neural adhesion molecules. They include myelin-associated glycoprotein (MAG)<sup>1</sup> (McGarry et al., 1983), neural cell adhesion molecule (N-CAM), L1 (Kruse et al., 1984), transiently-expressed axonal glycoprotein-1 (TAG-1) (Dodd et al., 1988), F3/F11 (Rathjen et al., 1987; Gennarini et al., 1989b), and P<sub>0</sub> (Bollensen and Schachner, 1987), all of which belong to the Ig superfamily. In addition, the HNK-1 epitope is present in fibronectin receptor  $\alpha$ -subunit (Pesheva et al., 1987), J1/tenascin (Kruse et al., 1985) and several proteoglycans (Hoffman and Edelman, 1987; Gowda et al., 1987), all of which are believed

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1. *Abbreviations used in this paper:* MAG, myelin-associated glycoprotein; N-CAM, neural cell adhesion molecule; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; TAG-1, transiently expressed axonal glycoprotein-1.

to be involved in cell-substratum interactions. The structure of the HNK-1 epitope has been determined as a 3-sulfated glucuronyl-substituted lactoseries oligosaccharide, an unusual carbohydrate structure in glycoproteins (Chou et al., 1986; Ariga et al., 1987). Several studies suggest that this carbohydrate structure is an important key in cell-cell interactions at various developmental stages in the nervous system (Riopelle et al., 1986; Schwarting et al., 1987; Mikol et al., 1988; Kunemund et al., 1988; Hynes et al., 1989; Jessell et al., 1990).

It has been reported that only a subpopulation of N-CAM expresses the HNK-1 epitope, while the rest of them lack this epitope (Kruse et al. 1984). This is also the case for MAG (Kruse et al. 1984; Poltorak et al. 1987), L1 (Faissner, 1987), and P<sub>0</sub> (Burger et al. 1990). The regulatory mechanism of expression of HNK-1 epitope on a specified molecule is poorly understood. In other words, it is still unclear at which developmental stage, in which brain region, and on what type of cell the HNK-1 epitope on a specified molecule is expressed. Immunohistochemical studies have been performed for the distribution of HNK-1 antigen in the nervous system (Schuller-Petrovic et al., 1983; Schwarting et al., 1987; Yamamoto et al., 1988), but it was impossible to look at the spatial expression of HNK-1 epitope on a specified protein because of a technical problem; that is, the HNK-1 epitope is present on a number of molecules, not only glycoproteins, but also proteoglycans and glycolipids. In the present study using the combination of membrane digestion by phosphatidylinositol-specific phospholipase C (PI-PLC) and Western blot analysis with mAb HNK-1, we were successful in surveying the regional distribution and developmental change of HNK-1 expression on a novel PI-anchored 150-kD membrane glycoprotein, PI-GP150.

## Materials and Methods

### Antibodies

The hybridoma clone secreting mAb HNK-1 (ATCC TIB 200 cells) was obtained from the American Type Culture Collection (Bethesda, MD). The hybridoma culture supernatants were used without purification in Western blot analysis. For preparation of immunoadfinity resin, mAb HNK-1 was partially purified by ammonium sulfate precipitation from ascites of hybridoma-injected mice (BALB/c).

Polyclonal antibody recognizing rat L1 was a generous gift from Dr. Hiroaki Asoh (Keio University, Tokyo, Japan). mAb 4D7 against rat TAG-1 was kindly donated by Dr. Miyuki Yamamoto (Tsukuba University, Ibaragi, Japan). mAb against N-CAM was purchased from Sigma Chemical Co. (St. Louis, MO).

### Preparation of Rat Brain Synaptosomal Membranes

Wistar rats ranging in age from embryonic day 16 to postnatal day 80 were sacrificed, and their brains were removed and immersed in ice-cold 0.32 M sucrose containing 5 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.1 mM leupeptin, 0.1 mM *p*-aminophenylmethane sulfonyl fluoride, and 5  $\mu$ M pepstatin A (buffer A). Five brain segments or several regions were dissected: telencephalon (olfactory bulb, cerebral neocortex, hippocampus, striatum, and amygdala), diencephalon (thalamus and hypothalamus), mesencephalon (midbrain), metencephalon (cerebellum and pons), and myelencephalon (medulla oblongata).

The dissected tissues were homogenized in 10 vol of buffer A with a Teflon-glass homogenizer. The homogenates were centrifuged at 1,000 *g* for 10 min, and the supernatant was further centrifuged at 10,000 *g* for 20 min. The pellet (crude synaptosomal fraction) was lysed in 10 mM Tris-HCl (pH 7.4) containing the enzyme inhibitors listed above (buffer B). After centrifugation at 30,000 *g* for 20 min, the synaptosomal membranes were suspended in buffer B, preincubated at 37°C for 30 min, and then recentrifuged. This washing procedure was repeated three times to remove materials spontaneously released from the membranes.

## PI-PLC Treatment of Brain Synaptosomal Membranes

In the standard experiment, the membranes (1.0 mg protein) prepared as described above were incubated in the presence or absence of 0.2 unit of PI-PLC from *Bacillus thuringiensis* (Sapporo Breweries Limited, Sapporo, Japan) in 200  $\mu$ l of buffer B. After incubation at 37°C for 30 min, the soluble and particulate fractions were separated by centrifugation at 100,000 *g* for 10 min.

## Electrophoresis and Western Blot Analysis

The supernatant fractions containing PI-PLC-released materials were concentrated with Centricon 30 (Amicon Corp., Danvers, MA). The membrane fractions were solubilized with 1% SDS. They were subjected to electrophoresis on 4–20% SDS-polyacrylamide gradient gels and blotted onto nitrocellulose sheets (Burnette, 1981). The remaining protein-binding capacity of the nitrocellulose was blocked for 30 min at room temperature with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20 (TTBS) containing 3% bovine serum albumin. Then, the blots were incubated with the first antibody for 1 h at room temperature or overnight at 4°C. After three 10-min washes with TTBS, the blots were incubated with the peroxidase-labeled second antibody and then processed with an ABC kit (Vector Laboratories, Inc., Burlingame, CA) using the avidin-biotin-peroxidase complex method (Hsu et al. 1981). The peroxidase products were visualized by immersion of the blots for 1–5 min in 0.05% 4-chloronaphthol and 0.01% H<sub>2</sub>O<sub>2</sub>.

For analysis of lectin binding properties of PI-PLC-released proteins, the blots were incubated with biotinylated lectins (Vector Laboratories, Inc.) and processed with the ABC kit. Tested lectins were erythroagglutinating phytohemagglutinin (PHA-E), leucoagglutinating phytohemagglutinin (PHA-L), Con A, wheat germ agglutinin (WGA), *Dolichos biflorus* agglutinin (DBA), peanut agglutinin (PNA), *Pisum sativum* agglutinin (PSA), *Ricinus communis* agglutinin I (RCA I), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA I), and *Vicia villosa* agglutinin (VVA), all of which were purchased from Vector Laboratories, Inc.

## Preparation of mAb HNK-1-Sepharose 4B

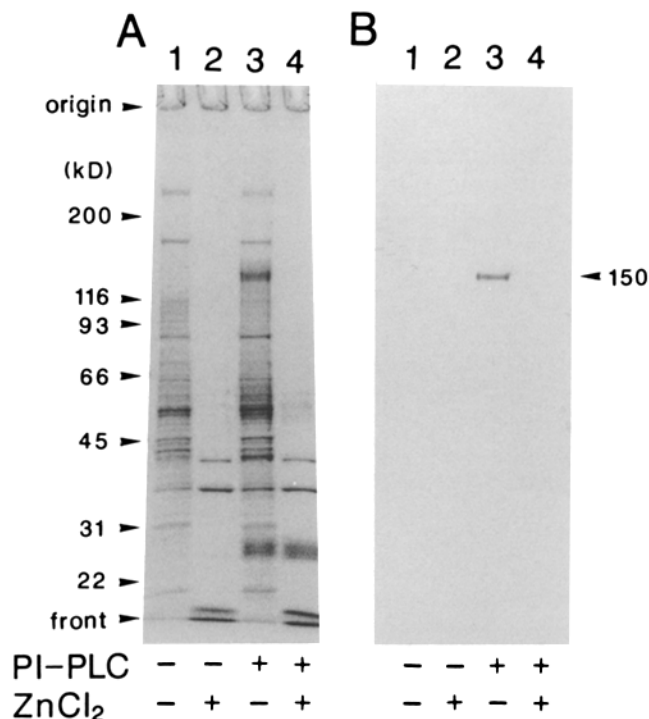
As the class of mAb HNK-1 is IgM (Abo and Balch, 1981), the usual procedures for coupling of antibody to protein A-Sepharose could not be used in this case. Therefore, anti-mouse IgM ( $\mu$  chain-specific, goat IgG fraction, 100 mg; Cappel Laboratories, West Chester, PA) was first coupled to activated CH-Sepharose 4B (20 ml; Pharmacia, Uppsala, Sweden) in 0.1 M NaHCO<sub>3</sub> (pH 8.0) containing 0.5 M NaCl at 4°C for 4 h. Then, the mAb HNK-1 partially purified from ascites of hybridoma-injected mice by precipitation with 50% ammonium sulfate was mixed with the anti-mouse IgM-Sepharose 4B in 0.1 M sodium borate (pH 8.0) and cross-linked with 0.2 M triethanolamine (pH 8.2) containing 20 mM dimethyl pimelimidate dihydrochloride (Pierce Chemical Co., Rockford, IL) according to the method of Schneider et al. (1982).

