Journal of Neuroscience Research 27:286–297 (1990)

Recombinant Peripheral Myelin Protein P_0 Confers Both Adhesion and Neurite Outgrowth-Promoting Properties

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To probe into the functional properties of the major peripheral myelin cell surface glycoprotein P_0, its ability to confer adhesion and neurite outgrowth-promoting properties was studied in cell culture. To this aim, P_0 was expressed as integral membrane glycoprotein at the surface of CV-1 cells with the help of a recombinant vaccinia virus expression system. Furthermore, the immunoglobulin-like extracellular domain of P_0 (P_0-ED) was expressed as soluble protein in a bacterial expression system and used as substrate coated to plastic dishes or as competitor in cell adhesion and neurite outgrowth-promoting assays. The adhesion of P_0-expressing CV-1 cells to P_0-ED substrate was specifically inhibitable by polyclonal P_0 antibodies (54% ± 6%). In addition, the specific interaction between P_0 molecules could be reduced (49% ± 8%) by adding soluble P_0-ED to the culture medium, demonstrating that the homophilic interaction between recombinant P_0 molecules can be mediated, at least on one partner of interacting molecules, by the unglycosylated Ig-like domain. Substrate-coated P_0-ED also conferred adhesion and neurite outgrowth ability to dorsal root ganglion neurons with neurites of a mean length of about 150 μm. This neurite outgrowth was specifically inhibitable by soluble P_0 (74% ± 14%) and P_0 antibodies (65% ± 9%). These observations indicate that P_0 is capable of displaying two different types of functional roles in the myelination process of peripheral nerves: The heterophilic interaction with neurons may be responsible for the recognition between axon and myelinating Schwann cell at the onset of myelination, whereas the homophilic interacton may indicate its role in the self-recognition of the apposing loops of Schwann cell surface membranes during the myelination process and in the mature compact myelin sheath.

Key words: immunoglobulin superfamily, peripheral nervous system, vaccinia virus, P_0

INTRODUCTION

Neuronal contacts are established with a considerable degree of precision during development and regeneration of the peripheral nervous system (PNS). Several cell surface molecules belonging to the gene families of cadherins, integrins, and immunoglobulin-like molecules contribute to adhesion and neurite outgrowth functions by the glial cells of the PNS, the Schwann cells (Bixby et al., 1988; Tomaselli et al., 1988; Seilheimer and Schachner, 1988; Johnson et al., 1989). The adhesion molecule L1 has been described to play a major role in the Schwann cell-dependent neurite outgrowth of dorsal root ganglion neurons (Seilheimer et al., 1989a) and in the onset (Seiheimer et al., 1989b) and process of myelination (Wood et al., 1990). L1 and N-CAM are expressed by nonmyelinating Schwann cells already before the onset of myelination, whereas myelin-associated glycoprotein (MAG) and P_0 become detectable after myelination has been initiated (Trapp et al., 1981; Martini and Schachner, 1986; Martini et al., 1988). The PNS-specific glycoprotein P_0 is the most abundant peripheral myelin glycoprotein and accounts for over 50% of the protein present in purified peripheral myelin (Greenfield et al., 1973). P_0 has an apparent molecular weight of 28–30 kD and is one of the smallest members of the immunoglobulin superfamily, consisting of 122 amino acids (aa) of a V-like Ig-domain carrying one canonical acceptor sequence for N-linked glycosylation, 24aa of a transmembrane region and 69aa of an intracellular part with a net charge of 15 positive amino groups (Lemke and Axel, 1985; Lemke et al., 1988; Uyemura et al., 1987). P_0 has been suggested to act as homotypic adhe-

Received July 16, 1990; accepted July 26, 1990.

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sion molecule compacting the adjacent membranes of myelin (Braun, 1984; Lemke and Axel, 1985), but direct experimental evidence was missing. In an attempt to define the functional role of P_0 at the cell surface, P_0 was transfected into host cells and found to induce the formation of desmosome-like structures between adhering cells (D'Urso et al., 1990; Filbin et al., 1990) and to enhance aggregation over mock-transfected cells (Filbin et al., 1990). These observations suggested an involvement of P_0 in homophilic interactions but failed to give conclusive evidence, since specific inhibition experiments of this interaction by antibodies to P_0 or by soluble fragments of the P_0 molecule were not performed. In the present study, we show by specific inhibition and competition experiments using recombinant DNA technology that P_0 not only acts as homophilic adhesion molecule but mediates heterophilic interactions with neurons.

MATERIALS AND METHODS

Analytical Procedurse

For Western blot analysis of the vaccinia virus clones, tissue culture dishes (3 cm in diameter) covered with a monolayer of CV-1 cells were infected with a multiplicity of infection (moi) of 0.1 for 48 hr. Cells were harvested with a cell scraper and solubilized in 400 μl gel loading buffer (125 mM Tris, pH 6.8, 50 mM β-mercaptoethanol, 10% glycerol, 2.5% sodium dodecyl sulfate [SDS], 0.2 mg/ml bromophenol blue). Lysates (40 μl per lane) were separated by SDS-PAGE on 12% slab gels (Laemmli, 1970), electrophoretically blotted onto nitrocellulose filters (Towbin et al., 1979), and stained with rabbit polyclonal antibodies raised against the extracellular domain of P_0 obtained by expression in a bacterial system. Protein determinations were performed as described (Bradford, 1976) or by comparing with bovine serum albumin (BSA) standards by SDS-PAGE. Staining of protein bands was performed by the reducing silver method (Oakley et al., 1980).

Antibodies and Indirect Immunofluorescence

Polyclonal antibodies against the extracellular domain of P_0 (P_0-ED) were prepared in rabbits by immunization with FPLC-purified P_0-ED (see below) (100 μg in complete Freund's adjuvant for the first injection and 50 μg in incomplete Freund's adjuvant for the subsequent injections). Rabbits were bled 7 days after the last injection, starting with the third injection. An IgG fraction was obtained by passing the $(NH_4)_2SO_4$ cut of the serum over a protein A Sepharose (LKB/Pharmacia) column. Polyclonal antibodies to mouse liver membranes (Lindner et al., 1983) reacting with various cell surface constituents of rat cells were used as controls.

