Homophilic Adhesion of the Myelin Po Protein Requires Glycosylation of Both Molecules in the Homophilic Pair

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Abstract. The myelin Po protein is glycosylated at a single site, asparagine 93, within its only immunoglobulin (Ig)-like domain. We have previously shown that Po behaves like a homophilic adhesion molecule (Filbin, M. T., F. S. Walsh, B. D. Trapp, J. A. Pizzey, and G. I. Tennekoon. 1990. *Nature (Lond.)*. 344:871–872). To determine if the sugar residues of this molecule contribute to its adhesiveness, the glycosylation site was eliminated by replacing asparagine 93 with an alanine, through site-directed mutagenesis of the Po cDNA. The mutated Po cDNA was transfected into CHO cells and surface expression of the mutated Po was assessed by immunofluorescence, limited trypsinization and an ELISA. A cell line was chosen which expressed approximately equivalent amounts of the unglycosylated Po (UNGPo) at the cell surface as did a cell line expressing the fully glycosylated Po (GPo); the adhesive properties of these two cell lines were compared. It was found that when a single cell suspension of the UNGPo cells were incubated, by 60 min, unlike the GPo cells, they had not formed large aggregates; they were indistinguishable from the control transfected cells. This suggests that the UNGPo protein does not behave like an adhesion molecule. To establish if only one molecule in the Po:Po homophilic pair must be glycosylated for adhesion to occur, the ability of UNGPo cells to adhere to GPo cells was assessed both qualitatively and quantitatively. The results of both types of assay imply that, indeed, both Po molecules in the homophilic pair must be glycosylated for adhesion to take place.

The immunoglobulin (Ig) superfamily is a large family of proteins distinguished by molecules containing amino acid sequence similarity with regions of immunoglobulins, and by a putative common function in adhesion/recognition (Edelman, 1970; Williams, 1982; Williams and Barclay, 1988). The myelin Po protein is included in this family of molecules because, first, it contains one Ig-like domain (Lai et al., 1987; Lemke and Axel, 1985) and, second, we (Filbin et al., 1990) and others (D'Urso et al., 1990; Schneider-Schaulies et al., 1990) have established that Po can behave like an adhesion molecule through interactions of its extracellular domains. Direct demonstration of an adhesive function for Po is in accord with the long-held hypothesis that this molecule is responsible for holding the spiral of myelin membranes compact at the intraperiod line by interactions of its extracellular domains (Kirschner and Ganser, 1980; Braun, 1984; Lemke et al., 1988). Indeed a requirement for Po in the formation of compact myelin has been demonstrated in mice in which no Po is expressed after disruption of the Po gene by homologous recombination (Giese et al., 1992). Therefore, it is of considerable interest that this protein is one of the simplest members of the Ig superfamily and hence, is most amenable to structure-function studies.

Our interest in Po stems from attempting to understand its role in maintaining the compactness of the myelin sheath in the peripheral nervous system, where Po is the most abundant myelin protein (Ishaque et al., 1980). The Po protein contains one V-like Ig domain, which is glycosylated at a single site, asparagine 93 (Lemke and Axel, 1985; Sakamoto et al., 1987). Because of this simple protein structure and the exon distribution of the nucleotide sequences coding for its Ig domain (Lemke et al., 1988), Po has been proposed to be the closest relative to the ancestral gene of the whole Ig superfamily (Barclay et al., 1987; Williams and Barclay, 1988). Hence, mapping the functional regions of the Ig domain of Po may have broader implications for the entire family of molecules.

The assay system we used to establish Po as a homophilic adhesion molecule (increased adhesiveness of CHO cells after transfection of Po cDNA) is readily manipulable and, as already stated, the simplicity of the Po protein is ideally suited to structure-function study. We therefore planned a systematic analysis of the role of the Ig domain, in particular the sugar residues, in Po adhesion. We have already shown that Po carrying high-mannose rather than complex type sugar residues is not adhesive (Filbin and Tennekoon, 1991).

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The focus of the present study was to determine whether Po protein retained its adhesive properties when its sugar residues were completely eliminated, and also whether glycosylation of both molecules in the Po:Po homophilic pair is required for adhesion to occur. We provide strong biochemical evidence that Po must be glycosylated to be adhesive, and also that for homophilic adhesion, glycosylation of both Po molecules is necessary. Such information should provide further insight not only on how Po functions in the myelin membrane, but also on the role of carbohydrates in the functioning of Ig molecules with multiple Ig domains.