## Purification of HNK-1-positive PI-GP150

PI-PLC-released materials (100 ml) from 30 rat brain crude synaptosomal membranes were concentrated to 5 ml with Centriprep 30 (Amicon Corp.) and applied onto a Sephacryl S-300 gel filtration column (4 × 120 cm; Pharmacia) preequilibrated with 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. 5 ml fractions were collected, and aliquots were analyzed by SDS-PAGE followed by Western blotting with mAb HNK-1. Immunoreactive fractions were pooled, desalted with Centriprep 30, and incubated with 1.2 ml of mAb HNK-1-Sepharose 4B in 10 mM Tris-HCl (pH 7.4) at room temperature with gentle shaking. After 60 min, the material was packed in a disposable gravity column, washed exclusively with the same buffer, and finally eluted with 10 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl. The eluate, which contained most of the HNK-1 immunoreactivity, was finally applied to a Mono Q HR 5/5 anion exchange column (5 × 50 mm; Pharmacia) after having been desalted with Ampure™ SA (Amersham Corp., Arlington Heights, IL). The adsorbed materials were eluted with a linear gradient of NaCl from 0 to 1 M. The final eluate contained highly purified PI-GP150.

## Production of Polyclonal Antibody against HNK-1-negative PI-GP150

Polyclonal antibody was directed against HNK-1-negative PI-GP150 in the pass-through fractions of mAb HNK-1 Sepharose 4B immunoadfinity chro-



**Figure 1.** Analysis of PI-PLC-released materials from rat brain synaptosomal membranes. Membranes were prepared from adult rat brain and incubated at 37°C for 30 min in buffer B in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PI-PLC. After centrifugation, the supernatant was subjected to SDS-PAGE, and the gels were either stained with CBB (A) or transferred to nitrocellulose and probed with mAb HNK-1 (B). In lanes 2 and 4 4 mM ZnCl<sub>2</sub> (an inhibitor of PI-PLC) was added to the incubation buffer. Molecular mass standards (in kilodaltons) are indicated by arrowheads.

matography. The 150-kD protein in fractions 1–3 (Fig. 3) was excised from the polyacrylamide gel and electroeluted with a Centrifuter apparatus (Amicon Corp.). A Japanese white female rabbit was immunized subcutaneously at multiple sites with 30 µg of electroeluted 150-kD protein at fortnightly intervals over 2 mo. Complete Freund's adjuvant was used for the first injection, and incomplete adjuvant was used for the second and third injections. For the last immunization, 20 µg of purified protein without adjuvant was injected intravenously and 30 µg subcutaneously. Blood was collected 10 d after the last injection.

### PI-PLC Treatment of Brain Primary Cultures and Immunofluorescence Labeling

Primary cultures of neurons and glial cells were prepared from telencephalon or cerebellum of postnatal day-5 rat according to Saji and Miura (1982). They were plated onto 6-well plates (Coaster, Cambridge, MA) or Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) precoated with 2.5 mg/ml poly-D-lysine, maintained in DME supplemented with 10% FCS under 5% CO<sub>2</sub>, and used 2–7 d after plating.

On the day of the experiment, the plates were centrifuged at 1,500 rpm for 10 min, and the culture medium was discarded. PI-PLC (0.1 U/ml) in DME without FCS was added to the cells and incubated at 37°C for 30 min. After incubation, the medium and cells were separated by centrifugation and subjected to electrophoresis followed by Western blot analysis with mAb HNK-1 as described above.

Primary cultures of neurons and glial cells on chamber slides were treated with either vehicle or PI-PLC for 1 h at 37°C. Cells were washed twice with TBS, fixed with 3% paraformaldehyde in TBS for 15 min, and incubated with rabbit anti-PI-GP150 antiserum diluted 1:1,000. Bound antibodies were detected by FITC-conjugated goat anti-rabbit IgG (Caltag, South San Francisco, CA). The coverslips were mounted on chamber slides

in TBS/glycerol (1:9 [vol/vol]) and the cells were examined under an epifluorescence microscope.

## Results

### Release of HNK-1-immunoreactive 150-kD Glycoprotein from Rat Brain Membranes by PI-PLC

Incubation of adult rat brain crude synaptosomal membranes with PI-PLC from *Bacillus thuringiensis* resulted in the release of several proteins into the medium, as assessed by Coomassie brilliant blue staining after SDS-PAGE (Fig. 1 A, lane 3). When the proteins in another gel were transferred to a nitrocellulose membrane and processed with mAb HNK-1, a strongly positive band was detected at 150 kD (Fig. 1 B, lane 3). This HNK-1 immunoreactive protein was released exclusively by PI-PLC, and the release was completely abolished by the addition of ZnCl<sub>2</sub>, an inhibitor of PI-PLC (Fig. 1 B, lane 4). We tentatively termed this 150-kD PI-anchored glycoprotein PI-GP150.

### Purification of HNK-1-positive PI-GP150

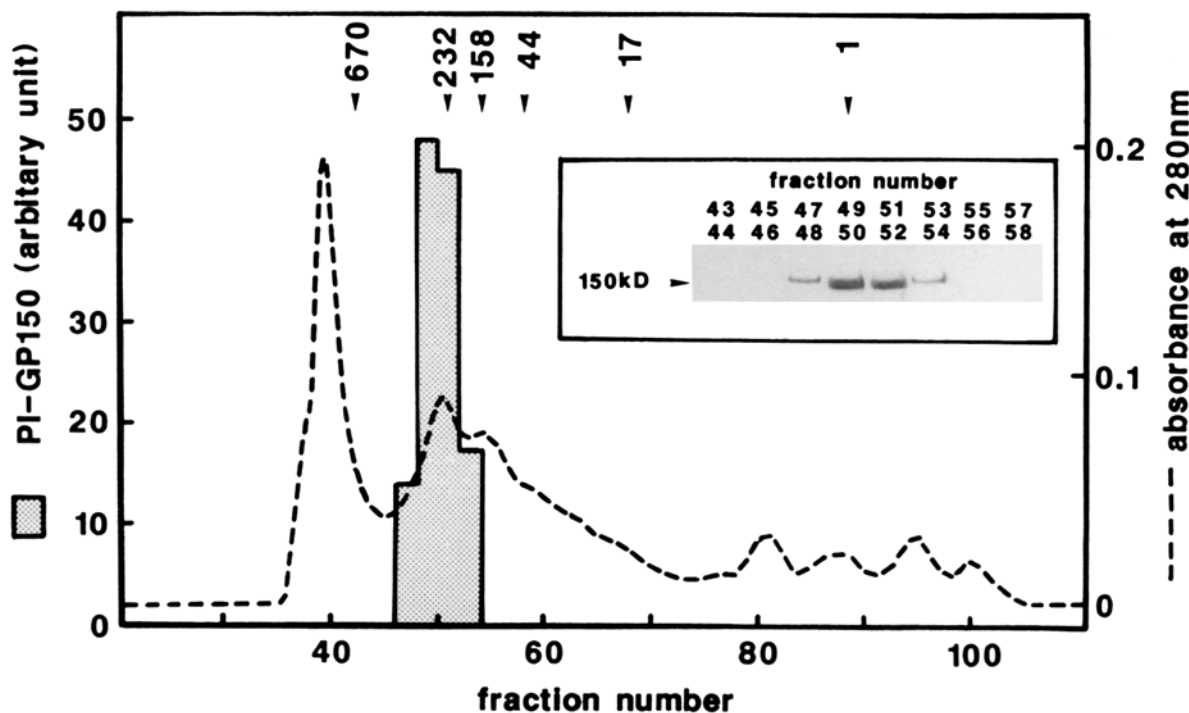
We purified PI-GP150 from adult rat telencephalon by employing column chromatographies and Western blot analysis with mAb HNK-1. The materials released from telencephalon membranes by PI-PLC treatment were concentrated and first subjected to Sephacryl S-300 gel filtration chromatography. Western blot analysis of each fraction with the mAb HNK-1 resulted in the detection of strong immunoreactivity of the 150-kD protein in a single peak at ~300 kD (Fig. 2).

Fractions 49–52 from the Sephacryl S-300 column were pooled, desalted, and then applied to an immunoaffinity column of mAb HNK-1-Sepharose 4B. As shown in Fig. 3, A and B, the pass-through fractions (lanes 1–3) that did not react with the mAb HNK-1 contained the majority of the 150-kD proteins; while HNK-1-positive PI-GP150 specifically adsorbed to the column and was eluted with 0.5 M NaCl (lane 9). Rabbit antiserum was raised against the 150-kD protein in the pass-through fractions. This polyclonal antibody reacted with not only the HNK-1-negative 150-kD protein in the pass-through fractions but also the HNK-1-positive PI-GP150 in the eluate (Fig. 3 C). This result suggests that the 150-kD proteins in both pass-through and high-salt eluate fractions are immunologically identical and that a subpopulation of PI-GP150 is HNK-1 positive, while the rest is HNK-1 negative.

HNK-1-positive PI-GP150 was further purified by Mono Q anion-exchange column chromatography. The eluate from the immunoaffinity column was desalted and applied onto a Mono Q HR 5/5 column. The adsorbed materials were eluted with a linear gradient of NaCl (0–1 M). HNK-1-positive PI-GP150 appeared at 0.2 M NaCl (data not shown) and was homogenous, as assessed by SDS-PAGE followed by CBB staining (Fig. 4).