Indirect immunofluorescence of CV-1 cells infected with P_0 expressing vaccinia virus recombinants (moi = 0.1) for 48 hr was essentially carried out as described (Schnitzer and Schachner, 1981). In brief, after a 5 min fixation with 4% formaldehyde cells were incubated for 30 min with the IgG fraction (100 μg/ml) of polyclonal rabbit antibodies to P_0-ED, all at room temperature, and visualized by fluorescein-coupled goat antirabbit immunoglobulin antibodies (1:100 diluted) (Cappel).

Expression of P_0 by Vaccinia Virus-Infected CV-1 Cells

The EcoRI/XbaI fragment of plasmid pSN63c (Lemke and Axel, 1985) containing the complete coding region of the cDNA of P_0, was cloned into the recombination plasmids p7.5k131 and p11KATA+18 (Mackett et al., 1984; de Magistris and Stunnenberg, 1988; v. Brunn, 1989). After transfection of recombination plasmids and wild-type vaccinia virus DNA into $HuTK^-$ cells by the calcium phosphate precipitation method (Gorman, 1985) and superinfection with temperature-sensitive vaccinia virus mutant ts7 (Drillien and Spehner, 1983), recombinants were selected by growth at 39.5°C. Recombinants were pooled and reselected by addition of 100 μg/ml bromodeoxyuridine (BrdU) to the medium. Several recombinants were cloned by three rounds of plaque purification using low melting point agarose (Seaplague agarose; Biozym Diagnostik), which was overlayed on monolayers of CV-1 cells. Over 90% of the clones were strongly positive for P_0 as indicated by Western blotting.

Production of P_0-ED in a Prokaryotic Expression System

The 399 bp BalI/DpnI fragment of P_0 cDNA (Lemke and Axel, 1985) containing the Ig-like extracellular domain of P_0 without leader sequence and transmembrane region was ligated to the 123 bp Pst/XbaI fragment of nontranslated sequences of P_0 cDNA. Ligation of the DpnI blunt end to the PstI end, made blunt-ended by T4-DAN polymerase, leads to five additional artificial amino acids (Ser, Glu, Ile, Pro, Glu) in frame of translation followed by the stop codon at position 913 of the original cDNA (Lemke and Axel, 1985). The recombinant P_0-ED cDNA was then ligated to BamHI linkers and cloned in the right orientation into the BamHI site of pET3b (Rosenberg et al., 1987), hereafter designated $pETP_0$-ED. The in frame cloning was verified by DNA sequencing. Expression of the 17.1 kD recombinant product was carried out as described elsewhere (Rosenberg et al., 1987). In brief, 100 ml culture of plasmid $pETP_0$-ED-containing bacteria (BL21[DE3]) from an overnight culture were added to 900 ml LB medium (1% bactotryptone, 0.5% bactoyeast extract, 1% NaCl) and

induced with 10 mM isopropylthio-β-D-galactoside (IPTG) for 6 hr to express high levels of T7-polymerase transcribing the recombinant P_o-ED mRNA. A crude lysate from 1 liter bacteria contained approximately 10 mg P_o-ED protein as determined by SDS-PAGE.

Purification of P_o-ED

P_o-ED-expressing bacterial cultures (1,000 ml) were centrifuged at 4°C and 3,000*g* for 10 min and fractionated by three cycles of freeze-thawing (liquid nitrogen/37°C) and centrifuged at 40,000*g* for 20 min to isolate the insoluble P_o-ED in form of inclusion bodies. The insoluble bacterial pellet was incubated overnight at 4°C with 40 ml extraction buffer (4 M urea, 150 mM Tris, pH 7.5). After addition of 100 mM β-mercaptoethanol, the protein extract was size fractionated on a Superose 12 column using the FPLC system. P_o-ED-containing fractions were then concentrated by filtration through Amicon filters (PM 10) to give a final protein concentration of 5 mg/ml. The fraction was denatured at room temperature with 300 mM dithioerythritol (DTE), 6 M guanidinium HCl, 100 mM Tris HCl, pH 8.0, and reoxidized for 24 hr by 5 mM oxidized glutathione (Calbiochem) in 100-fold excess volume to allow refolding of the molecule (Buchner and Rudolph, 1989). After this renaturation procedure, P_o-ED remained soluble in physiological buffers. SDS-PAGE using 15% slab gels revealed one major band (see Fig. 1B).

Adhesion Tests

The aggregation of vaccinia virus-infected CV-1 cells was studied in the presence of 0.1 (low Ca^{2+}) and 1 (high Ca^{2+}) mM Ca^{2+} ions in Hank's balanced salt solution (HBSS) containing 40 mM HEPES, pH 7.2, 0.1% BSA, and 0.1 mg/ml DNase. Number and size of aggregates were quantified by Coulter counter analysis as described (Kadmon et al., 1990a,b). Cell-to-substrate adhesion tests were carried out as described (Keilhauer et al., 1985). The following substrates were used: FPLC-purified P_o-ED, rat tail collagen (Boehringer Mannheim), and poly-L-lysine, all at a concentration of 100 μg/ml in 100 mM phosphate buffer, pH 7.2. Substrate-containing solutions were applied to plastic tissue culture dishes (3 cm in diameter) in 5 μl droplets (three spots each) incubated for 16 hr at 37°C and washed three times with Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS). Unsaturated protein binding sites were blocked by incubating the dishes with RPMI-1640 medium (Gibco) containing 10% fetal calf serum for 30 min at room temperature. For the determination of cell adhesion to the coated substrates, CV-1 cells infected for 20 hr with P_o-expressing recombinant vaccinia virus (moi = 1.0) or wild-type vaccinia virus (moi = 1.0) were used. Infected cells were removed from the culture dishes by gentle pipetting with CMF-HBSS and incubated in the substrate-coated dishes at densities of 5×10^{-5} cells/ml in 2 ml RPMI 1640 medium containing 10% fetal calf serum for 16 hr at 37°C in a CO_2 incubator in the absence or presence of the following compounds at a concentration of 300 μg/ml: IgG fraction of antibodies to P_o-ED or mouse liver membranes and FPLC-purified P_o-ED. Culture dishes were then washed carefully three times with CMF-HBSS and remaining cells were fixed with 4% formaldehyde for 5 min at room temperature. Adhering cells were counted in photomicrographs. For every spot, three microscopic fields corresponding to an area of 1 mm^2 were evaluated. Percentage inhibition of adhesion was calculated using the formula

$$\% \text{ inhibition} = \frac{\text{number of adhering cells (control)} - \text{number of adhering cells (with additive)}}{\text{number of adhering cells (control)}} \times 100$$

Statistical evaluation was performed using Student's t test.

