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CARBOHYDRATE ANTIGENS RECOGNIZED BY ANTI-HORSERADISH PEROXIDASE ANTISERUM ARE EXPRESSED ON MAMMALIAN CELLS

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Summary: Antiserum raised against horseradish peroxidase (HRP) recognizes neural specific carbohydrate antigens in *Drosophila* and other insects. The occurrence of the antigens in mammalian cells was investigated. Proteins were extracted from rat pheochromocytoma cells, PC12, and various tissue of rat and bovine origin, and further purified by successive chromatography with Con A-Sepharose and anti-HRP glycopeptide antibody-Sepharose. The proteins were separated by SDS-PAGE and subjected to immunoblot analysis of the antigens. When the Con A- and antibody-Sepharose bound fractions were examined, four glycoproteins with carbohydrate antigens recognized by anti-HRP antiserum were identified in all the tissue. They are low molecular weight glycoproteins that are not membrane-bound. We demonstrated that the carbohydrate antigens recognized by the anti-HRP antibodies are not confined to insects but are expressed on mammalian cells in several tissue.

Antibodies that react specifically with neural cells have been a powerful tool in studying the structure and function of brains. Antiserum raised against HRP is a good example of such an antibody. It is widely used to stain the surface of all axon pathways in the central and peripheral nervous system of *Drosophila* or grasshoppers (1, 2).

The anti-HRP antibodies recognize the carbohydrate chains of insect glycoproteins, some of which have been identified as adhesion molecules and are expected to play an important role in recognition between cells and molecules (3, 4). Recently we determined the epitopic structure for the anti-HRP antibodies and showed that Man and Fuc residues, unique to plant glycoproteins, are involved in binding with the antibodies (5).

So far, all the carbohydrate antigens reported to be recognized by anti-HRP antibodies have been confined to insects. To find out whether the antigens are expressed only in insect neural tissue, we investigated the presence of antigens by immunoblot analysis on various tissue of rat

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The abbreviations used are: HRP, horseradish peroxidase; Man, mannose; Fuc, fucose; Xyl, xylose; GlcNAc, N-acetylglucosamine; Con A, concanavaline A; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

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and bovine origin. We found that the carbohydrate antigens are expressed on mammalian cells and they are carried by four common low molecular weight proteins that are widely distributed , unlike the antigens on insect neural cells.

Materials and Methods

Materials -- HRP (type VI) was purchased from Sigma, St. Louis. HRP glycopeptides were prepared according to the procedure of Snow *et al.* (4). Con A-Sepharose, ECH Sepharose and CNBr-activated Sepharose were obtained from Pharmacia, Uppsala. Goat anti-HRP antiserum and swine anti-goat IgG antibody were from Cooper, West Chester, and Tago, Burlingame, respectively.

Cells and Cell Culture -- PC12, a gift from Dr. M. K. Seo of Kyoto Sangyo University, was cultured in RPMI1640 medium supplemented with 5% normal horse serum and 10% fetal calf serum.

Immunoblot Analysis -- Samples were dissolved in buffer for electrophoresis and subjected to SDS-PAGE (12.5 % gel). The proteins were then electrophoretically blotted onto a polyvinylidene difluoride membrane (Atto, Tokyo). The blot was blocked with 3 % BSA solution and incubated with goat anti-HRP antiserum. Immunodetection was done by incubating the membrane with alkaline phosphatase labeled swine anti-goat IgG antibodies and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, successively. To prevent non-specific binding of the second antibodies, the anti-goat IgG antibodies were incubated with rat brain acetone powder and centrifuged. The supernatant was used for immunodetection. For the inhibitory reaction, HRP glycopeptides or HRP was added to the anti-HRP antiserum to give final concentrations of 0.4 mg/ ml and 1 mg/ml, respectively.

Preparation of Anti-HRP Glycopeptide Antibody-Sepharose -- HRP glycopeptides were coupled with ECH Sepharose 4B. Anti-HRP glycopeptide antibodies were purified from the anti-HRP antiserum with the above HRP glycopeptide column and then they were coupled with CNBr-activated Sepharose 4B (1 mg antibody/ml resin).