Materials and Methods

Cell Cultures

Dihydrofolate reductase mutant CHO cells (Urlaub and Chasin, 1980) were maintained on MEM supplemented with 10% FCS and proline (11 μg/liter) at 37°C in 5% CO_2. For untransfected cells, thymidine (0.73 mg/liter), glycine (7.5 mg/liter), and hypoxanthine (4.1 mg/liter) were added. For transfected cells dialyzed FCS was used, hypoxanthine was omitted, and 100 nM $CdCl_2$ was added.

Mutation of Po-cDNA at Asn 93

The 1.08 Kb ECoRI to XbaI fragment of the Po cDNA (Lemke and Axel, 1985) was subcloned into M13 mp19. In vitro mutagenesis was carried out as previously described (Taylor et al., 1985; Nakamaye and Eckstein, 1986). The single-stranded DNA template was annealed with the phosphorylated oligonucleotide 5'-GAC.TAC.AGT.GCC.ACT.TTC-3' and the M13 sequencing primer. The heteroduplex was completed with the Klenow fragment of DNA polymerase I and T4 DNA ligase. In the extension step, the thionucleotide dCTPα5 replaced dCTP in the nucleotide mixture. The heteroduplex was then nicked with NciI, and because the mutant strand contained dCTPαS, all sites on this strand were protected. Further digestion of the nonmutant strand was accomplished using exonuclease III, after which the digested DNA was repolymerized by DNA polymerase I and the heteroduplex was ligated with T4 DNA ligase. The mutated Po cDNA was subcloned into bluescript and the mutation was confirmed by dideoxy sequencing (Sanger et al., 1977).

Ligation of Po-cDNA into a Suitable Plasmid

The plasmid used for expression of the mutated Po cDNA has been described previously (Lee and Nathans, 1988; Filbin and Tennekoon, 1990). Briefly, the mutated Po cDNA was ligated into the plasmid at a unique XhoI cloning site downstream from the mouse metallothionein promoter and upstream from the poly (A) tail of the SV40 t-antigen gene. The plasmid also contained the mini genes for G418 resistance and dihydrofolate reductase (*dhfr*). The orientation of the Po cDNA in the plasmid was confirmed by restriction enzyme digestion.

Transfection

CHO cells were transfected with 1–2 μg of DNA per 10-cm plate, by calcium phosphate precipitation (Graham and van der Eb, 1973) followed by a glycerol shock (Frost and Williams, 1978). The cells were passed, 1:2, the following day, and 3 d after transfection, 400 μg/ml of active G418 was added to the culture medium. Colonies appeared after ~3 wk and a number were picked, expanded, and single cell cloned by limiting dilution. Several of these single-cell clones were screened for expression of Po by Western blot analysis and used for gene amplification.

Gene Amplification

Cells with multiple copies of the *dhfr* gene were selected by growing the cells in increasing concentrations (0.05–2.0 μM) of methotrexate (MTX).[1] Cells were plated at 5×10^5 cells per T75 flask, and those surviving after 2–3 wk at each concentration of MTX were allowed to multiply before being replated on the higher concentration of MTX. At different stages in the gene amplification procedure, cells were monitored for Po expression by Western blot analysis and again single-cell cloned.

Immunodetection of Po Immobilized on Nitrocellulose

Cells (80–90% confluent) were lysed in 0.5 M Tris-HCl (pH 7.5) containing 2% SDS, 4% β-mercaptoethanol and the following antiproteases: leupeptin 1 μg/ml; antipain, 2 μg/ml; benzamidine, 10 μg/ml; chymostatin, 1 μg/ml; pepstatin, 1 μg/ml; and phenylmethylsulfonylfluoride, 1 μg/ml. The lysate was homogenized by passage through a 23-gauge syringe, and centrifuged in a microfuge at maximum speed for 10 min. The supernatant fraction was removed (protein was measured with a Bio-Rad (BioRad Labs, Hercules, CA) kit before the addition of β-mercaptoethanol) and incubated at 95°C for 3 min, after which lysates were subjected to SDS-PAGE on a 12 or 18% acrylamide gel (Laemmli, 1970). The proteins were transferred to nitrocellulose and immunostained (Filbin and Poduslo, 1986) with rabbit anti-bovine Po IgG at a dilution of 1:3,000. Secondary antibody was alkaline phosphate-conjugated goat anti-rabbit IgG (1:2,000) (Sigma Chemical Co., St. Louis, MO). 5-Bromo-4-chloro-3-indolyl-phosphate was used as a substrate and nitro blue tetrazolium as a chromogen, according to the manufacturer's instructions (Kirkegaard and Perry Lab., Gaithersburg, MD).