Preparation of Dorsal Root Ganglion Neurons

Cultures of dorsal root ganglion neurons were established from 1-day-old NMRI mice as described (Seilheimer and Schachner, 1988). After incubation of dorsal root ganglia with 0.03% collagenase (Sigma) and 0.5% trypsin (Sigma) for 45 min at room temperature, cells were mechanically dissociated by pipetting up and down in a fire-polished Pasteur pipette and centrifuged through a cushion of 35% Percoll (Sigma) in CMF-HBSS for 20 min at 200*g* to separate large neurons from other contaiminating cells. Neurons were plated into eight-well plastic chamber slides (Nunc) at a cell density of 1×10^3/well in 200 μl basal medium Eagle's (BME) containing 10% horse serum and 50 ng/ml NGF (7s NGF from mouse submaxillary gland; Boehringer Mannheim). The cultures contained 90–95% cells with large, round cell bodies and typically neuronal morphology.

Neurite Outgrowth Test

To determine the extent of neurite outgrowth, eight-well plastic chamber slides were coated overnight at 37°C with collagen, purified P_o-ED, poly-L-lysine, BSA, IgG fraction of polyclonal antibodies to P_o-ED or combinations of these substrates at concentrations of 100 mg/ml each in 100 mM phosphate buffer, pH 7.2. After three washings with CMF-HBSS, dorsal root ganglion neurons were seeded at a concentration of 1×10^3/well in 200 μl BME containing 10% horse serum and 50 ng/ml nerve growth factor (NGF) into the substrate-coated wells. After 20 hr at 37°C, slides were gently washed three times with PBS, fixed with 2.5% glutaraldehyde in PBS, and stained with 0.5% toluidine blue O

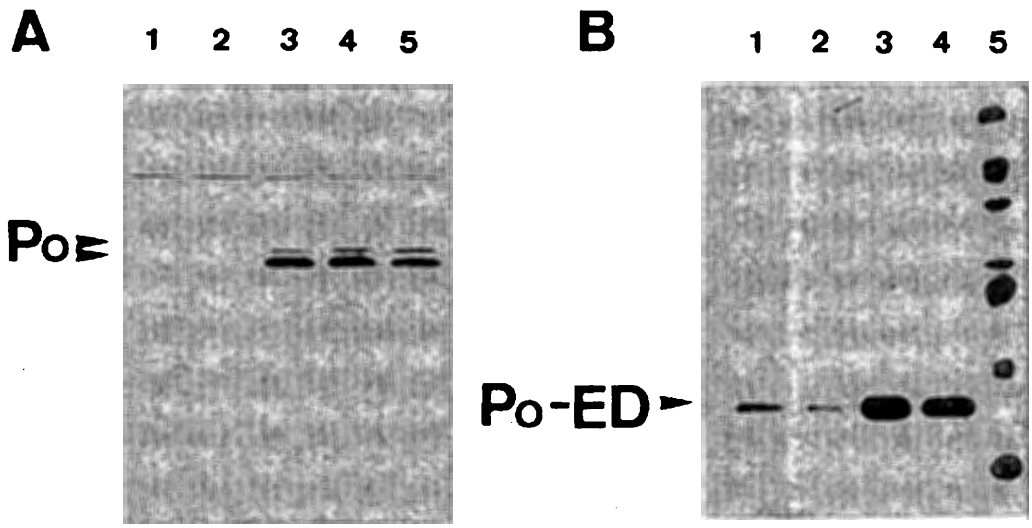

Fig. 1. Expression of recombinant P_0 by vaccinia virus infected CV-1 cells (**A**) and by the bacterial expression system (**B**). A: Western blot analysis of total homogenate of CV-1 cells infected with recombinant P_0 expressing vaccinia virus (100 μg total protein per lane). **Lane 1:** CV-1 cells infected with wild type vaccinia virus 24 hr after infection; **lanes 2–5:** CV-1 cells infected with recombinant P_0 vaccinia virus 6 (lane 2), 20 (lane 3), 30 (lane 4) and 48 (lane 5) hr, after infection. Proteins were separated by SDS-PAGE using a 12% slab gel and visualized after electroblotting onto nitrocellulose sheets by developing with an IgG fraction of polyclonal antibodies to P_0 (300 μg/ml). B: SDS-PAGE separation of FPLC-purified recombinant extracellular domain of P_0 (P_0-ED) using a 15% slab gel and staining by the reducing silver method. **Lanes 1, 2:** 0.5 μg each. **Lanes 3, 4:** 5 μg each of two independent preparations of P_0-ED, with an apparent molecular weight of 17.1 kD. **Lane 5:** Low-molecular-weight marker proteins with molecular weights of 66, 45, 36, 29, 24, 20.1, and 14.2 kD.

(Sigma) in a 2.5% Na_2CO_3 solution to visualize cell bodies and neurites. The total length of neurites, number of processes, and number of branch points per neuron were measured with a computer equipped with a graphic tablet and cursor, TV camera, color monitor, and image analysis program (VIDS III, AI tektron, Meerbusch, Federal Republic of Germany). Forty cells each from three independent experiments were measured for statistical evaluation, and for estimation of significance levels the U test of Mann and Whitney was used.

RESULTS

Expression of Recombinant P_0 in Eukaryotic Cells

Since several attempts to incorporate P_0 from human sciatic nerve (Bollensen and Schachner, 1987) into liposomes in functionally active form and to direct surface expression of P_0 in transfected cells in amounts detectable by immunofluorescence had not been successful, we chose the vaccinia virus expression system because of the possibility to express high amounts of glycosylated recombinant protein at the surface of live cells. The vaccinia virus system also has the advantage of infecting virtually all recipient cells in a culture dish at the appropriate moi. The synthesis of the P_0 glycoprotein by CV-1 cells infected with vaccinia virus recombinants was determined by Western blot analysis. The time course of glycoprotein expression reveals detectable P_0 at 28.5 kD apparent molecular mass already at 20 hr (Fig. 1A, lane 3) and high levels of P_0 at 30 and 48 hr (Fig. 1A, lane 3) after infection. In some experiments, a less prominent band at 29 kD specifically recognized by P_0 antibodies was also detected (Fig. 1A). This band probably represents the intermediate high-mannose-type oligosaccaride chain containing P_0 before it is biosynthetically trimmed and terminal sugars are added (Poduslo et al., 1985; D'Urso et al., 1990).