### Biochemical Characterization of HNK-1-positive PI-GP150

The mobility on SDS-PAGE of the purified HNK-1-positive PI-GP150 was compared under both nonreducing and reducing conditions. The molecular mass of the purified protein was 150 kD in the presence of DTT, while it was 135 kD under nonreducing condition (Fig. 4).



**Figure 2.** Purification of HNK-1-positive PI-GP150 from PI-PLC-released materials of adult rat telencephalon on Sephacryl S-300 chromatography. PI-PLC-released materials from 30 rat telencephalon membranes were applied onto a Sephacryl S-300 gel filtration column ( $4 \times 120$  cm). Aliquots were analyzed by SDS-PAGE followed by Western blot analysis with mAb HNK-1 (*inset*). Hatched column indicates the immunoreactivity of PI-GP150 with mAb HNK-1, quantified with a densitometer. Dashed line indicates the absorbance at 280 nm. Molecular mass standards (in kilodaltons) are represented by arrowheads.

The susceptibility of HNK-1-positive PI-GP150 to several sugar-degrading enzymes was examined. The subunit size of HNK-1-positive PI-GP150 was reduced from 150 to 130 kD by N-glycanase digestion (data not shown), while O-glycanase was ineffective. This suggests that the carbohydrate moiety of PI-GP150 is linked to asparagine residues, but neither to serine nor threonine residues, of the polypeptide. Treatment with chondroitinase ABC, heparitinase, and hyaluronidase resulted in no change in PI-GP150 mobility on SDS-PAGE (data not shown).

To determine whether PI-GP150 is related to the previously described HNK-1-positive molecules, we performed Western blot analysis of purified PI-GP150 with mAbs against N-CAM, L1 and TAG-1. As shown in Fig. 5, though antibodies against these three adhesion molecules recognized the corresponding protein bands in rat brain membrane (*B, C, and D, lane 1*) they did not react with isolated PI-GP150 (*B, C, and D, lane 2*). This result suggests that PI-GP150 is immunologically distinct from N-CAM, L1, and TAG-1.

#### **Developmental Change in Expression of HNK-1-positive PI-GP150**

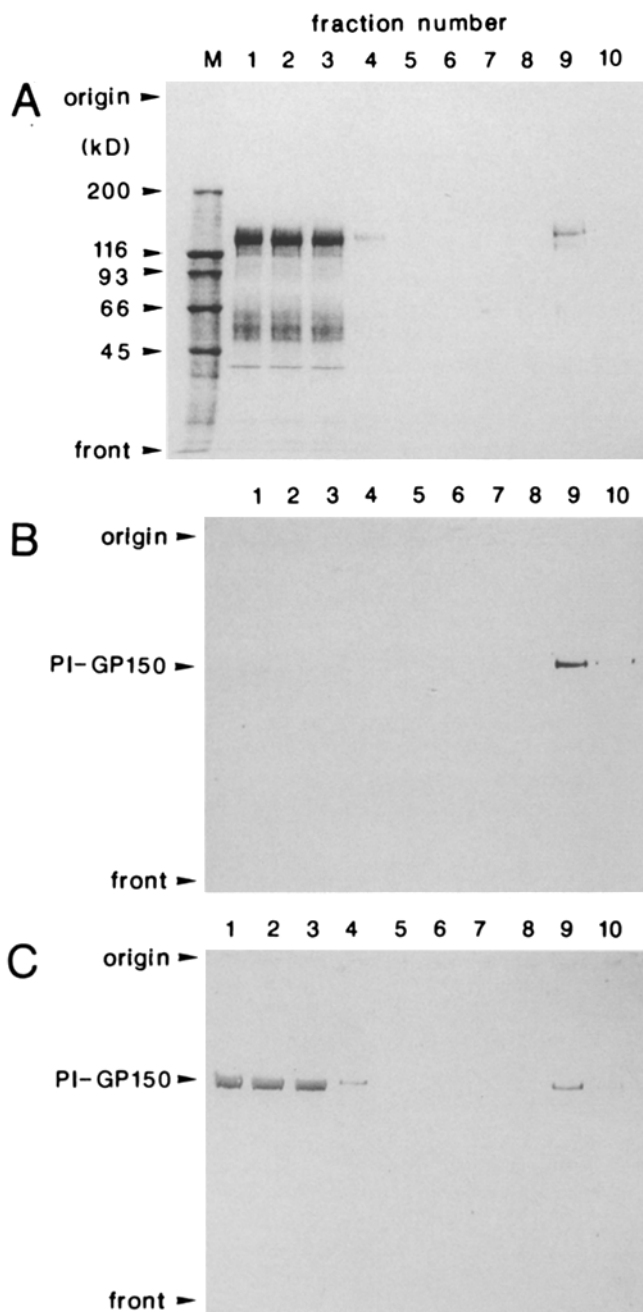
Fig. 6 shows the developmental change in PI-GP150 expression from embryonic to newborn rat brain. Crude synaptosomal membranes were prepared from whole brain of embryonic day-16, -19, and postnatal day-1 rats, and PI-PLC-released materials were examined by Western blot analysis with either anti-PI-GP150 antiserum or mAb HNK-1. PI-

GP150 was detected in the membranes from day-16 embryo, and increased progressively in concentration toward birth (Fig. 6 *A*). HNK-1 immunoreactivity of PI-GP150 showed a similar developmental change from embryo to newborn rat (Fig. 6 *B*).

The postnatal development in expression of HNK-1-positive PI-GP150 was investigated in the forebrain and hindbrain of postnatal day-0, -3, -7, -14, and -80 rats. Although several bands were observed in young rats by Western blot analysis with mAb HNK-1, only HNK-1-positive PI-GP150 was released by exogenous PI-PLC and had the strongest immunoreactivity with mAb HNK-1. In the forebrain, HNK-1-positive PI-GP150 was constitutively expressed from postnatal day 0-80, with the highest level around postnatal week 1-2 (Fig. 7 *A*). On the other hand, the expression of the HNK-1 epitope on PI-GP150 was developmentally regulated in the hindbrain. That is, HNK-1-positive PI-GP150 was detected in the hindbrain from postnatal day 0-7, but it gradually disappeared thereafter and was completely absent in adulthood (Fig. 7 *B*).

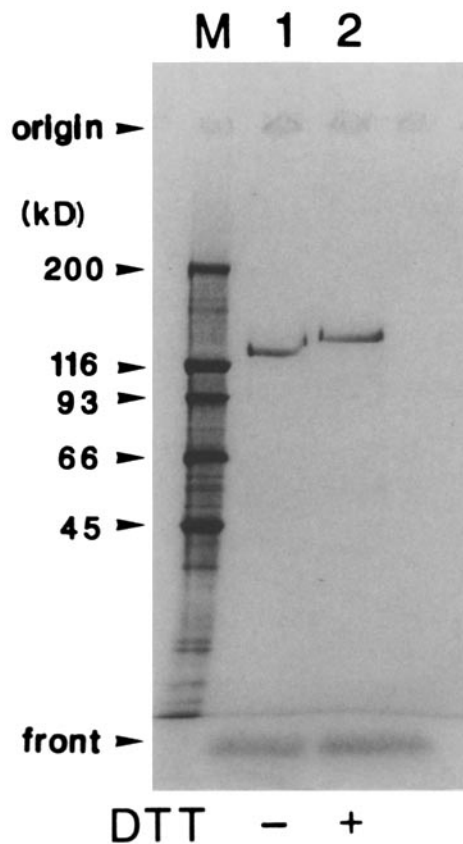
#### **Regional Distribution of PI-GP150 in Adult Rat Brain**

Western blot analysis with anti-PI-GP150 antiserum (Fig. 8 *A*) and mAb HNK-1 (Fig. 8 *B*) of PI-PLC-released proteins from five different segments of adult rat brain revealed an interesting feature of PI-GP150. Anti-PI-GP150 antiserum recognized PI-PLC-released 150-kD protein in all brain segments (Fig. 8 *A*). On the other hand, the HNK-1 epitope on PI-GP150 was expressed most densely in the telencephalon



**Figure 3.** Purification of HNK-1-positive PI-GP150 by mAb HNK-1-Sepharose 4B immunoaffinity chromatography. Immunoreactive fractions from Sephacryl S-300 chromatography were desalted and applied onto an immunoaffinity column of mAb HNK-1-Sepharose 4B (1.2 ml). The adsorbed materials were eluted at fraction 9 (lane 9) with 0.5 M NaCl. (A) CBB staining pattern; (B) Western blot analysis with mAb HNK-1; (C) Western blot analysis with polyclonal antibody raised against the 150-kD protein in pass-through fractions (fractions 1-3). Lane M in A shows the molecular mass standards.

