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Glycopeptide of P0 protein inhibits homophilic cell adhesion

Competition assay with transformants and peptides

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Expression of major myelin glycoprotein P0 by P0 cDNA transfection into C6 glioma cells promoted homophilic cell adhesion of the cells. After the dissociated cells were incubated for various times, the number of particles at each time point was measured. The total number of particles decreased to 24% in 60 min for transformant (C6P0) cells, in contrast to only 68% for control (C6P0') cells. To confirm the homophilic mechanism of adhesion, mixed-cell aggregation experiments were performed. Among the four synthetic peptides corresponding to a part of the P0 sequence used, only peptide 3 (residues 90–96), which contained a carbohydrate attaching site, caused considerable inhibition of cell aggregation (approximately 50%). In addition, the glycopeptide (residues 91–95) obtained from bovine P0 markedly inhibited cell aggregation (by approximately 85%).

P0; Homophilic adhesion; Immunoglobulin superfamily; Glycopeptide; Myelin

1. INTRODUCTION

P0 protein, a major myelin glycoprotein in mammalian peripheral nervous system (PNS), has an apparent molecular weight of 28,000 Da and is a member of the immunoglobulin superfamily [1–5]. Several molecules of the immunoglobulin superfamily, including L1, N-CAM, myelin-associated glycoprotein (MAG) and P0, play important roles in the development and regeneration of the nervous system. L1 and N-CAM are expressed by non-myelinating Schwann cells before the onset of myelination, whereas MAG and P0 are expressed only by myelinating cells [6]. P0 appears at the initial stage of myelination and contributes to the formation and maintenance of myelin compaction as an adhesion molecule. Recently direct evidence of homophilic adhesion of P0 protein has been reported on the basis of recombinant P0 expression studies [7–9]. We have also constructed a P0 expression vector under the control of the β-actin promoter, transfected this plasmid into C6 cells, and reported neurite outgrowth-promoting activity of P0 protein [10]. In this study, we prepared short synthetic peptides from different regions within P0 and the glycopeptide of P0 protein, and carried out competition analysis of the reaggregation assay of P0, in order to determine functional domains in the P0–P0 binding mechanism.

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2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The *Eco*RI site of expression vector pBact-STneoB [11] was methylated with *Eco*RI methylase. This plasmid was cleaved with *Sal*I, and blunt ended by Klenow enzyme. After joining an *Eco*RI linker to this site, the *Eco*RI fragment of the pSN63e plasmid, containing the full-length rat P0 cDNA [3], was isolated and cloned into the expression vector, pBactSTneoB (which possesses a strong chick β-actin promoter to promote inserted genes, and a neomycin-resistant gene driven by the SV40 promoter). The resultant plasmid is pBACTP0. The vector in which rat P0 cDNA was inserted in the opposite direction is referred to as pBACTP0'.

2.2. *DNA transfection*

C6 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For each dish, 10 μg of pBACTP0 or pBACTP0' was co-precipitated with calcium phosphate according to standard procedures [12]. After 3 h of incubation, the cells were glycerol-shocked (15% glycerol in HEPES) for 1–2 min at room temperature. Stable transformants were selected in medium containing 600g μg/ml G418 (Gibco). After limiting dilution of G418-resistant clones, cells were further screened for P0 expression by immunostaining with anti-P0 polyclonal antibody. Stable clone C6P0 and C6P0' are C6 cell transformants with pBACTP0 and pBACTP0', respectively.

2.3. *Production of antibodies*

Purified bovine P0 protein [4] was mixed with Freund's complete adjuvant (Difco) in PBS and injected into 3 rabbits at a dose of >300 μg of protein/injection. Rabbits were boosted with purified protein mixed with incomplete adjuvant in PBS at 3 and 6 weeks, and bled at 8 weeks.