Indirect Immunofluorescence of Intact Cells

Cells were grown on glass coverslips coated with poly-L-lysine (Sigma Chemical Co.). The cells were rinsed with MEM, fixed for 10 min with 4% paraformaldehyde, and again rinsed with MEM. Nonspecific binding sites were blocked by incubation of cells for 30 min with 3% normal goat serum in MEM. Cells were then incubated overnight at 4°C with rabbit anti-Po-peptide 74–82, affinity purified, (1:50) in MEM containing 1% normal goat serum, rinsed twice with MEM, and again incubated for 30 min with 3% normal goat serum in MEM followed by incubation for 1 h with fluorescein-conjugated goat anti-rabbit $F(ab)_2$ fragments (1:500) (Jackson Laboratories, West Grove, PA) in MEM containing 1% normal goat serum. The cells were then rinsed twice with MEM and post-fixed for 10 min in 4% paraformaldehyde. All incubations were at room temperature unless stated otherwise. The cells were rinsed, mounted in glycerol, and viewed with a Zeiss fluorescence microscope. Controls consisted of control transfected cells incubated with Po antibody and Po-expressing cells incubated with preimmune serum.

Protease Treatment of Intact Cells

Cells, 80–90% confluent, in 6-cm dishes, were rinsed twice with calcium- and magnesium-free PBS (CMF PBS). 250 μl of trypsin (5 U/ml) were added to each dish and the cells were triturated with an 18-gauge syringe to obtain a single cell suspension. The cells were incubated as a single cell suspension with the trypsin for 1 h and then washed by centrifugation and resuspension, once with 5 ml of CMF PBS and once with 5 ml of MEM containing 10% FCS. The final pellet was resuspended in 100 μl of SDS sample buffer containing antiproteases, as above, heated for 3 min at 95°C, subjected to PAGE in an 18% acrylamide gel, transferred to nitrocellulose and immunostained for Po-protein as described above. After the trypsin treatment, the permeability of the cells was assessed by their ability to take up the dye trypan blue and also by the ability of a Po-peptide antibody directed against cytoplasmic sequences of Po to bind to the cells.

Quantitation of Po Expressed at the Cell Surface

An ELISA was carried out as previously described by Doherty et al. (1990*b*), modified as follows. Between 2,000 and 3,000 cells per well were plated in a 96-well ELISA plate and allowed to attach for 2 d. The cells were rinsed twice with PBS, fixed for 30 min with 4% paraformaldehyde, and then rinsed with PBS. Nonspecific binding sites were blocked for 30 min with 3% normal goat serum in MEM, and the cells were then incubated overnight at 4°C with rabbit Po-peptide antibody directed against sequences in Po's extracellular domain (1:200) in MEM containing 1% normal goat serum. The cells were rinsed, nonspecific binding sites were again blocked for 30 min with 3% normal goat serum in MEM, and the cells were incubated for 1 h with HRP-conjugated goat anti-rabbit IgG (1:250) (Sigma Chemical Co.). The cells were rinsed with PBS and then with water. Color was developed by the addition of 50 μl of 0.2% (wt/vol) o-phenylenediamine (Sigma Chemical Co.) and 0.02% (vol/vol) H_2O_2 in citrate buffer (pH 5.0) to each well. The reaction was stopped after 15 min by the addition of 50 μl of 4.5 M H_2SO_4 and the optical density at 490 nm was determined with

1. *Abbreviations used in this paper*: CFDA, 6-carboxyfluorescein diacetate; GPo, glycosylated Po; MTX, methotrexate; UNG Po, unglycosylated Po.

a 96-well plate reader. All incubations were at room temperature unless stated otherwise. Controls consisted of control transfected cells incubated with Po antibodies, Po-expressing cells incubated with Po antibody against peptide sequences in Po's cytoplasmic domain, and Po-expressing cells with no primary antibody. For each experiment, 40 wells were assayed for each cell line at least three times. The average number of cells per well was estimated by counting cells using a Coulter counter after their removal with trypsin, from five separate wells for each 96-well plate. Results were standardized to absorbance units per cell.