Isolation of Glycoproteins Recognized by Anti-HRP Antibodies -- PC12 or tissue was homogenized in 10 mM Tris/HCl buffer, pH 7.4, containing 0.15M NaCl, 1 mM EDTA, 10 μg/ml aprotinin (when detergents were used for the extraction of proteins, 0.1 % sodium deoxycholate, 0.1 % SDS, and 0.5 % NP40 were added to the buffer). Lysate was obtained by centrifuging the homogenates (10,000 x g, 20 min, 4°C). The above buffer was used to equilibrate and wash the Con A-Sepharose and anti-HRP glycopeptide antibody-Sepharose columns. For elution from the columns, 0.5 M α-methylmannoside and 50 mM diethylamine were used for Con A- and antibody-Sepharose, respectively.

Results

Reactivity of Anti-HRP Antibodies with Con A-Bound Glycoproteins from PC12 Cells -- In order to find out whether carbohydrate antigens recognized by anti-HRP antibodies are present in mammalian cells, solubilized proteins from mammalian cells with detergent-containing buffer were separated by SDS-PAGE, and then subjected to immunoblot analysis as described in Materials and Methods. The specific binding of the antibodies toward carbohydrate chains was monitored by adding HRP glycopeptides to the anti-HRP antiserum during the incubation with the membrane. The HRP glycopeptides inhibited the binding reaction between the anti-HRP antibodies and sugar moieties of glycoproteins blotted onto the membrane.

We tested PC12 cells first because PC12 cells differentiate into neural cells on the addition of growth factors to the culture media. When the lysate from PC12 cells was analyzed, many bands were observed on the membrane but no inhibition was detected with HRP glycopeptides. HRP also failed to inhibit the binding of the anti-HRP antiserum, indicating that most of the detected bands were non-specific. We purified the glycoproteins with lectin to remove

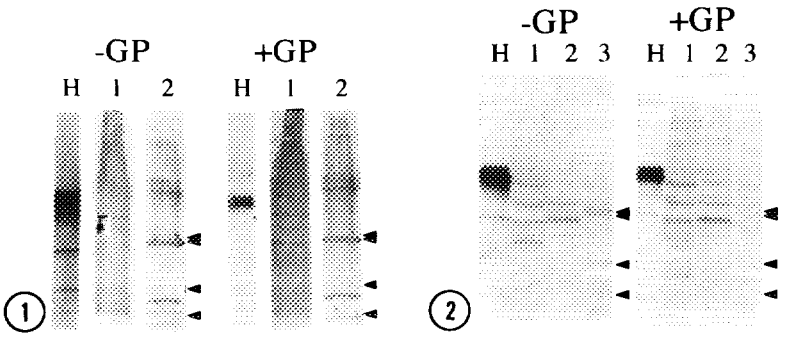

Fig.1. Immunoblot Analysis of Proteins from PC12 Cells. H, HRP 0.5 μg; 1, Con A-Sepharose unbound fraction 100 μg; 2, Con A-Sepharose bound fraction 50 μg. +GP, and -GP indicate that immunoblot analysis was carried out with and without HRP glycopeptides, respectively. Arrows indicate the glycoproteins carrying carbohydrate antigens recognized by the anti-HRP antibodies.

Fig. 2. Immunoblot Analysis of Proteins from Rat Brains and Livers. H, HRP 0.5 μg; 1, Lysate 100 μg; 2, Con A-Sepharose unbound fraction 100 μg; 3, Con A-Sepharose bound fraction 50 μg. +GP, and -GP indicate that immunoblot analysis was carried out with and without HRP glycopeptides, respectively. Arrows indicate the glycoproteins carrying carbohydrate antigens recognized by the anti-HRP antibodies.