2.4. *Immunocytochemistry*

C6 cells were plated on glass coverslips coated with poly-*L*-lysine. Cells were pre-fixed with 4% paraformaldehyde and then post-fixed with 5% acetic acid in ethanol. The cell preparation was incubated with anti-P0 polyclonal antibody (1:200). After PBS washing, the

samples were treated with secondary antibody (fluorescein isothiocyanate-labelled anti-rabbit IgG). These samples were examined with a Nikon fluorescence microscope.

2.5. *Western blot analysis*

Membrane fractions were obtained according to the method described by Miyawaki et al. [11]. The membrane fractions were boiled in SDS sample buffer (2% SDS, 25 mM Tris-HCl (pH 6.8), 5% mercaptethanol, 10% glycerol) for 5 min. Proteins were separated with 12.5% SDS-PAGE and transfected to an Immobilon filter (Millipore). The blots were processed by sequential incubation with blocking buffer (1% skimmed milk in PBS), anti-P0 polyclonal antibody (1:50 dilution), and alkaline phosphatase-conjugated anti-rabbit IgG. Final staining was carried out in 0.1M Tris-HCl (pH 9.5), containing 330 μg/ml Nitro blue tetrazonium, 165 μg/ml 5-bromo-4 chloro-3-indonyl phosphate, 10 mM $MgCl_2$ and 0.15 M NaCl.

2.6. *Synthesis of peptides and preparation of P0 glycopeptide*

The peptides (residues 43–50, 83–90, 90–96 and 100–107) of the rat P0 protein were synthesized with an ABI Model 430A peptide synthesizer by the Fmoc. method (sequences are presented in Table 1). Purification of the synthesized fragments was performed by HPLC (Jasco) with ODS-column (5 μ, 4.6 mm × 25 cm, Beckman) and the purity was determined with an ABI 473A protein sequencer [13]. P0 glycopeptide was purified from bovine P0 by Sephadex G-25 and G-5 columns. [14].

2.7. *Cell aggregation and competition assays*

Cells in monolayer culture were incubated with 2 mM EDTA in PBS for 15 min at room temperature and then dispersed by gentle pepetting. The cells were suspended in complete medium at 1×10^6 cells/ml and then transferred to polystyrene tubes. The cell suspension was incubated at 37° C, and aliquots were taken at 15-min intervals after mixing by several gentle inversions. The number of cell aggregates (particles) was counted in a hemocytometer. Aggregation was represented by the index N_t/N_0, where N_t and N_0 are the total number of particles at incubation times t and 0, respectively. For competition assay, one of the four synthetic peptides or P0 glycopeptide suspended at either 50, 100, 200 or 500 μg/ml was added in advance of making the cell suspension. To test homophilic binding of cells, control C6 cells (C6P0′) and P0-transfected C6 cells (C6P0) were labelled by incubation with 15 μg/ml DiI and DiO, respectively (Molecular Probes), for 2 h [15]. Cells were dispersed and suspended in complete medium as described above and then mixed at a 1:1 ratio in which total cell number was 5×10^5/ml. After 45 min of incubation at 37° C, aliquots were removed from polystyrene tubes, spotted on glass coverslips, and then fixed in 4% paraformaldehyde in PBS for 10 min.