(Fig. 8 B, lane 2), followed by diencephalon (lane 4), and mesencephalon (lane 6). No expression of HNK-1-positive PI-GP150 was observed in metencephalon (Fig. 8 B, lane 8) and myelencephalon (lane 10), or in spinal cord and sciatic nerve (data not shown). Thus, the expression of HNK-1-

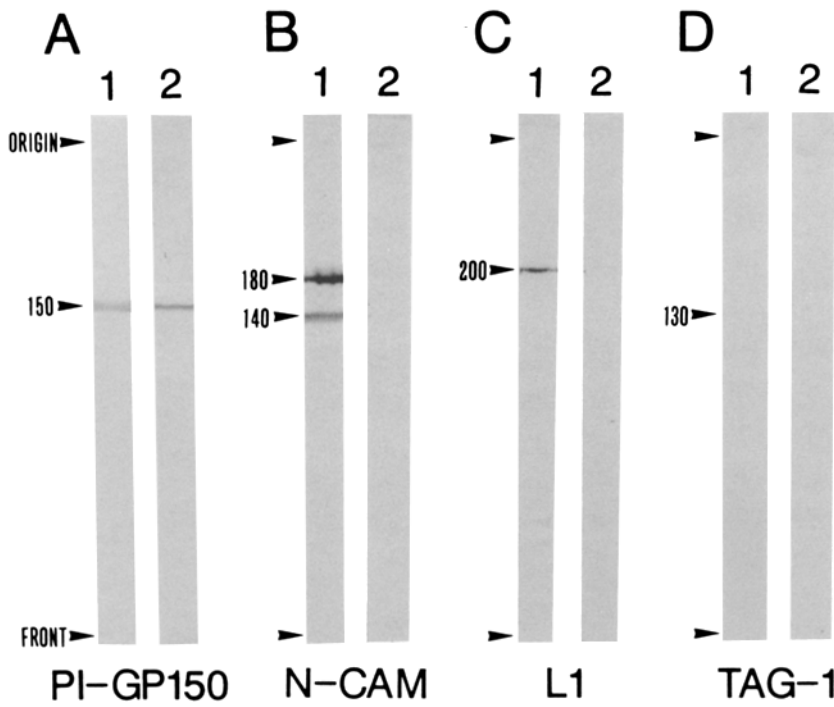


**Figure 4.** Homogeneity of purified HNK-1-positive PI-GP150. The eluate from the mAb HNK-1-Sepharose 4B column was desalted and applied onto a Mono Q HR 5/5 anion-exchange column ( $5 \times 50$  mm). The adsorbed materials were eluted with a linear gradient of 0-1.0 M NaCl. HNK-1 immunoreactive fraction was subjected to SDS-PAGE under both nonreducing and reducing conditions, and the gel was stained with CBB. Lane M, molecular mass standards; lane 1, purified HNK-1-positive PI-GP150 under nonreducing condition; lane 2, purified HNK-1-positive PI-GP150 under reducing condition.

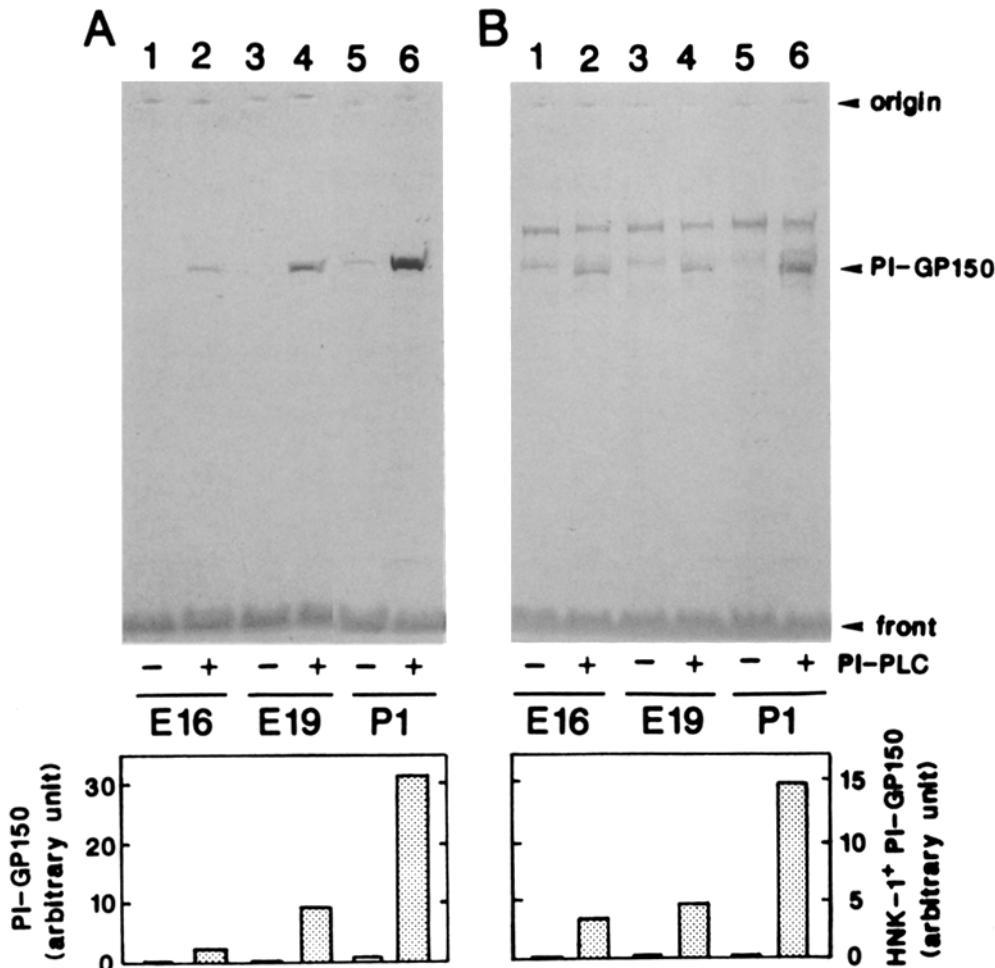
positive PI-GP150 formed an apparent rostro-caudal gradient in the adult rat brain. This result raises the possibility that the polypeptide moiety of PI-GP150 recognized by polyclonal antibody is expressed in all brain regions, whereas the carbohydrate moiety, especially HNK-1 epitope, is expressed in a segment-specific manner.

Detailed distribution of HNK-1-positive PI-GP150 was examined in various telencephalic and diencephalic regions from adult rat brain. The concentration of HNK-1-positive PI-GP150 varied greatly in different telencephalic regions; it was highest in the amygdala, followed by cerebral neocortex, hippocampus, olfactory bulb, and striatum (data not shown). Thus, within telencephalon, the HNK-1 determinant is not expressed to form a rostro-caudal gradient, indicating that the apparent rostro-caudal gradient is observed only when the brain was divided into rough segments, but not when into small regions.

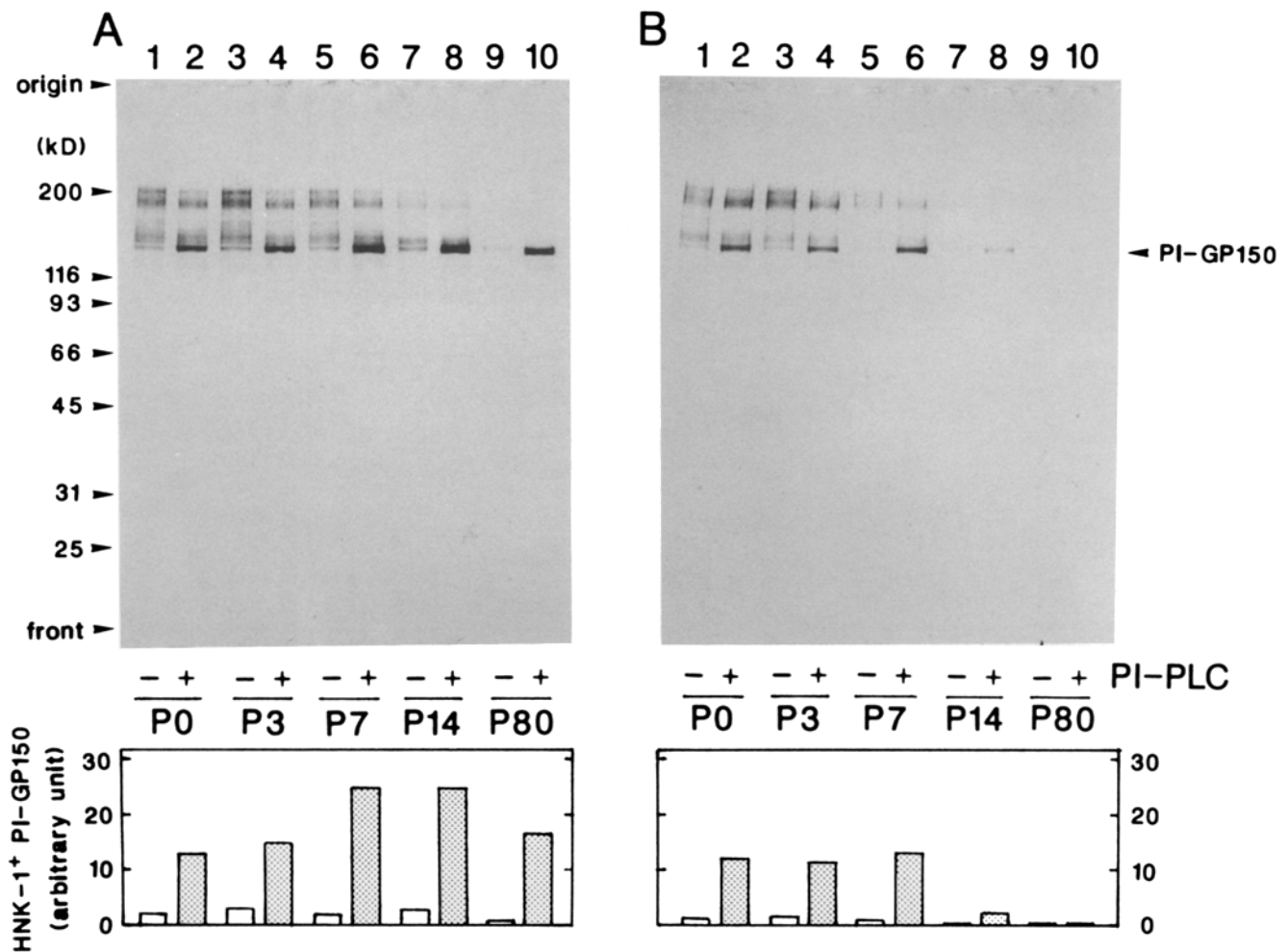
For analyzing the carbohydrate moiety of PI-GP150, we examined the lectin-binding properties of PI-PLC-released materials from five brain segments. Among 10 lectins tested, PI-GP150 reacted strongly with PHA-E (Fig. 9 B), as well