Adhesion Assay

The adhesion/aggregation assay was carried out as previously described (Filbin et al., 1990) with the following modification. Cells at 80–90% confluence were washed with PBS and incubated with 5 U/ml trypsin (GIBCO-BRL, Gaithersburg, MD) for 2–3 min at room temperature, washed twice with MEM, and finally resuspended in MEM containing 10% FCS by three passages through an 18-gauge syringe. Suspensions, containing a minimum of 95% single cells, were diluted to a final concentration of 1.5–2 × 10^6 cells per ml and allowed to aggregate at 37°C in 5% CO_2 with gentle rocking at 5 rpm. Before sampling, the tubes were gently inverted and aliquots were removed at intervals, examined under the microscope, and the total particle number was determined with a Coulter counter. At least three separate incubations were performed for each experiment, and duplicate samples were withdrawn at each time point and counted three times each.

Labeling Cells with 6-Carboxyfluorescein Diacetate

Cells were vitally labeled by incubating a single cell suspension (0.5 ml; 2.5 × 10^5 cells/ml) in MEM containing 10% FCS with 10 μl of 0.5% 6-carboxyfluorescein diacetate (CFDA), in acetone for 15 min at 37°C. Cells were then washed twice with 5 ml of MEM containing 10% FCS and resuspended in the same medium at 1.5–2 × 10^6 cells/ml.

Preformed Aggregate Mixed Adhesion Assay

CHO cells expressing glycosylated Po were allowed to aggregate for 45 min as described above. A single cell suspension of cells prelabeled with CFDA as described above, expressing either glycosylated or unglycosylated Po protein was then added to the preformed aggregates and the incubation was carried out for a further 45 min, after which aliquots were examined by both phase and fluorescence microscopy (see Fig. 7) (Snow et al., 1989).

Mixed Adhesion Assay

Single cell suspensions of cells expressing glycosylated Po protein were mixed with an equal number of cells expressing unglycosylated Po protein to give a final concentration of 1.5-2 × 10^6 cells/ml. One population of cells was prelabeled with the dye CFDA as described above. After 20 min, aliquots were removed and for each experiment 50 four-cell aggregates were scored for glycosylated and unglycosylated Po-expressing cells by use of phase and fluorescence microscopy and statistically evaluated as previously described (Sieber and Roseman, 1981; Pizzey et al., 1988).

Results

Expression of Unglycosylated Po-Protein in CHO Cells

Although we had demonstrated that Po behaved like a homophilic adhesion molecule after expression of Po cDNA in CHO cells, this protein was glycosylated. To determine whether glycosylation of Po was necessary for adhesion, we expressed in CHO cells a Po cDNA in which the single glycosylation site, asparagine 93, was mutated to code for an alanine, a relatively conservative substitution. Fig. 1 shows expression of the mutated Po cDNA by one single cell clone after growth in increasing concentrations of MTX to a final concentration of 1 μM. The molecular weight of the unglycosylated Po (Fig. 1, *arrowhead*) is, as would be expected, ~6% smaller than the glycosylated Po protein (Fig. 1, lane *c*, *arrow*). A second, fainter band, ~2,000 D smaller