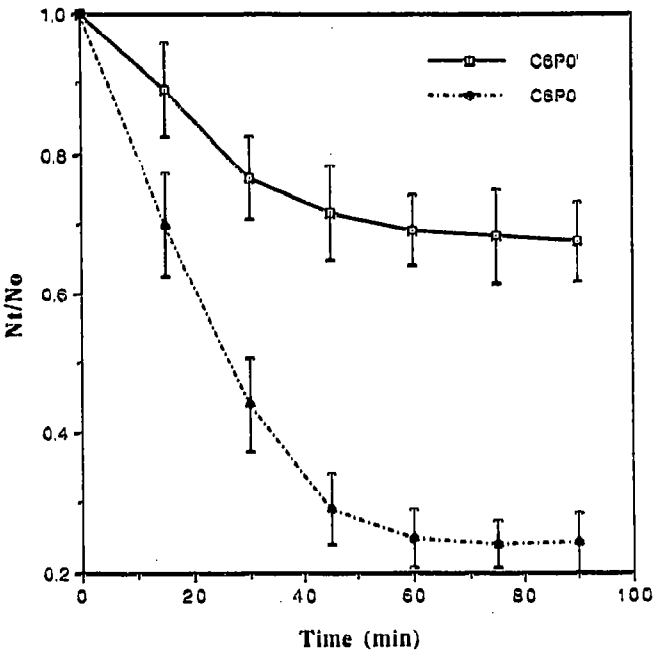

Fig. 1. Short-term aggregation of C6P0′ and C6P0 cells. Values are the mean ± S.E.M. for three independent experiments. N_t and N_0 are the total number of particles at incubation times t and 0, respectively. Rate of aggregation is represented by the index N_t/N_0. The total number of particles decreased 24% in 60 min for C6P0 cells, in contrast to only 68% for C6P0′ cells.

3. RESULTS

3.1. *Expression of rat P0 in glioma C6 cells*

A rat P0 expression vector (pBACTP0) was constructed by cloning the full-length sDNA of rat P0 into a vector (pBact-STneoB). Rat glioma C6 cells were chosen at the recipient cells because they do not produce P0 activity and also grow independently from other adhesive molecules in subconfluent culture. The expression of P0 on the membrane surface of stable clones was confirmed immunocytochemically using anti-P0 polyclonal antibody diluted 1:200, and FITC-conjugated anti-rabbit IgG diluted 1:200 as the second antibody. A distinct positive band, with an apparent molecular weight of 30 kDa was found in the membrane fraction of P0 transformants by Western blot analysis, using anti-P0 polyclonal antibody, as reported previously [10].

3.2. *P0 expression correlates with cell adhesion*

Whether or not the expression of P0 in C6 cells would promote cell–cell adhesion in these cells was tested in this study. In the first series of experiments, single cell suspensions were prepared by pipetting the cells in the presence of EDTA. Dissociated cells were resuspended in complete medium and incubated for various times, and the number of particles at each time point was measured. A decrease in the number of particles indicates increased aggregate formation. A significant difference was observed in the aggregation kinetics between C6P0 and C6P0′ cells (Fig. 1). The total number of particles decreased to 24% in 60 min for transformant (C6P0) cells, in contrast to only 68% for control (C6P0′) cells (Fig. 2a,b). The results presented here demonstrate a significant correlation of P0 expression with cell aggregation.

3.3. *P0 mediates homophilic cell adhesion*

Cell binding activities, in a homophilic manner, have been demonstrated by transfection experiments with some adhesion molecules in the immunoglobulin superfamily, including P0. However, the possibility remains that C6 cells express other molecules interacting with P0