**Figure 5.** Immunological characterization of purified PI-GP150 with antibodies against PI-GP150, N-CAM, L1, and TAG-1. Crude synaptosomal membranes from adult rat brain (lane 1) or purified PI-GP150 (lane 2) was electrophoresed, transferred to nitrocellulose sheet, and processed with antibodies against PI-GP150 (A), N-CAM (B), L1 (C), and TAG-1 (D). The antibody against N-CAM recognized two major bands of 140 and 180 kD, L-1, a single band of 200 kD, and TAG-1, a faint but significant band of 130 kD in the crude membranes (lane 1), but they did not react with the purified PI-GP150 (lane 2). The antibody against PI-GP150 recognized a major band of 150 kD in both lanes.



**Figure 6.** Developmental change in PI-GP150 expression from embryonic to newborn rat brain. Crude synaptosomal membranes were prepared from whole brain of embryonic day-16 (E16), -19 (E19), and postnatal day-1 (P1) rats. The membranes (1 mg protein) were incubated at 37°C for 30 min in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 0.1 unit of PI-PLC. After centrifugation, supernatants were subjected to SDS-PAGE followed by Western blot analysis with anti-PI-GP150 antiserum (A) or mAb HNK-1 (B). Upper panels show the electrophoretic patterns of immunoreactive proteins. Lower panels show the relative concentration of PI-GP150 (A) and HNK-1-positive PI-GP150 (B), as quantified with a densitometer.



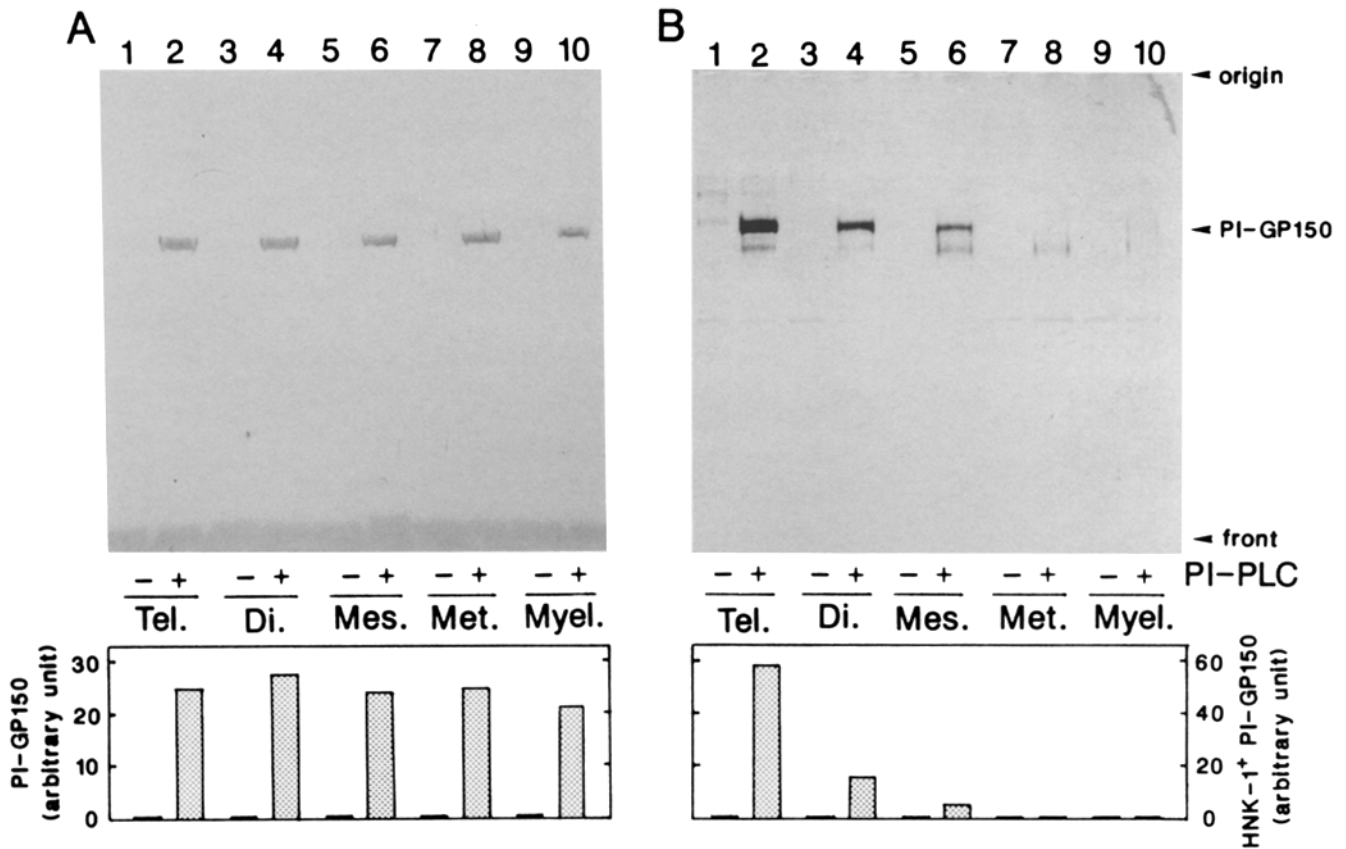
**Figure 7.** Developmental change in HNK-1-positive PI-GP150 expression in postnatal rat forebrain and hindbrain. Crude synaptosomal membranes were prepared from forebrain (A) and hindbrain (B) of postnatal day-0 (P0), -3 (P3), -7 (P7), -14 (P14), and -80 (P80) rats. The membranes (1  $\mu$ g protein) were incubated at 37°C for 30 min in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 0.1 unit of PI-PLC. Upper panels show the electrophoretic patterns of HNK-1-immunoreactive proteins assessed by Western blot analysis with mAb HNK-1. Lower panels show the relative concentration of HNK-1-positive PI-GP150, as quantified with a densitometer. Molecular mass standards (in kilodaltons) are indicated by arrowheads on the left.

as Con A, WGA, and VVA (data not shown), and weakly but significantly with PHA-L (Fig. 9 C) and RCA-I (data not shown). PNA (Fig. 9 D), as well as SBA, DBA, and UEA-I (data not shown), did not bind to PI-GP150. The important points are that these lectin-binding properties of PI-GP150 were observed equally in all brain segments (Fig. 9, B and C) and that only HNK-1 epitope was expressed in a segment-specific manner (Fig. 9 A). Thus, it is likely that the protein portion and overall carbohydrate moiety, as far as the lectin-binding properties are concerned, of PI-GP150 are expressed throughout the brain, however, only the expression of the HNK-1 epitope at glycan terminus of PI-GP150 is regulated to form the apparent rostro-caudal gradient in the adult rat brain.

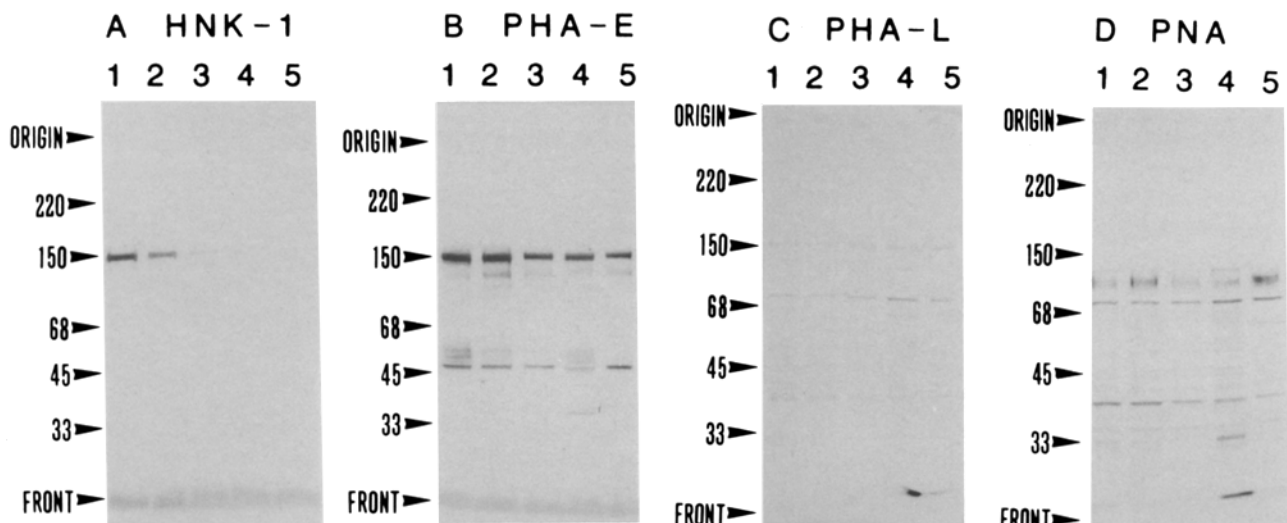
#### **Developmental and Spatial Regulation of HNK-1 Expression on Membrane Glycoproteins**