CV-1 cells infected for 48 hr with recombinant vaccinia virus expressed P_0 at levels detectable by indirect immunofluorescence staining with antibodies to P_0 (Fig. 2B). The specific expression of P_0 on the cell surface enabled us to investigate its function in cell-to-cell or cell-to-substrate interactions. Cells infected with wild-type vaccinia virus did not show any immunofluorescence staining with P_0 antibodies (Fig. 2D).

Expression of the Extracellular Domain of Recombinant P_0 in Prokaryotic Cells

The Ig-like extracellular domain of P_0 (P_0-ED) was expressed in a bacterial expression system to use it as culture substrate and soluble competitor. Analysis of the FPLC-purified bacterial expression product P_0-ED by

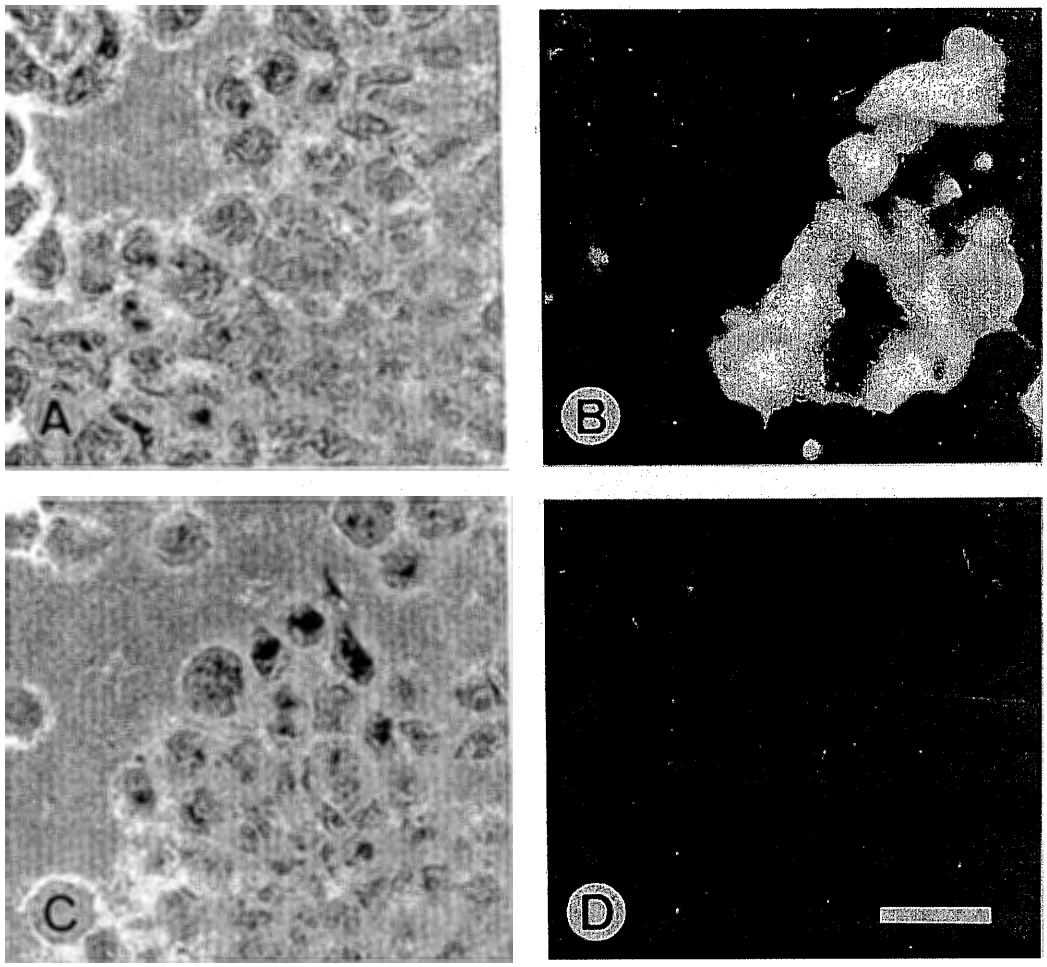

Fig. 2. Indirect immunofluorescence localization of P_o on CV-1 cells infected with recombinant vaccinia virus. Phase-contrast micrograph (**A**) and corresponding immunofluorescence image (**B**) of CV-1 cells 48 hr after infection with recombinant vaccinia virus (moi 0.1). Phase-contrast micrograph (**C**) and corresponding immunofluorescence image (**D**) of CV-1 cells 48 hr after infection with wild-type vaccinia virus (moi 0.1). Cells were processed in parallel with A and B. Bar = 10 μg.

SDS-PAGE revealed a single band at 17 kD, which is in accordance with the calculated molecular weight of 17.1 kD (Fig. 1B). P_o-ED purified in 4 M urea by Superose 12 column chromatography did not remain soluble when dialyzed against aqueous solutions without urea or detergents and could, therefore, not be used in experiments with living cells. To obtain P_o as a molecule soluble in physiological buffer solutions and with proper folding of its single Ig-like domain, we used a denaturation-renaturation procedure (Buchner and Rudolph, 1989) similiar to procedures established for the preparation of active antibody fragments expressed in prokaryotes (Huston et al., 1988; for review, see Schein, 1989). After applying this denaturation-renaturation procedure, P_o-ED was soluble in physiological buffers.

Demonstration of a Homophilic Adhesion Mechanism

To determine whether P_o is a homophilic adhesion molecule, recombinant P_o vaccinia virus-infected CV-1 cells were allowed to aggregate with each other and size and number of aggregates quantified by Coulter counter analysis. Aggregation was measured in the presence of low and high Ca^{2+} concentrations to distinguish between Ca^{2+}-dependent and -independent adhesion mechanisms. For control, wild-type vaccinia virus-infected CV-1 were allowed to aggregate. But particle sizes and numbers of aggregates detectable after 30 min and 2 and 16 hr were not found to be different for P_o vs. wild-type infected CV-1 cells (data not shown). It is likely that

other adhesion molecules present on the surface of uninfected or wild-type vaccinia virus-infected cells are functionally so dominant that an increment contributed by P_o to the aggregation may not have been detectable. We therefore tried to measure the adhesion of P_o vaccinia virus-infected cells to P_o-ED coated as substrate onto tissue culture dishes.