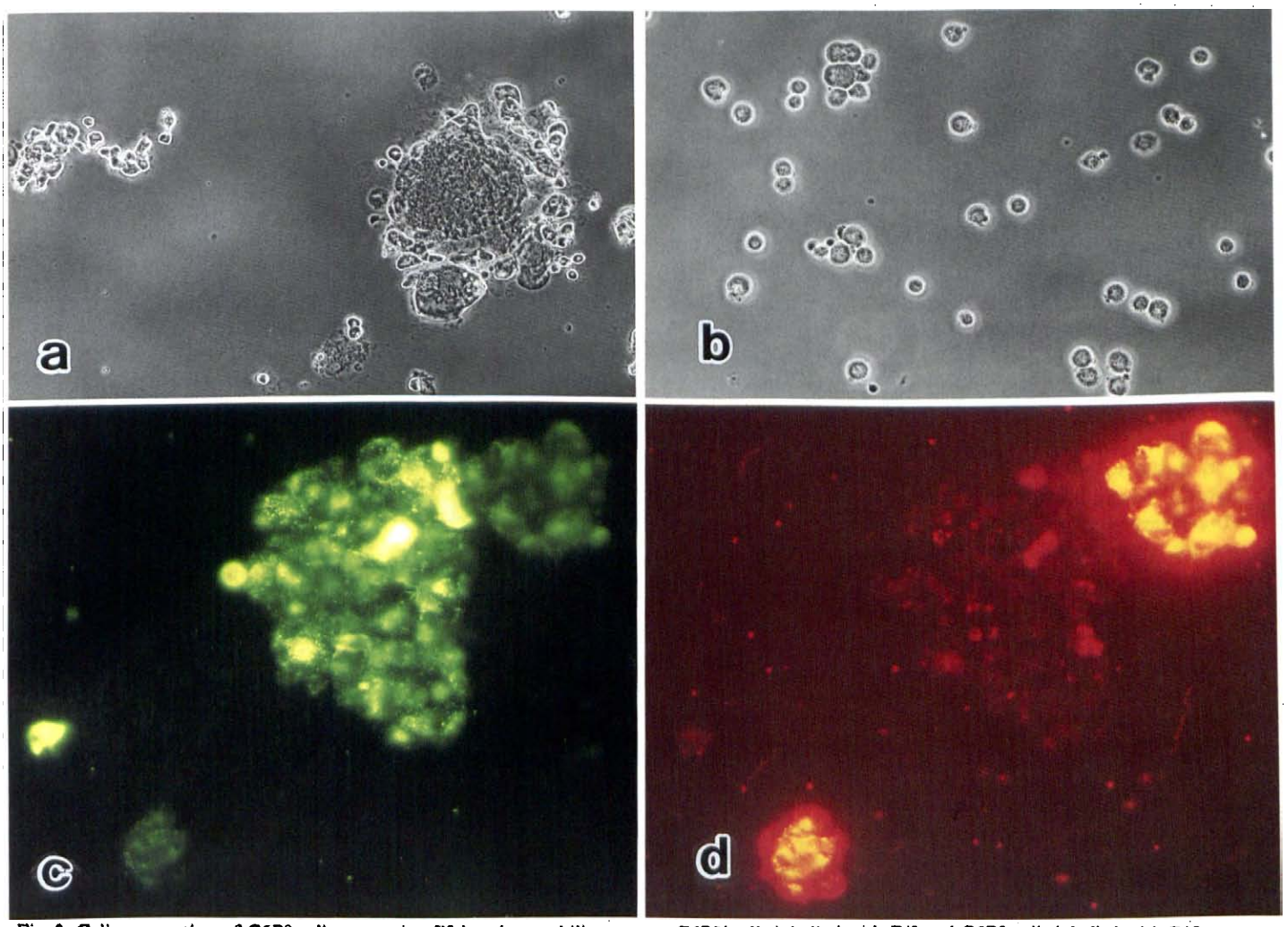

Fig. 2. Cell aggregation of C6P0 cells expressing P0 in a homophilic manner. C6P0′ cells labelled with DiI and C6P0 cells labelled with DiO were mixed and incubated for 45 min in complete medium. (a) Lower power photograph of C6P0 aggregated cells. (b) Lower power photograph of C6P0′ non-aggregated cells. (c) Fluorescence photograph using a B-2A filter cassette (Nikon). Fluorescence of both DiI- and DiO-positive cells is shown. (d) Fluorescence photograph using a G-2A filter cassette (Nikon). Only DiI-labelled cells were detected.

heterophilically to cause cell aggregation. To test this hypothesis, mixed-cell aggregation experiments with DiI-labelled C6P0′ cells and DiO-labelled C6P0 cells were performed. These cells were mixed at a 1:1 ratio and then allowed to aggregate. C6P0 cells expressing P0 adhered predominantly to each other to form aggregates, with the exception of attachment to a few C6P0′ cells at the periphery (Fig. 2c,d).