To investigate whether similar temporal change and segmental difference of HNK-1 expression could be seen in other

HNK-1-positive proteins, we subjected the membrane fractions of rat brains of different ages to Western blot analysis with mAb HNK-1. As shown in Fig. 10, several protein bands ranging from 120 to 250 kD expressed HNK-1-epitope. However, an obvious difference in the timing of disappearance of HNK-1 epitope was observed among brain segments. The HNK-1 epitope was detected in both forebrain and hindbrain of the postnatal 2-d rat, with higher amount in the hindbrain (Fig. 10 A). In the postnatal 7-d brain, the HNK-1 epitope in caudal segments (metencephalon plus myelencephalon) decreased and the highest level was seen in the diencephalon plus mesencephalon (Fig. 10 B). On the postnatal day 14, the HNK-1 epitope was hardly detectable in metencephalon (Fig. 10 C). In the adult brain, the HNK-1 epitope is expressed most densely in telencephalon, but is completely absent in the caudal segments, metencephalon and myelencephalon (Fig. 10 D). Thus, the developmental change in expression of the HNK-1 epitope is not limited to PI-GP150, but is applicable to a number of HNK-1-positive glycoproteins.

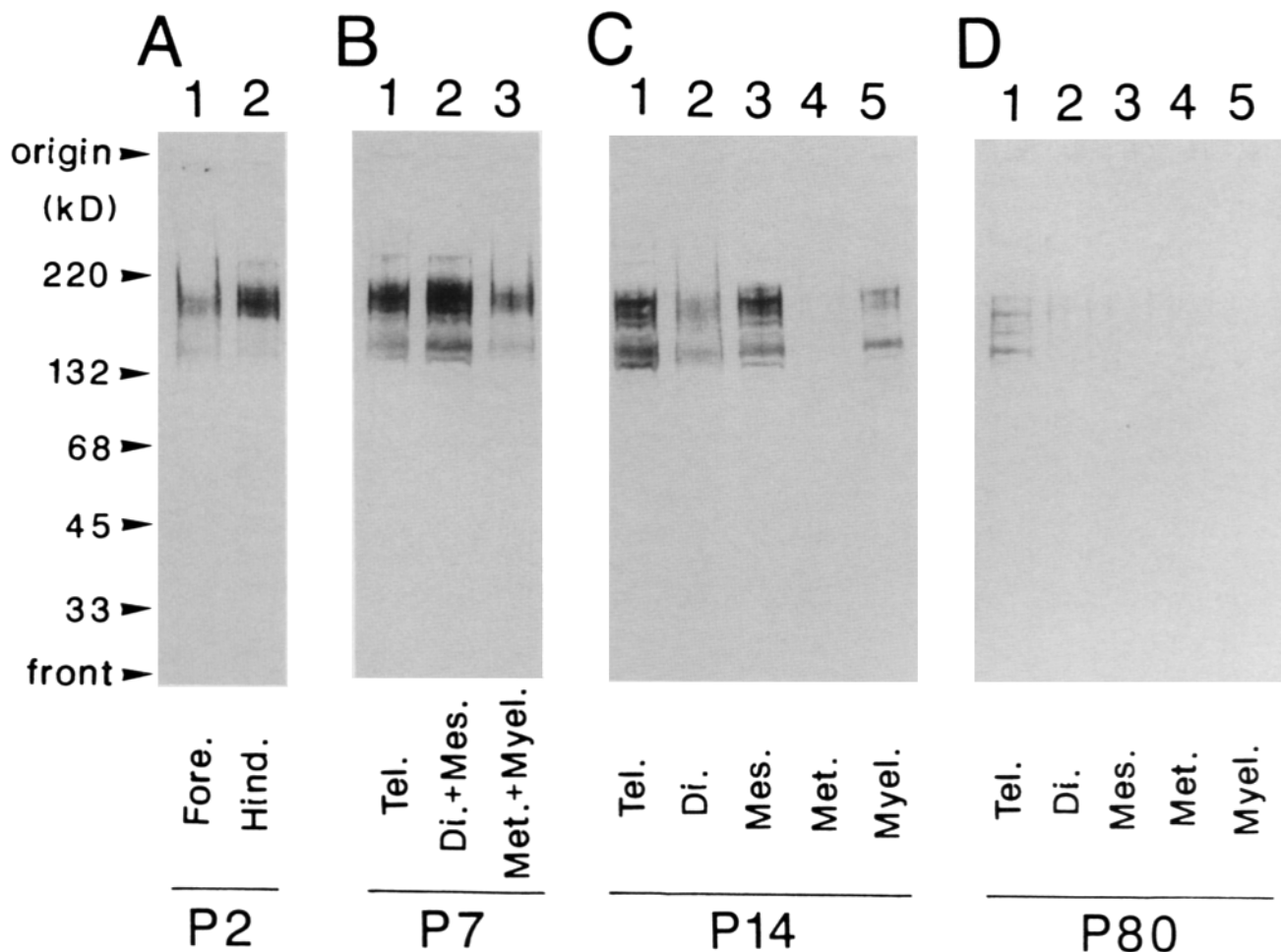


**Figure 8.** Segmental distribution of PI-GP150 in adult rat brain assessed by Western blot analysis with anti-PI-GP150 antiserum (A) and mAb HNK-1 (B). Crude synaptosomal membranes (1 mg protein) prepared from five brain segments (*Tel.*, telencephalon; *Di.*, diencephalon; *Mes.*, mesencephalon; *Met.*, metencephalon; *Myel.*, myelencephalon) were incubated at 37°C for 30 min in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 0.1 unit of PI-PLC. After centrifugation, the supernatants were subjected to SDS-PAGE followed by Western blot analysis with anti-PI-GP150 antiserum (A) and mAb HNK-1 (B). Upper panels show the electrophoretic patterns of immunoreactive proteins. Lower panels show the relative concentrations of PI-GP150 and HNK-1-positive PI-GP150, as quantified with a densitometer.



**Figure 9.** Lectin binding properties of PI-PLC-released proteins from membranes of adult rat brain segments. Membranes (1 mg protein) from five segments of adult rat brain were incubated with 0.1 unit of PI-PLC. PI-PLC-released materials were subjected to SDS-PAGE followed by Western blot analysis with mAb HNK-1 or various biotinylated lectins. (A) mAb HNK-1; (B) PHA-E (erythrocytoagglutinating phytohemagglutinin); (C) PHA-L (leukoagglutinating phytohemagglutinin); (D) PNA (peanut agglutinin). In all panels, lane 1 indicates telencephalon; lane 2, diencephalon; lane 3, mesencephalon; lane 4, metencephalon; lane 5, myelencephalon. Molecular mass standards (in kilodaltons) are indicated by arrowheads on the left.





**Figure 10.** Developmentally and spatially regulated expression of HNK-1 epitope on glycoproteins in rat brain membranes. Membranes (100  $\mu$ g protein) from rat brains of different ages were subjected to SDS-PAGE followed by Western blot analysis with mAb HNK-1. (A) Postnatal day 2; lane 1, forebrain; lane 2, hindbrain. (B) Postnatal day 7; lane 1, telencephalon; lane 2, diencephalon plus mesencephalon. (C) Postnatal day 14. (D) Postnatal day 80. In C and D, lane 1, telencephalon; lane 2, diencephalon; lane 3, mesencephalon; lane 4, metencephalon; lane 5, myelencephalon. Molecular mass standards (in kilodaltons) are indicated by arrowheads on the left.

### Subcellular Distribution of HNK-1-positive PI-GP150 in Adult Rat Brain

Homogenate of adult rat telencephalon was subfractionated by differential centrifugation according to Whittaker (1959), and the amount of HNK-1-positive PI-GP150 released from membranes of each subfraction was measured. 80% of HNK-1-positive PI-GP150 was recovered in the crude synaptosomal fraction, followed by 17.6% in the microsomal fraction and 2.4% in the nuclear fraction. The crude synaptosomal fraction was further separated into the myelin, synaptosome, and mitochondria subfractions. The highest amount was observed in the synaptosome subfraction, representing 96.4% of the HNK-1-positive PI-GP150 in the crude synaptosomal membrane. This result suggests the localization of HNK-1-positive PI-GP150 in synaptosomal membranes.