P_o-expressing cells were allowed to attach to P_o-ED, rat tail collagen, or poly-L-lysine for 16 hr at 37°C (Fig. 3). This cell-to-substrate adhesion test clearly revealed specific and significant homophilic adhesion of P_o expressing cells (Table I). Polyclonal P_o antibodies specifically inhibited adhesion of P_o expressing cells to the P_o-ED substrate by 54% ± 6% (n = 6), whereas adhesion to collagen of poly-L-lysine was only weakly inhibited by 15% ± 8% (n = 6) or even slightly enhanced by −5% ± 6% (n = 3) (Fig. 4, columns 1, 2 and 3, respectively). Soluble P_o-ED also specifically inhibited the interaction of P_o-expressing CV-1 cells to substrate bound P_o-ED by 49% ± 8% (n = 6) (Fig. 4, column 8), whereas soluble P_o-ED did not affect the adhesion to collagen (Fig. 4, column 7) or poly-L-lysine (Fig. 4, column 9) by more than 9%. Addition of polyclonal antibodies raised against mouse liver membranes did not exhibit P_o-specific inhibition of adhesion but rather showed a more generalized effect by inhibiting adhesion on collagen by 23% ± 8% (n = 6), on P_o-ED by 22% ± 10% (n = 6), and on poly-L-lysine by 24% ± 4% (n = 3) (Fig. 4, columns 13, 14, 15, respectively). Wild-type vaccinia virus-infected CV-1 cells showed no significant P_o-specific inhibition of cell-to-substrate adhesion (Table I and Fig. 4, columns 4–6, 10–12, and 16–18). Thus inhibition of adhesion of P_o-expressing cells to a P_o-ED substrate by P_o antibodies and soluble P_o-ED indicates a homophilic binding mechanism for P_o.

Demonstration of a Heterophilic Adhesion Mechanism

Since P_o is expressed on the Schwann cell surface at the onset of myelination and might, therefore, contribute to Schwann cell–axon interactions via heterophilic binding mechanisms (Martini and Schachner, 1988), we decided to study this mechanism by the sensitive neurite outgrowth promotion assay. Purified P_o-ED was used as substrate for determination of neurite outgrowth from dorsal root ganglion neurons. Rat tail collagen, poly-L-lysine, and BSA served as control substrates (Fig. 5).

For quantitative evaluation of neurite outgrowth patterns, single neurons without contacts to other cells were measured using a semiautomated image analysis system. Mean values of total length of neurites, neurite length per neuron, number of neurites, and number of neurite branch points per neuron are shown in Table II. Neurite outgrowth on P_o-ED was as extensive as on poly-L-lysine. Essentially no outgrowth of neurites was measurable when P_o antibodies or BSA were coated as substrates. Outgrowth of neurites on the P_o-ED substrate was drastically reduced in the presence of P_o antibodies, whereas these antibodies did not interfere with neurite outgrowth on collagen or poly-L-lysine. Addition of soluble P_o-ED to the culture medium did not inhibit the outgrowth of neurites of collagen or poly-L-lysine but reduced neurite outgrowth on the P_o-ED substrate. The number of processes per neuron was essentially the same on the collagen and P_o-ED substrates, with most neurons bearing one or two processes (1.38 ± 0.66 and 1.15 ± 0.58, respectively), whereas poly-L-lysine gave rise to a significantly higher number of processes per neuron, with most neurons bearing three neurites (2.73 ± 0.63). The number of neurite branch points per neuron showed a tendency for straight, unbranched neurites on the P_o-ED substrate (0.81 ± 0.31), whereas on collagen or poly-L-lysine neurites showed a more elaborate branching pattern (1.60 ± 0.40 and 1.75 ± 0.56, respectively).

Diagrammatic representations of these experiments are shown in the cumulative frequency distribution plot, where the percentage of neurons with neurites greater than a certain length (≥ length x) is given as a function of neurite length (Fig. 6). Neurite outgrowth on P_o-ED was similar to that of poly-L-lysine. The inhibition of neurite outgrowth on P_o-ED by antibodies to P_o was reflected by a curve significantly shifted to lower values of neurite lengths. The reduction of the mean neurite length per neuron on P_o-ED from 151 μm ± 27 μm to 44 μm ± 13 μm in the presence of P_o antibodies corresponded to an inhibition of neurite outgrowth by 65% ± 9%. Soluble P_o-ED also strongly reduced neurite outgrowth on substrate-bound P_o-ED from 151 μm ± 27 μm to 39 μm ± 21 μm, corresponding to an inhibition of 74% ± 14%. Soluble P_o-ED did not significantly inhibit neurite outgrowth on collagen or poly-L-lysine. Thus P_o also interacted with neurons via a heterophilic binding mechanism.

DISCUSSION

The vaccinia virus expression system provided high levels of surface expression of P_o and was, therefore, exceptionally useful for probing the functional role of this glycoprotein. We used cells that were harvested in the time window of 20–30 hr after infection for the cell-to-substrate adhesion test, avoiding longer times of infection (≥48 h), which would have led to lysis of cells. Western blot analysis showed the expected expression of a processed and glycosylated mature P_o with a molecular weight of 28.5 kD. This protein band was also recognized by polyclonal antibodies to the extracellular domain of P_o expresed in bacteria. Furthermore, P_o could