3.4. *Time-course of competition of cell–cell adhesion by P0 peptides*

To define the cell binding site of P0, we have prepared four synthetic peptides comprising 7 or 8 amino acids. Peptides 1, 2, 3 and 4 correspond to rat P0 amino sequence between Gly-43 and Ile-50, Ile-83 and Tyr-90, Tyr-90 and Phe-96 and Val-100 and Val-107, respectively (Table I). If the cell binding site of P0 resides within these sequences, these peptides would be expected to compete for P0 binding. A competition assay was used to examine the effect of the four synthetic peptides on P0–P0 binding. Only peptide 3 was distinctly capable of competing for cell binding; the other peptides had no effect on P0-expressed cell adhesion, except peptide 1 which had a slight effect. We then prepared the glycopeptide of P0 protein since peptide 3 includes a carbohydrate domain (Asn-93) of P0. This glycopeptide of P0 protein markedly inhibited cell adhesion of C6P0 cells (Fig. 3).

3.5. *Dose-dependent inhibition of cell–cell adhesion by P0 peptides*

If any of the synthetic peptides or the glycopeptide can recognize the homophilic binding site of P0, the binding of these peptides to the cell surface P0 molecules should interfere with cell–cell adhesion. To test this property, aggregation-stage cells were re-associated in

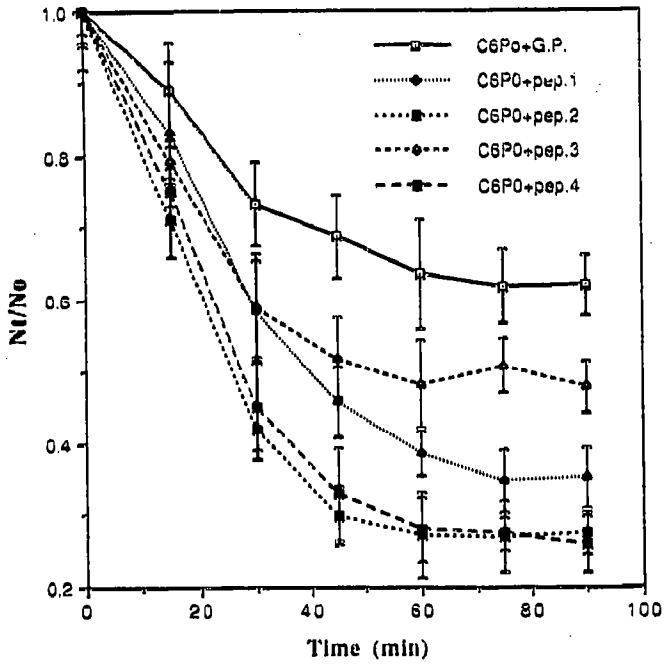

Fig. 3. Effect of synthetic peptides and purified P0 glycopeptide on P0–P0 binding. Four synthetic peptides or P0 glycopeptide were suspended at 500 μg/ml in advance of making the cell suspension. Of the four synthetic peptides, only peptide 3 (residues 90–96), which contained a carbohydrate attaching site, showed considerable inhibition (approximately 50%) of the cell aggregation. The glycopeptide markedly inhibited (by approximately 85%) the cell aggregation. In addition, peptide 1 (residues 43–50) had a slight inhibitory effect.