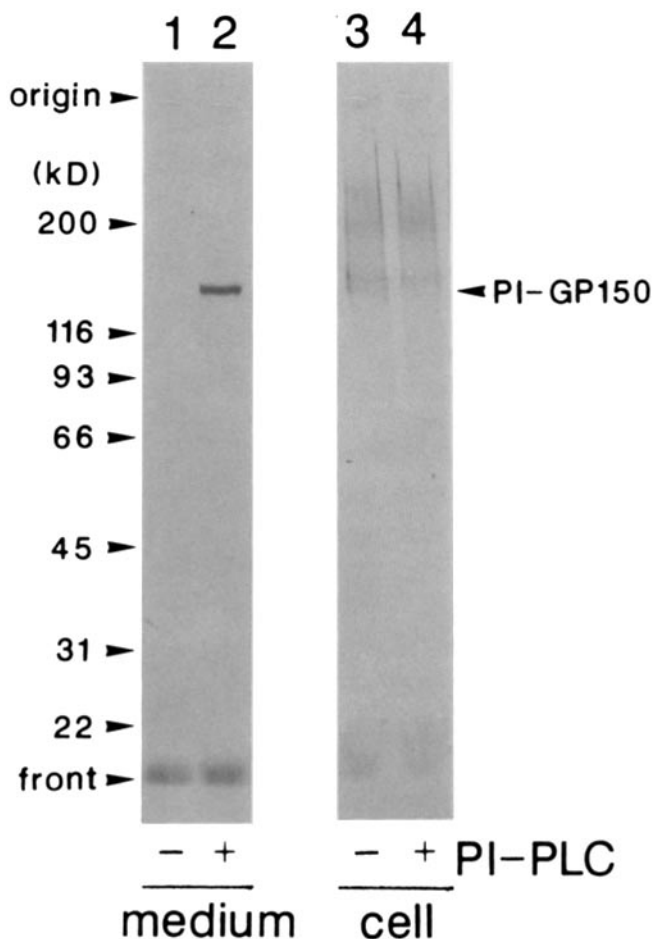
### Release of PI-GP150 from Primary Cultures of Brain

To ascertain that PI-GP150 is released from the extracellular side of the membranes, we prepared primary cultures of the telencephalic cells from postnatal day-10 rats. Seven days after plating, the cultures were incubated in the presence or ab-

sence of PI-PLC at 37°C for 30 min. The medium and cells were separated by centrifugation and subjected to Western blot analysis with mAb HNK-1. As shown in Fig. 11, HNK-1-positive PI-GP150 released by PI-PLC treatment was observed in the culture medium (lane 2). Simultaneously, the mAb HNK-1-reactive 150-kD protein almost disappeared from the cells (Fig. 11, lane 4).

### Immunofluorescence Labeling of Primary Cultures of Brain

As shown in Fig. 12, anti-PI-GP150 antiserum labeled cultured cells prepared from postnatal day-5 rat cerebellum. Surface of cell bodies and their processes of neuronal morphology were labeled (Fig. 12, A, B, and C), while the astroglial cells underlying these neurons showed no immunoreactivity. The PI-GP150 immunoreactivities on the neuronal processes were observed in the form of clusters (Fig. 12 C, arrowheads). The preabsorption of the antiserum with the purified PI-GP150 resulted in the loss of the fluorescent labeling of the neurons (data not shown), confirming that this antiserum is highly specific to PI-GP150. In addition, the treatment of cultured cells with PI-PLC for 1 h caused a



**Figure 11.** PI-PLC-induced release of PI-GP150 from rat brain primary cultures. Primary cultures prepared from telencephalon of postnatal day-10 rats were treated with (lanes 2 and 4) or without (lanes 1 and 3) PI-PLC. The supernatants (lanes 1 and 2) and cells (lanes 3 and 4) were subjected to SDS-PAGE followed by Western blot analysis with mAb HNK-1. The molecular mass standards (in kilodaltons) are indicated by arrowheads on the left.

complete loss of the PI-GP150 immunoreactivity from the cells, suggesting that virtually all PI-GP150 on the surface membranes are released by PI-PLC.

## Discussion

This paper describes identification, purification and characterization of PI-GP150, a novel member of HNK-1 family molecules. The mode of HNK-1 expression on PI-GP150 was investigated by use of a combination of PI-PLC treatment of brain membranes and Western blot analysis with mAb HNK-1 and was shown to be precisely controlled temporally and spatially in the developing rat brain.

### A Novel PI-anchored Molecule, PI-GP150

PI-GP150 is a PI-anchored membrane glycoprotein identified as an HNK-1-positive 150-kD band on SDS-PAGE. The molecular mass of PI-GP150 in its native form was estimated to be ~300 kD by gel filtration, suggesting that PI-GP150 is a dimeric protein of 150 kD subunits. The mobility of PI-GP150 on SDS-PAGE differed under reducing and nonreduc-

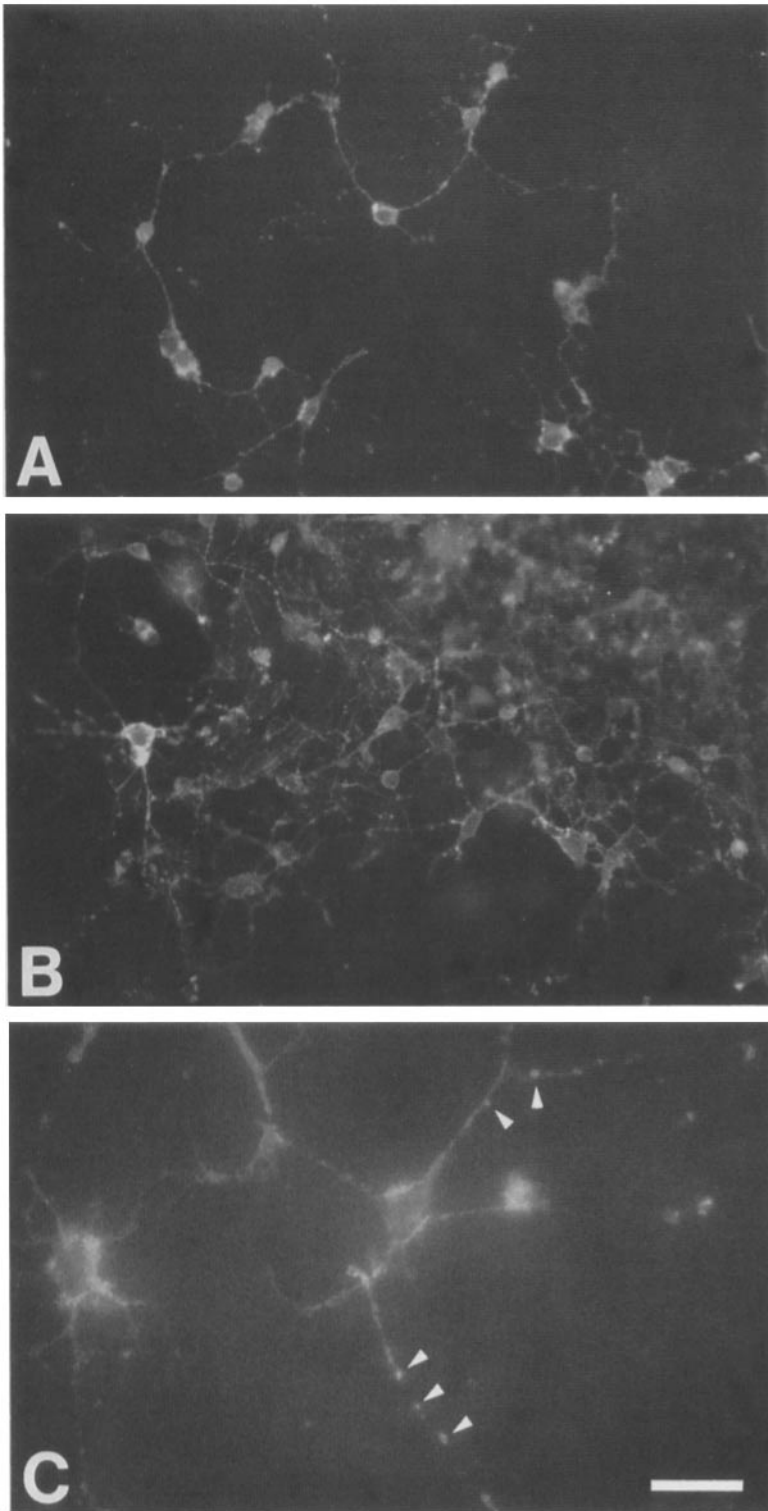
ing conditions. The smaller molecular mass in the absence of DTT suggests the presence of intramolecular disulfide bond(s), as is seen in the case of Ig superfamily molecules.

PI-GP150 belongs to the group of glycoproteins that express the HNK-1 epitope, but it appears to be distinct from previously known members of the family, such as N-CAM, L1, TAG-1, and F3/F11, on the basis of following observations. (a) The immunoblot analysis described in Fig. 5 revealed that PI-GP150 is immunologically different from N-CAM, L1, and TAG-1. (b) The molecular mass and the developmental change in expression of PI-GP150 do not correspond to those of F3/F11. F3/F11 is a 130-kD glycoprotein whose expression peaks during the first two postnatal weeks followed by an eightfold drop into adulthood (Gennarini et al., 1989a). (c) As a direct evidence, we determined the NH<sub>2</sub>-terminal amino acid sequence of purified PI-GP150. The sequence is NH<sub>2</sub>-Val-Phe-Arg-Pro(or Trp)-Trp-Ala-Ala-Val-(Asp?)-Lys-Met-Leu-Val-. This does not appear in any other proteins in the Protein Sequence Data Bank (National Biomedical Research Foundation, Washington, DC). All of above results are consistent with an interpretation that PI-GP150 is a novel HNK-1 family molecule.