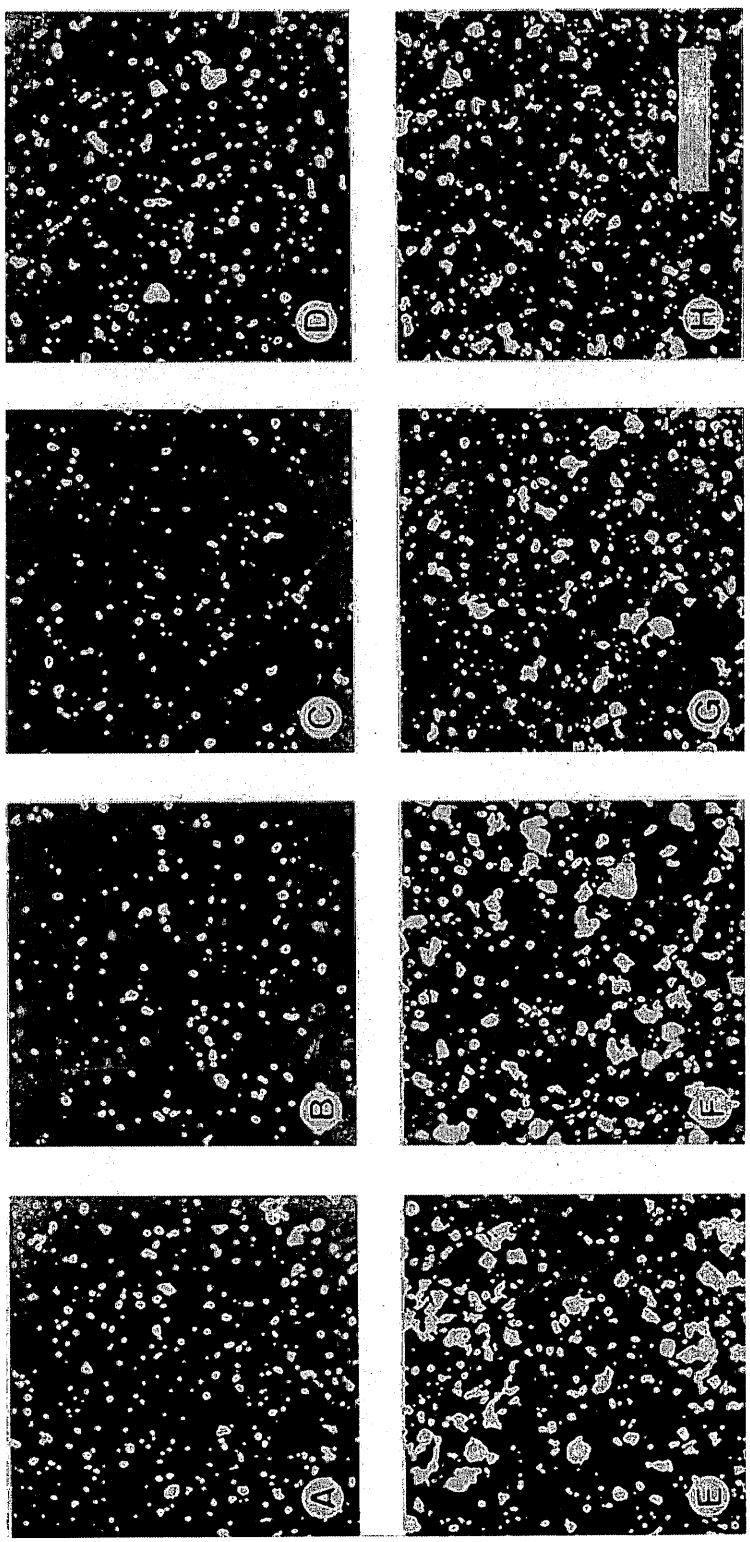

Fig. 3. Binding of CV-1 cells to substrate bound P_o-ED and collagen 16 hr after infection with recombinant P_o vaccinia virus. P_o expressing CV-1 cells were allowed to attach to the following substrates: P_o-ED (**A, B, C, D**) and collagen (**E, F, G, H**). A, E, no additives; B, F, in the presence of 300 μg/ml polyclonal P_o antibodies; C, G, in the presence of 300 μg/ml soluble recombinant P_o-ED; D, H, in the presence of 300 μg/ml polyclonal antimouse liver membrane antibodies. Sixteen hours after addition of cells to the substrates, cells were fixed with 4% formaldehyde, and photographs were taken for quantitative evaluations. Bar = 400 μm.

TABLE I. Adhesion of Vaccinia Virus-Infected CV-1 Cells to Various Substrates†

Substrates	CV-1 cells infected with vaccinia virus	Percent of cells bound[a]	Percent inhibition of adhesion in the presence of [b]		
			Anti-P_o IgG	Soluble P_o-ED	Anti-liver IgG
Collagen	P_o	68 ± 18	15 ± 8	8 ± 11	23 ± 8
P_o-ED	P_o	51 ± 10	54 ± 6**	49 ± 8**	22 ± 10*
PLL	P_o	100 ± 22	−5 ± 6	7 ± 6	24 ± 4
Collagen	wt	79 ± 6	−8 ± 8	3 ± 11	5 ± 8
P_o-ED	wt	41 ± 9	14 ± 9	−3 ± 8	6 ± 7
PLL	wt	118 ± 19	3 ± 9	11 ± 9	18 ± 3

†Numbers are mean values from three independent experiments ± standard deviations.
[a]The percentage of cells bound from total number of input cells per well (1×10^6) was 23% ± 8%.
[b]For each substrate, values are referred to the adhesion values without additives. P_o-ED, recombinant extracellular domain of P_o; PLL, poly-L-lysine; wt, wild-type.
*Significance by Student's t test is $0.05 < \alpha < 0.1$.
**Significance by Student's t test is $0.005 < \alpha < 0.01$.

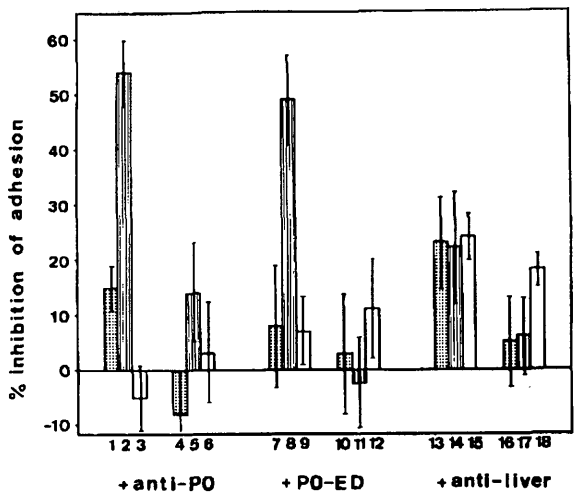

Fig. 4. Inhibition of adhesion of infected CV-1 cells by anti-P_o IgGs and soluble P_o-ED. The percentage of inhibition of adhesion of infected CV-1 cells by P_o antibodies and soluble P_o-ED was investigated on the following substrates: collagen (dotted bars), P_o-ED (lined bars), and poly-L-lysine (open bars). CV-1 cells were infected with recombinant P_o vaccinia virus (columns 1–3, 7–9, and 13–15) or with wild-type vaccinia virus (columns 4–6, 10–12, and 16–18). Additives in the adhesion assay were IgG fraction of polyclonal P_o antibodies (300 μg/ml) (columns 1–6), soluble P_o-ED (300 μg/ml) (columns 7–12), and IgG fraction of polyclonal antibodies to mouse liver membrane (300 μg/ml) (columns 13–18). Values were referred to adhesion values obtained without additives for each substrate and set to 100% bound cells (=0% inhibition). Mean values are from three independent experiments ± standard deviations.

be localized by indirect immunofluorescence on the cell surface of recombinant P_o vaccinia virus-infected CV-1 cells. Since these cells did not show any significant P_o-dependent interaction in an aggregation assay, an adhesion test was used, in which purified P_o was used as substrate for P_o-expressing CV-1 cells. The observation that this adhesion could be inhibited by P_o antibodies and by soluble P_o-ED provided unequivocal evidence for a homophilic binding mechanism for P_o. The ability of P_o to mediate homophilic adhesion between apposing surface membranes could contribute to the formation of desmosome-like structures (D'Urso et al., 1990; Filbin et al., 1990) and may also lead to the close membrane contact between the turning loops of myelinating Schwann cells which is essential for the compaction of PNS myelin in vivo.