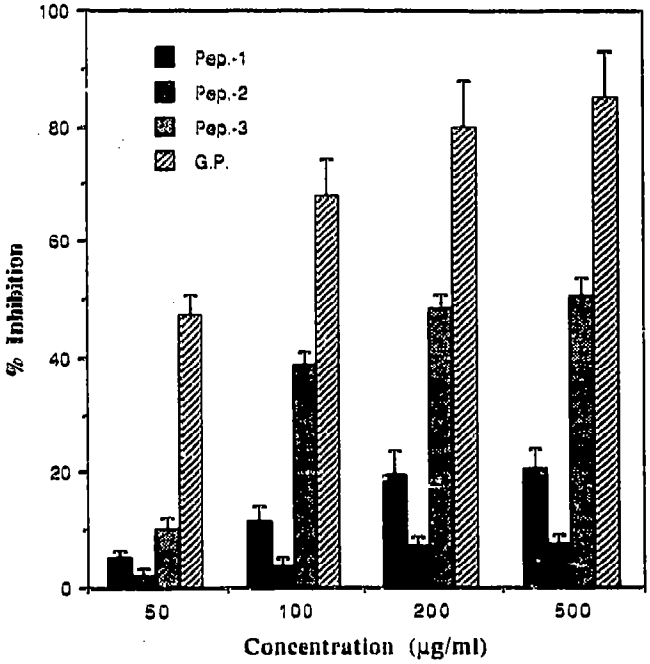

Fig. 4. Dose-dependent effect of synthetic peptides and P0 glycopeptide. Aggregation cells were re-associated in the presence of different concentrations of peptide 1, 2, 3 or glycopeptide. Dose dependency of cell adhesion inhibition to P0 peptides and glycopeptide is demonstrated.

the presence of different concentrations of peptide 1, 2, 3 or glycopeptide. The effect of peptide 1 and 3 and the glycopeptide on cell re-association were dose dependent, and cell aggregation was inhibited by approximately 20%, 50 and 85%, respectively, when these peptides were included in the assay medium, at 500 μg/ml. On the other hand, peptide 2 had no appreciable effect even at 500 μg/ml (Fig. 4).

4. DISCUSSION

The primary structures of rat [3], shark [16], chicken [17] and human [18] P0 proteins were deduced from the nucleotide sequences of their dDNAs, and the bovine P0 sequence was determined directly by protein chemical methods [4]. The P0 of 219 residues in mammalian peripheral myelin has a single transmembrane segment (125–150) with an amino-terminal domain containing an Asn (93)-*N*-linked carbohydrate unit [4]. Several post-translational modifications of P0 are known, such as glycosylation, acylation [19], phosphorylation [20] and sulfation. Because it is one of the simplest molecules in the immunoglobulin cell adhesion molecule superfamily [5], P0 presents an attractive model for defining the molecular details of adhesive interaction.

Transfection experiments of several neural cell adhesion molecules of the immunoglobulin superfamily, such as N-CAM [21, 22], F3/F11 [23], Fasciclin III [24], SC1 [25], and L1 [26], have demonstrated that these molecules adhere in a homophilic manner. To address the function of P0, several transfection experiments have been reported. Filbin et al. showed that Chinese hamster ovary (CHO) cells expressing P0, by transfection of P0 cDNA, showed at least twice the log value of magnitude for adhesion than control transfected cells [8, 9]. They also demonstrated that the adhesion was most likely to have been a result of P0 homophilic interaction, and not P0 interaction with some ubiquitous component in the membrane of opposing cells. D'Urso et al. [7] expressed P0 cDNa in HeLa cells under the

Table I

Sequence of synthetic peptides and the glycopeptide of P0 and effect on cell–cell adhesion

Peptide	Sequence	% inhibition
None		0
1	(43)GGRDAISI(50)	20.3
2	(83)IVIHNLDY(90)	7.6
3	(90)YSDNKGTF(96)	50.2
4	(100)VKNPPDIV(107)	5.1
	CHO	
5	(91)SDNGT(95)*	85.1