proteins that bind the antibodies non-specifically. Con A-Sepharose was used because the insect antigens recognized by anti-HRP antibodies are known to bind Con A. Fig. 1 shows the binding of anti-HRP antibodies to HRP and the Con A-Sepharose unbound and bound fractions. HRP was detected as diffuse bands because of the microheterogeneity of the carbohydrate chains. When HRP glycopeptides were added, the bands became weaker but still stained well. This indicates that HRP glycopeptides inhibited the binding of antibodies recognizing HRP carbohydrates but the binding of peptide-binding antibodies was unaffected. For the Con A-Sepharose unbound fraction, only non-specific bands were detected. On the other hand, several bands were observed for the Con A-Sepharose bound fraction and the HRP glycopeptides prevented the antibody binding to four of them. They are relatively small molecules with molecular weight of 34,000, 32,000, 21,000 and 15,000, respectively. These findings demonstrate that PC12 cells contain carbohydrate antigens on four glycoproteins and that these antigens are retained on Con A-Sepharose and recognized by the anti-HRP antiserum. Addition of basic fibroblast growth factors to the culture media did not affect the expression of these four glycoproteins.

Carbohydrate Antigens from Mammalian Brain Tissue -- We prepared lysate from rat brains with the same buffer containing detergents and analyzed the presence of the antigens (Fig. 2). We obtained essentially the same results to those from PC12 cells. Four bands with the same molecular weight were observed for Con A-Sepharose eluate and they disappeared when the HRP glycopeptides were added as inhibitors. These four carbohydrate antigens were expressed in rat brain tissue as well as rat cultured cells.

Purification of Carbohydrate Antigens with Anti-HRP Glycopeptide Antibody-Sepharose from Brains -- To purify the antigens further, an immunomatrix column packed with the anti-HRP glycopeptide antibodies bound to Sepharose, was prepared. Rat brain

glycoproteins, retained on a Con A-Sepharose column, were applied to the anti-HRP glycopeptide antibody column. Although most of the proteins passed through the column, a small but significant amount (~1% of applied proteins) was retained on and eluted from the column. Fig. 3 shows that all the four bands were retained on the column and detected as major bands. The HRP glycopeptides clearly inhibited the binding of the antibodies to those glycoproteins. Only one faint non-specific band was observed when the HRP glycopeptides were added, indicating that these four bands were highly purified by this glycopeptide-specific antibody chromatography. We carried out the same experiments for bovine brains and detected the four antigens with the same molecular weight (data not shown).

The Occurrence of the Antigens in Rat Tissue -- To investigate the occurrence of the antigens in rat tissue other than brain, rat liver and stomach tissue were analyzed in the same way. Only the rat liver results are shown (Fig. 4) as both tissue behaved similarly. The four glycoproteins with the same molecular weight were also observed for these tissues (lane 2), and all of them were retained on the anti-HRP glycopeptide antibody column (data not shown).

Extraction of the Antigens without Detergents -- To investigate whether these four antigens were bound to the cell membranes, lysates were prepared from rat brains with detergent-free buffer, further purified with Con A-Sepharose, and subjected to immunoblot analysis (Fig. 5). Essentially the same patterns were obtained. Detergents were not required to extract these antigens, indicating that these antigens are not membrane-bound glycoproteins.

Discussion

We demonstrated for the first time that the antigens are not confined to insects but are expressed by mammalian tissue, such as rat brain, liver, and stomach, and bovine brain. Although we could not isolate enough antigens to perform the structural analysis because of the limited amount of antigens in the tissue, the sugar chains of the antigens are expected to share the structural features of HRP sugar chains. In our previous paper, we showed that the anti-HRP antibodies recognize the major sugar unit of HRP, Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4 GlcNAcβ1,4(Fucα1,3)GlcNAc, and α1,6-linked Man and α1,3-linked Fuc are predominantly involved in the epitopic structure (5). The major HRP sugar chain has a structure characteristic of plant glycoproteins, *i.e.*, it is highly processed with two exposed mannose residues and has additional Xyl and Fuc residues (6-8). It is interesting to find mammalian cells contain sugar chains sharing structural features with sugar units of plant glycoproteins.