During development of the mammalian PNS, P_o is expressed before the formation of compact myelin and first detectable at the time when Schwann cells sort out individual axons on the basis of a 1:1 ratio (Martini et al., 1988). The presence of P_o on the periaxonal membrane surface of the Schwann cell has led to the suggestion that P_o also mediates heterologous interactions as a prelude to myelination (Martini et al., 1988). Our observation that the extracellular domain of P_o promotes interaction with neurons strongly supports this view. That P_o confers neurite outgrowth-promoting properties is noteworthy, since neurite outgrowth in peripheral nerves has largely ceased at the time when P_o becomes first detectable. However, elongation of axonal membranes occurs during postnatal times of animal growth, and it is possible that P_o aids in the stabilization of such processes. Not only P_o but also the myelin-associated glycoprotein (MAG) has been implicated in neurite outgrowth (Johnson et al., 1989) and may play a similar role. Furthermore, it is tempting to speculate that P_o and MAG may be useful for neurite outgrowth under conditions of regeneration, where the degenerating myelin may serve as substrate for regrowing axons at the myelin-basal lamina interface. That P_o may be indeed characteristic of neural structures capable of supporting neurite outgrowth has been suggested by

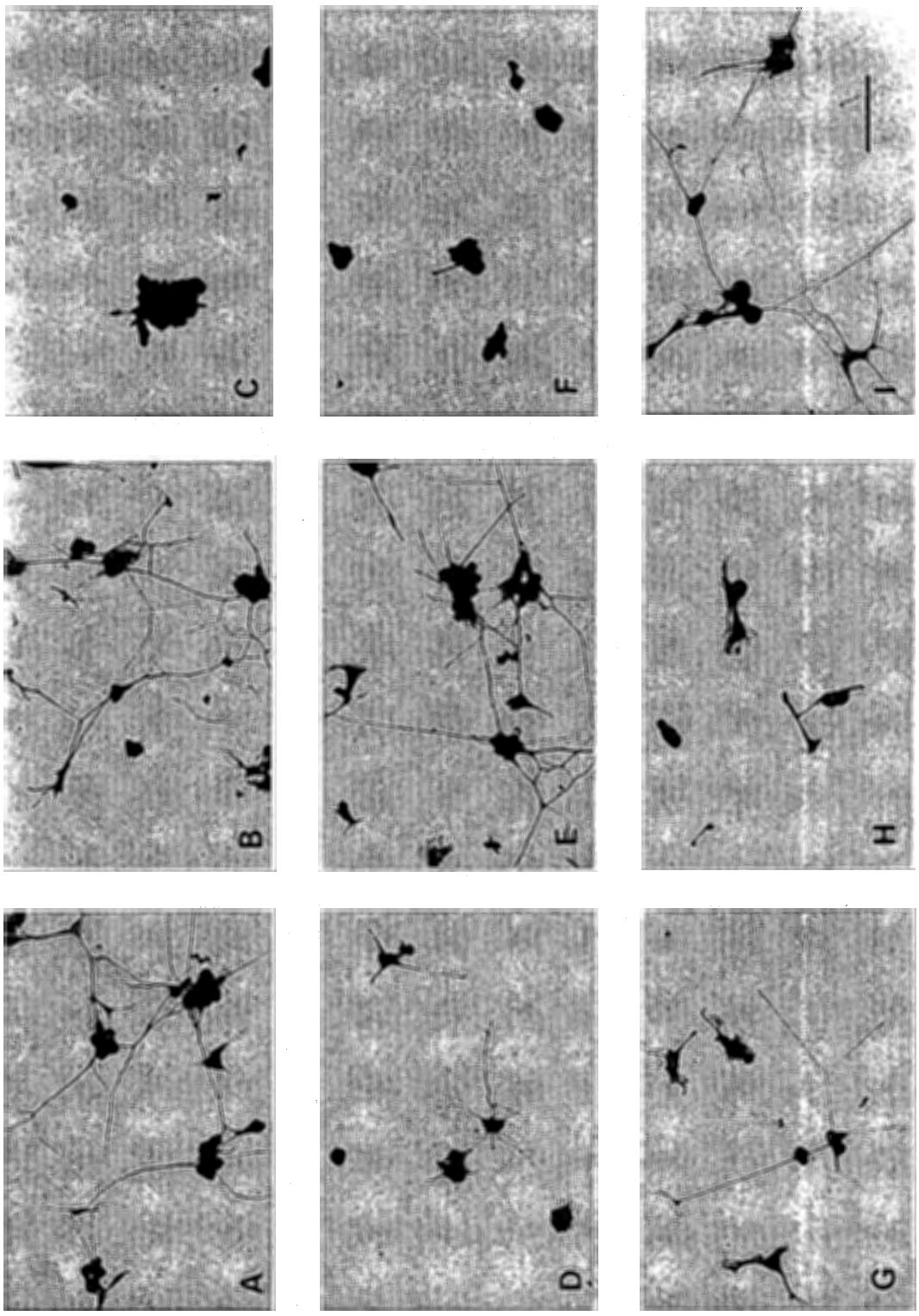

Fig. 5. Neurite outgrowth of dorsal root ganglion neurons on recombinant P_0-ED and control substrates. Typical shapes of neurites are shown on rat tail collagen (100 µg/ml) **(A)**; on collagen in the presence of an IgG fraction of polyclonal P_0 antibodies (300 µg/ml) **(B)**; on IgG fraction of polyclonal P_0 antibodies coated as substrate (100 µg/ml) **(C)**; on poly-L-lysine (100 µg/ml) **(D)**; on collagen in the presence of soluble P_0-ED (300 µg/ml) **(E)**; on BSA (100 µg/ml) **(F)**; on substrate-coated P_0-ED (100 µg/ml) **(G)**; on substrate-coated P_0-ED in the presence of an IgG fraction of polyclonal P_0 antibodies (300 µg/ml) **(H)**; and on collagen in the presence of soluble P_0-ED and IgG fraction of polyclonal P_0 antibodies **(I)**; Twenty-four hours after cell plating cultures were fixed and stained with toluidin blue. Bar = 100 µm.