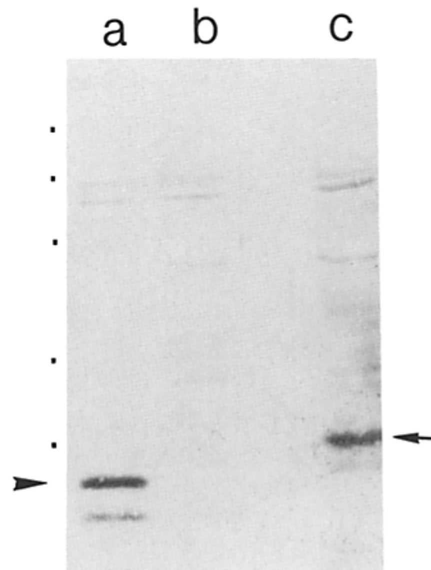

Figure 1. Western blot of transfected CHO cells grown in 1 μM of MTX and immunostained for Po. CHO cells transfected with plasmids containing the Po DNA mutated at the nucleotides coding for an 93, were selected in G418 and single cell cloned. Individual clones were grown in sequentially increased concentrations of MTX. Proteins (100 μg/lane) from cells expressing UNGPo grown in a final concentration of 1 μM MTX (lane *a*), control transfected cells (lane *b*) and cells expressing GPo on 1 μM MTX (lane *c*) were separated by SDS-PAGE, transferred to nitrocellulose, and immunostained for Po (anti-bovine Po antibody, 1:3,000), followed by alkaline phosphatase-conjugated goat anti-rabbit $F(ab)_2$ fragments (1:2,000). Arrow refers to GPo, arrowhead to UNGPo and dots to molecular weight standards, from top to bottom as follows: 65, 58, 48.5, 36.5, and 30 kD.

than the unglycosylated Po protein was also apparent in the cells. We found that this band is recognized by a series of Po-peptide antibodies that span the whole molecule (results not shown) suggesting that it does not represent a proteolytic fragment of Po. This lower band probably represents an unreduced form of the Po protein, even though the samples were separated under reducing conditions, as reported by Cammer et al. (1980) for Po from rat sciatic nerve under similar conditions. This lower molecular weight band is also present at low levels, in cell lysates of CHO cells expressing the glycosylated Po, but is not easily detected because the two bands are not as well resolved when Po is glycosylated as they are when Po is unglycosylated. Of note, the levels of expression of glycosylated or unglycosylated Po per 100 μg of total protein for the two cell lines grown in a final concentration of 1 μM MTX, are comparable (Fig. 1, lanes *a* and *c*, respectively); a good indication that these two cell lines are appropriate for comparing their adhesive properties. Direct comparison of the adhesive properties of unglycosylated Po with the fully glycosylated molecule, however, requires that surface expression of each is equivalent for the cell lines chosen. Therefore, cell surface Po-protein was first visualized by (*a*) immunofluorescence of intact cells, then estimated by (*b*) limited trypsinization, and finally quantitated using (*c*) an ELISA assay.

Surface Expression of Po-Protein

(*a*) Expression of unglycosylated Po protein at the cell sur-

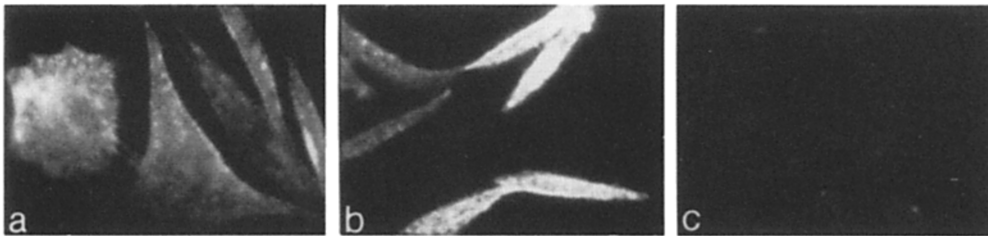

Figure 2. Surface immunofluorescence staining for Po of fixed CHO cells. (*a*) Cells expressing GPo. (*b*) Cells expressing UNGPo. (*c*) Control transfected cells.

face was detected by immunostaining the intact transfected cells using an affinity purified Po-peptide antibody directed against amino acids 74–82 in Po's extracellular domain. Fig. 2 shows immunofluorescent detection of Po-antiserum bound to fixed, unpermeabilized CHO cells expressing glycosylated or unglycosylated Po protein. The intensity of staining is comparable for both cell lines, indicating that equivalent amounts of Po, glycosylated (Fig. 2 *a*) or unglycosylated (Fig. 2 *b*), are reaching the surface for each cell line. Under the same conditions, no staining was apparent in the control transfected CHO cells, i.e., those not expressing Po (Fig. 2 *c*). Furthermore, when cells fixed in the same way and, again, not permeabilized were stained with an antibody directed against sequences in the cytoplasmic domain of Po, staining was apparent in <1% of the cells examined, adding further support to the notion that under the fixation conditions used the cells are intact and therefore only surface expression of Po protein was being monitored.