*Glycopeptide

control of the SV40 promoter, and traced the biosynthesis of P0 by analyzing the dynamics of P0 insertion into membranes. They further provided evidence that the extracellular domain of P0 insertion into membranes. They further provided evidence that the extracellular domain of P0 functioned homotypically to cause adjacent membrane surfaces of the P0-expressing HeLa cells to adhere. P0 was also distributed along the cell–cell borders in transformants, with the formation of desmosome-like structures between adhering cells occurring. A recent report of Filbin and Tennekoon demonstrated the importance of oligosaccharide moieties of P0 in its function as a homophilic adhesion molecule [27]. Expression of the complex form of P0 glycoprotein in transfected CHO cells greatly increased the adhesiveness of those cells, whereas expression of the high-mannose form of P0 glycoprotein in the carbohydrate-synthesizing, enzyme-deficient mutant cells did not. The importance of the structure of the oligosaccharide chain was emphasized in their report. Schneider-Schaulies et al. [29] demonstrated that the extracellular domain fragment of P0 was able to inhibit the adhesion of P0-expressing CV-1 cells to plates coated with the same extracellular domain. This extracellular P0 fragment lacked carbohydrate, because the soluble peptide was obtained using a bacterial expression system. P0 was able to mediate adhesion to unglycosilated immunoglobulin-like domains with other interacting P0 molecules. They presented two possibilities for this phenomenon; (i) the carbohydrate residue of P0 may play no role in adhesion, or (ii) the carbohydrate residues of one P0 molecule could interact with regions other than the carbohydrates of opposing P0 partner molecules. The existence of important domains other than the oligosaccharide chain, in the extracellular region of P0 protein, for cell adhesion, was suggested from their report. However, in the past, there has been no detailed analysis of the functional domain of P0 in the extracellular region by the competition experiment using synthetic peptides.

As a first step in this study, we confirmed the ability of P0 as an homophilic adhesion molecule using P0-expressing C6 cells prepared previously [10]. To determine the minimum sequence required for the P0 binding activity, four different synthetic peptides (peptide 1–4)) were prepared and used in our studies. All peptides were from the amino-terminal domain and only peptide 3 included the glycosylation site of Asn-93. We have presented here evidence, that peptide 3 considerably inhibited cell aggregation of transformants and peptide 1 showed slight inhibition by competition assay. Further, the glycopeptide fraction purifed from P0 had a much stronger ability to inhibit P0-P0 cell aggregation (Fig. 3). The effect of these peptides were compared on the same weight basis in this experiment. When we compared the abiility by molar basis, inhibition of the glycopeptide was much stronger, because the molecular weight of glycopeptide is about four times as large as those of other peptides. These results suggest that the oligosaccharide chain of P0 is the most important for the homophilic adhesion of P0 protein. In addition, the neighboring peptide of the oligosaccharide attaching site is also important in P0-P0 cell adhesion. Also, there remains the possibility that other domains, such as peptide 1, may assist cell–cell binding involving P0.

The carbohydrate chain of P0 reacted with HNK-1 /L2 antibody, and was separated into at least five subfractions by ion-exchange chromatography. The common carbohydrate structure of the glycopeptide is Gal1-4GlcNAc1-2Man1-3 (Man1-3Man1-6)Man1-4GlcNAc1-4(Fuc1-6)GlcNAc-Asn. The micro-heterogeneity appeared to be due to extra sialic acid, sulfate and glucuronic acid contents [28]. In further studies, we will be comparing the inhibition of cell adhesion by the addition of oligosaccharide subfractions or other peptides of the P0 extracellular region. The active domain in the carbohydrate chain will be also analyzed in the near future. On the other hand, we recently demonstrated the neurite outgrowth-promoting activity of P0 protein using P0-expressing C6 cells and co-cultured dorsal root ganglion (DRG) neurons [7]. Because DRG neurons do not express P0, it is suggested that the extracellular domain of P0 can bind with a receptor on neurons heterophilically. Kadmon et al. [30] proposed a cis-binding interaction between other molecules of the immunoglobulin superfamily (N-CAM and L1) naming it "assisted homophilic' interaction". Miura et al. [26] suspected that the L1-N-CAM type of adhesion plays important roles in the regulation of binding in cell interaction. It is tempting to speculate that P0 is not only involved in homophilic adhesion but also mediates a heterophilic trans-binding mechanism.

In conclusion, we have demonstrated that the interaction between one P0-oligosacchalide chain and another P0-oligosaccharide chain coupling with the neighboring peptides, is most important in its functioning as a homophilic adhesion molecule, and that a second molecule, such as peptide 1, could be involved in the stabilization of the P0–P0 interaction.

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