Recently, a growing number of PI-anchored proteins have been found in the nervous system as well as in the nonneural sites. PI-anchored proteins have two major advantageous characteristics, compared with membrane proteins with transmembrane regions (Ferguson, 1988; Low, 1989). (a) PI-anchored molecules are released into the extracellular space by PI-specific phospholipases. The cleavage of the anchor of cell adhesion molecules by PI-specific phospholipases might result in termination of adhesion events. Alternatively, proteins released from the cell surface might act as transmitters or hormones to bind to their specific receptors. An endogenous PI-specific phospholipase D, which was recently discovered and partially characterized from bovine brain by Hoener et al. (1990), could participate in these events. (b) PI-anchored proteins are relatively more mobile in the plane of the membrane than transmembrane polypeptides (Ishihara et al., 1987). This rapid lateral mobility due to the lipid anchor would make it possible for proteins to be recruited into areas where their functions are particularly required (Dustin et al., 1987). In the nervous system, for example, this recruitment of the cell surface proteins into areas of contact seems to be necessary at synapses in particular.

In the cell staining experiment, PI-GP150 immunoreactivity was detected along the neuronal processes in a discontinuous and dotted pattern (Fig. 12). Similar results were reported in the case of F3 (Gennarini et al., 1989a) and Thy-1 (Dotti et al., 1991) in cultured neurons. Both of them are also PI-anchored molecules and their immunoreactivities are present in the axons in the form of clusters. Therefore, it is likely that such a type of cellular localization is a common feature of PI-anchored proteins, resulting from their free mobility on the plasma membrane.

Unique functions of several identified PI-anchored molecules are worth noting, when we consider the function of PI-GP150. One example in the nervous system is rat TAG-1, an Ig superfamily molecule transiently expressed by the developing commissural axons. TAG-1 is thought to be involved in the axonal growth and guidance. Furley et al. (1990) suggested that, when released into the extracellular environment, TAG-1 can act as a substrate to promote the growth



**Figure 12.** Immunofluorescence staining of rat cerebellum primary cultures with anti-PI-GP150 antiserum. (A) A typical staining of cells with neuronal morphology that make contact with each other to form a neuronal network-like structure. (B) Most of the neuronal cell bodies and their processes are stained by the antiserum; however, astroglial cells underlying these neurons are not stained. (C) The antiserum stained neuronal processes in a characteristic dotted pattern (*arrowheads*). Bars: (A and B) 50  $\mu\text{m}$ ; (C) 25  $\mu\text{m}$ .

and guidance of later developing commissural growth cones. Another example is chick F11, an Ig superfamily molecule transiently expressed by the retinal and sympathetic axons during embryonic development. F11 is assumed to play roles in neurite fasciculation and elongation. Wolff et al. (1989) in their paper on F11 proposed that the selective detachability of PI-anchored molecules from the membranes may be an advantageous property for migrating axons to change subsets

of their cell recognition molecules, resulting in altered substrate specificity. PI-GP150 shares some properties with TAG-1 and F11, such as the PI-anchoring structure, the smaller molecular mass seen by SDS-PAGE under nonreducing conditions, the HNK-1 reactivity, and the expression on neuronal axons. This suggests that PI-GP150 may possess functions analogous to those of TAG-1 and F11. However, the expression of PI-GP150 is more widely distributed throughout

the brain, while that of TAG-1 and of F11 is restricted to certain regions. Accordingly, PI-GP150 seems to be a relatively ubiquitous molecule that is required in various regions of the central nervous system.

Walter et al. (1987) demonstrated in their *in vitro* model system of the retino-tectal projection that temporal retinal axons grow exclusively on anterior tectal membranes, avoiding posterior membranes. Recently, Stahl et al. (1990) found that this avoidance mechanism is due to a PI-anchored 33-kD glycoprotein in the posterior tectum that induces collapse of temporal retinal growth cones. Thus, during the development of the nervous system, PI-anchored proteins play crucial roles as plastic cell recognition molecules in various events finally leading to the formation of specific neural networks.

### **Precisely Controlled Expression of HNK-1 Epitope**

Monoclonal antibody HNK-1 was first raised against the cell surface antigen of human natural killer cells and identified to recognize a specific carbohydrate antigen on glycolipids there (Abo and Balch, 1981). Cross-reactivity of this mAb was later observed with a variety of neural cell surface and extracellular molecules, which are involved in cell-cell or cell-substrate interaction processes, such as many neural Ig superfamily molecules (McGarry et al., 1983; Kruse et al., 1984; Rathjen et al., 1987; Bollensen and Schachner, 1987; Dodd et al., 1988; Gennarini et al., 1989b), fibronectin receptor  $\alpha$ -subunit (Pesheva et al., 1987), J1/tenascin (Kruse et al., 1985), and several neural proteoglycans (Hoffman and Edelman, 1987; Gowda et al., 1989). It has been postulated that the HNK-1 epitope may play important roles in modifying the cell adhesion function of the protein moiety in these molecules. However, when we look at a specified glycoprotein, all the molecules do not react with mAb HNK-1. In other words, only a subpopulation of them is HNK-1 positive, the rest being HNK-1 negative (Kruse et al., 1984; Poltorak et al., 1987; Faissner, 1987). The HNK-1 epitope may appear at specific developmental stages, in the selective regions of the brain or on the specific types of cells, according to some regulatory mechanism of its expression. An immunohistochemical technique is not suitable for looking at the spatial expression of the HNK-1 epitope on a specified protein, since the HNK-1 epitope is present on a number of molecules, not only on glycoproteins but also on proteoglycans and glycolipids. In the present study using the combination of PI-PLC-digestion and Western blot analysis, we were able to successfully survey the mode of HNK-1 expression on a novel HNK-1 family molecule, PI-GP150.

PI-GP150 appears at embryonic day 16 and is present during all later developmental stages and throughout the brain. However, the expression of the HNK-1 epitope on the molecule undergoes a drastic change both temporally and spatially. The HNK-1 expression on PI-GP150 decreases after postnatal day 7 in the hindbrain and becomes completely absent in the adult myelencephalon and metencephalon. In contrast, it is constitutively observed in the telencephalon. This segmental difference in HNK-1 expression results in the formation of an apparent rostro-caudal gradient in the adult brain. The result shown in Fig. 6 suggests that the segment-specific regulation in HNK-1 expression is applicable for a large number of HNK-1-positive membrane glyco-

proteins. Thus, the present study indicates that the HNK-1 carbohydrate moiety is regulated independently of the expression of protein backbone of HNK-1 family molecules. One possible mechanism for this regional specificity is the regulation of glycosyltransferase activity which catalyzes the attachment of the HNK-1 determinant on glycan moiety. Studies on the temporal and spatial expression of the HNK-1 glycosyltransferase will provide some cues to this question.

The differential expression of the HNK-1 epitope in different brain segments is reminiscent of the expression pattern of mouse *Hox* and *Krox* genes. In mouse hindbrain, they are distinctly expressed at different axis levels or in specific rhombomeres (Kessel and Gruss, 1990). *Hox* and *Krox* are homeobox- and zinc-finger-containing genes, respectively, and considered to be transcriptional regulators. It has been reported that the expression pattern of the HNK-1 determinant is closely related to that of the *Krox-20* gene. Both of them appear in migrating neural crest cells and in specific rhombomeres (3 and 5) during overlapping developmental periods (Kuratani, 1991). Therefore, the expression of the HNK-1 glycosyltransferase may be strictly regulated both temporally and spatially by some transcriptional factors such as *Krox-20*.

Of interest is the functional importance of the modification of carbohydrate structures on glycoproteins. One of the best-known examples is the polysialic acid on N-CAM. N-CAM changes its degree of polysialylation from embryo to adult. The presence of large amounts of polysialic acid on N-CAM decreases the cell adhesion activity (Hoffman and Edelman, 1983; Sadoul et al., 1983; Rutishauser et al., 1985). Then, what is the functional difference between the HNK-1-positive and -negative forms of PI-GP150? Hynes et al. (1989) suggested the following possibilities for the function of the HNK-1 carbohydrate epitope: (a) the HNK-1 epitope itself may represent a ligand for  $\beta$ -galactoside-binding lectins; (b) the presence of the epitope may promote the adhesion process of the protein moiety; or (c) the HNK-1 epitope may play a role as a recognition signal involved in the targeting of glycoproteins to the cell surface. However, the defined functions of this carbohydrate structure have not yet been elucidated. Using several *in vitro* assay systems, we are now in the process of elucidating the functions of HNK-1-positive and -negative forms of PI-GP150, by which we expect to provide an answer to the long-standing question of the role of the HNK-1 epitope.

Though we do not know at present the precise function of PI-GP150, the involvement of the molecule in the cell-cell recognition process at or near the synaptic connections is speculated from the results in this paper. PI-GP150 was present on neuronal surfaces, localized in synaptosomal fractions, bound to membranes via a PI-anchoring structure, and a subpopulation of them contained the HNK-1 epitope, which is present on many cell-cell adhesion and recognition molecules. These findings suggest that PI-GP150 might play roles as one of the recognition molecules on neurons to make specific synaptic contacts with appropriate targets during development and to maintain synaptic plasticity in the adult brain. The selective detachability by PI-specific phospholipases and the rapid lateral mobility on plasma membranes would be convenient for dynamic processes such as axonal elongation, growth cone attraction and collapse, and synapse formation.

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