Sugar chains recognized by anti-HRP antibodies are expressed on at least 17 glycoproteins in grasshopper, all of which have a molecular weight larger than 50,000 (4). Some of them are identified as fasciclin I and II, a family of membrane-bound glycoproteins, which are expressed on different subsets on axon fascicles during development and are thought to be adhesion molecules specific to the nerve cell network (9). We also showed that mammalian cells express four glycoproteins carrying sugar chains recognized by anti-HRP antibodies. They are relatively small glycoproteins, which are not tightly associated to the membrane, *i.e.*, extractable from tissue without detergents. These physical differences indicate that the glycoproteins found in mammalian tissue are different from those in insects. Furthermore, whereas the occurrence of the

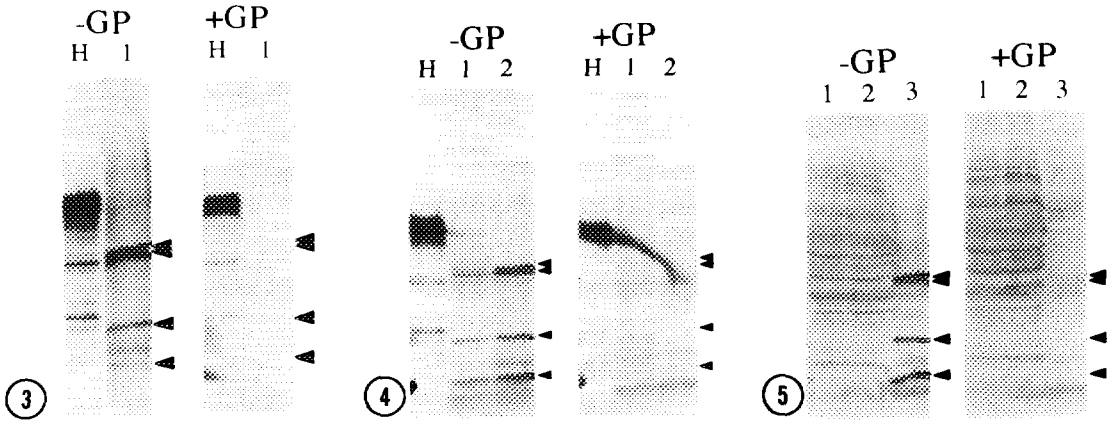

Fig. 3. Immunoblot Analysis of Proteins Purified from Rat Brains with Anti-HRP Glycopeptide Antibody Sepharose. H, HRP 0.5 μg; 1, Antibody column bound fraction 0.7 μg. +GP, and -GP indicate that immunoblot analysis was carried out with and without HRP glycopeptides, respectively. Arrows indicate the glycoproteins carrying carbohydrate antigens recognized by the anti-HRP antibodies.

Fig. 4. Immunoblot Analysis of Proteins from Rat Brains and Livers. H, HRP 0.5 μg; 1, Rat brain Con A-Sepharose bound fraction 50 μg; 2, Rat liver Con A-Sepharose bound fraction 50 μg. +GP, and -GP indicate that immunoblot analysis was carried out with and without HRP glycopeptides, respectively. Arrows indicate the glycoproteins carrying carbohydrate antigens recognized by the anti-HRP antibodies.

Fig. 5. Immunoblot Analysis of Proteins Prepared from Rat Brains without Detergents. 1, Lysate 100 μg; 2, Con A-Sepharose unbound fraction 100 μg; 3, Con A-Sepharose bound fraction 50 μg. +GP, and -GP indicate that immunoblot analysis was carried out with and without HRP glycopeptides, respectively. Arrows indicate the glycoproteins carrying carbohydrate antigens recognized by the anti-HRP antibodies.

antigens in insects is confined to neural cells, the antigens in mammalian cells are widely distributed and are carried by four common proteins with the same molecular weight. These four glycoproteins were found in every tissue we examined, although the amount of antigen in the tissue was low. It is tempting to speculate that these four glycoproteins are expressed in most tissue and are involved in the maintenance of mammalian cells. To fully characterize these molecules, large scale preparation is required. Purification of these antigens and structural analysis of sugar and peptide moieties are in progress in our laboratory.

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