TABLE II. Neurite Outgrowth of Dorsal Root Ganglion Neurons on Various Substrates†

Substrates	Soluble competitors	Total length of neurites (% of control)[a]	Neurite length per neuron (μm)	Number of neurites per neuron	Number of neurite branch points per neuron
Collagen	—	100 ± 12	328 ± 43	1.38 ± 0.66	1.60 ± 0.40
Collagen + P_O-ED	—	103 ± 9	339 ± 41	1.30 ± 0.57	1.33 ± 0.43
Collagen	Anti-P_O IgG	104 ± 19	341 ± 57	1.65 ± 0.64	1.73 ± 0.47
Collagen	P_O-ED	111 ± 18	366 ± 48	1.18 ± 0.43	2.18 ± 0.62
P_O-ED	—	46 ± 6	151 ± 27	1.15 ± 0.38	0.81 ± 0.31
P_O-ED	Anti-P_O IgG	13 ± 4	44 ± 13	0.63 ± 0.49	0.13 ± 0.10
P_O-ED	P_O-ED	12 ± 2	39 ± 21	1.61 ± 0.54	0.05 ± 0.05
PLL	—	50 ± 3	164 ± 22	2.73 ± 0.63	1.75 ± 0.56
PLL + P_O-ED	—	71 ± 6	235 ± 36	3.46 ± 0.81	1.28 ± 0.43
PLL	P_O-ED	34 ± 3	124 ± 25	3.56 ± 0.76	0.73 ± 0.48
Anti-P_O IgG	—	2 ± 1	8 ± 5	0.05 ± 0.01	0.06 ± 0.05
BSA	—	<1	<5	0	0

†Numbers are mean values from three independent experiments ± standard deviations. BSA, bovine serum albumin; P_O-ED, recombinant extracellular domain of P_O; PLL, poly-L-lysine.

[a]Collagen was chosen as the control for neurite outgrowth and the total length of neurites from 120 neurons measured in three independent experiments was set to 100%.

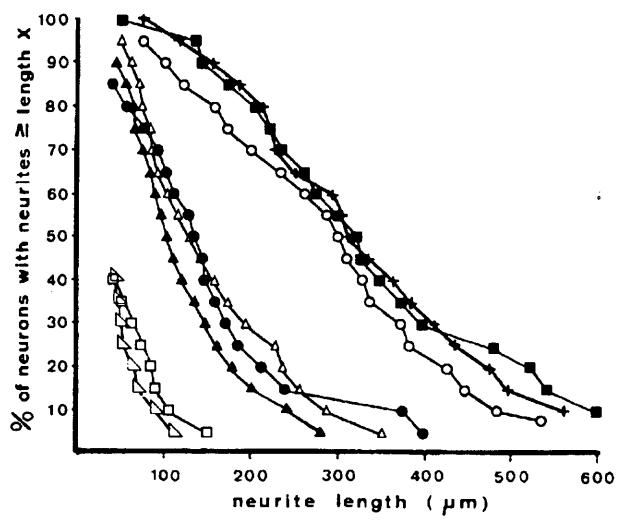

Fig. 6. Cummulative frequency distribution plot of neurite lengths of dorsal root ganglion neurons on various substrates. Total lengths of neurites per neuron were determined by the image analysis and plotted as percentage of neurons with neurites longer than a certain length x (vertical axis) as a function of neurite length (horizontal axis). Values smaller than 30 μm or 5% are not shown. The neurites were grown on collagen (○), on collagen in the presence of an IgG fraction of polyclonal P_o antibodies (300 μg/ml) (■), on collagen in the presence of soluble P_o-ED (300 μg/ml) (+), on poly-L-lysine (△), on poly-L-lysine in the presence of soluble P_o-ED (▲), on substrate-coated P_o-ED (100 μg/ml) (●), on substrate-coated P_o-ED in the presence of polyclonal P_o antibodies (300 μg/ml) (⊡), and on substrate-coated P_o-ED in the presence of soluble P_o-ED (□). One hundred twenty neurons from three indepen-dent experiments were traced by computer for each condition. The inhibition of neurite outgrowth on P_o-ED by P_o antibodies, and soluble P_o-ED was highly significant ($\alpha = 0.001$) by the U test by Mann and Whitney.

the observation that it is present in the central nervous system (CNS) of adult fish (Saavedra et al., 1989). Since P_o can be functional both in homo- and in heterophilic binding mechanisms, the question of how one Ig-like domain mediates these diverse features remains an intriguing problem.

P_o isolated from the PNS expresses various carbohydrate epitopes, among them L2/HNK-1 and L3 (Bollensen and Schachner, 1987; Bollensen et al., 1988; Fahrig et al., 1990). CV-1 cells also express the L2/HNK-1 epitope on their surfaces, both in the uninfected state and after vaccinia virus infection (data not shown), and it is likely that P_o expressed in this system may be at least partially glycosylated as the native P_o. Since the bacterial expression product of P_o is not glycosylated, our results may suggest a carbohydrate-independent mechanism of adhesion. However, it is also possible that a carbohydrate-bearing P_o molecule on one side of the partner pair may interact with unglycosylated P_o mediating a lectin-like function on the other side. Since carbohydrate epitopes, such as L2/HNK-1 and L3 are also present on other adhesion molecules, such as L1 and N-CAM (Kruse et al., 1984; Antonicek et al., 1987; Kücherer et al., 1987), a lectin-like function could be an explanation for possible heterophilic interactions to other adhesion molecules that may underlie the neurite outgrowth-promoting properties of P_o. Whether this is indeed the case can now be tested with the recombinant P_o products.

ACKNOWLEDGMENTS

We thank Iris Bahnmüller for technical assistance, Greg Lemke (Salk Institute, La Jolla) for the P_o cDNA, Johannes Buchner (University of Regensburg) for his advice in renaturation of bacterial P_o, and Deutsche For-

schungsgemeinschaft (fellowship to J.S.-S.), Schweizerischer Nationalfonds, and Kommission zur Förderung der wissenschaftlichen Forschung for support.

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