(*b*) To confirm that Po protein was reaching the cell surface in cell lines expressing glycosylated or unglycosylated Po, limited trypsinization of the intact cells was carried out. By 1 h for both the glycosylated and unglycosylated Po, a species with lower molecular weight (∼15,000) is apparent (Fig. 3, *arrowhead*). This Po species corresponds in size to that predicted for a Po molecule without the extracellular domain and thus indicates that only the extracellular sequences of Po are digested by limited trypsinization. When scanned with a densitometer, it was estimated that ∼40% of both glycosylated or unglycosylated Po proteins are sensitive to trypsin under these conditions, indicating that 40% of the total Po expressed by these cell lines reaches the cell surface. This estimate was made from the amount of Po undigested after trypsin treatment (Fig. 3, *arrows*), because the band representing digested Po-protein (Fig. 3, *arrowheads*) appears more concentrated than it actually is because of poor resolution in the lower regions of the gel thus making the bands appear more diffuse and darker. After 1 h exposure to trypsin, albeit at low levels, it was possible that some of the cells became permeable, thus allowing the trypsin to enter the cell where digestion of Po, not at the surface, can occur. This does not appear to have taken place for several reasons. First, if trypsin were digesting intracellular Po, it would be expected to remove the cytoplasmic domain of the molecule which would result in a Po species of ∼20,000 mol wt and not 15,000. Moreover, removal of the cytoplasmic domain of glycosylated and unglycosylated Po would result in Po fragments of different sizes as a result of the presence or absence of sugar residues. This is not the case as a species of 15,000 appears after digestion of either glycosylated or unglycosylated Po. Second, after 1 h in the presence of trypsin, the cells still exclude the dye, trypan blue, and do not bind a Po-peptide antibody directed against the cytoplasmic sequences of the molecule, both strong indicators that the cells are still intact (results not shown). Furthermore, the lower molecular weight species of Po, present in the undigested cell lysate, also decreases in size when the cells are exposed to trypsin indicating that this species of Po also reaches the cell surface.

(*c*) For quantitative comparison of the surface expression of glycosylated-Po and unglycosylated Po in the two cell lines, an ELISA assay was carried out on fixed, unpermeabilized cells. Approximately equivalent amounts of unglycosylated and glycosylated Po are reaching the surface in the two cell lines (Fig. 4, columns *1* and *2*). Only background staining was apparent in the control transfected cells when the same antibody was used (Fig. 4, column *3*), or when the ELISA was carried out with a Po-peptide antibody directed against sequences in the cytoplasmic domain of Po (Fig. 4, column *4*). This confirms that the majority of cells are indeed intact and that under these conditions of fixation, only

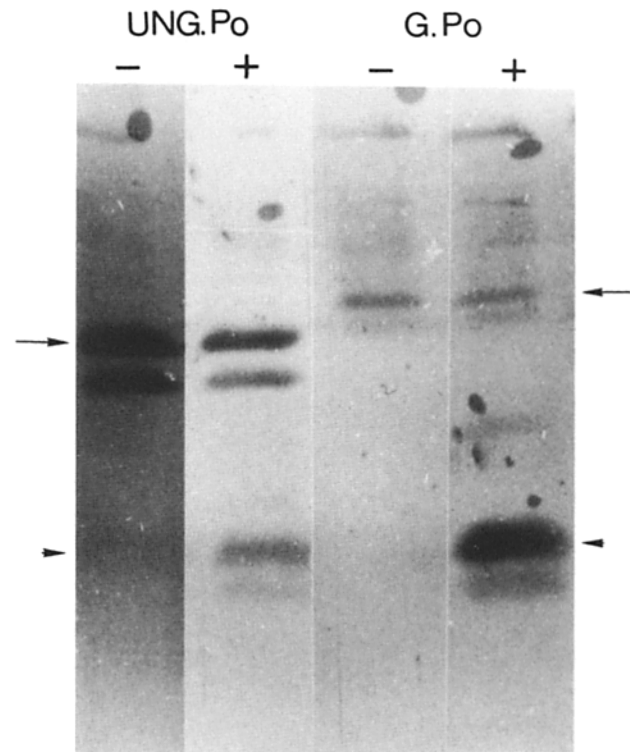

Figure 3. Limited trypsinization of Po expressed at the surface of CHO cells. Cells expressing either UNGPo or GPo, lanes as marked, were treated with 5 U/ml trypsin for 1 h at 37°C (+ sign indicates trypsin treatment), subjected to SDS-PAGE and immunostained for Po. Arrows refer to undigested Po and arrowheads to digested Po.

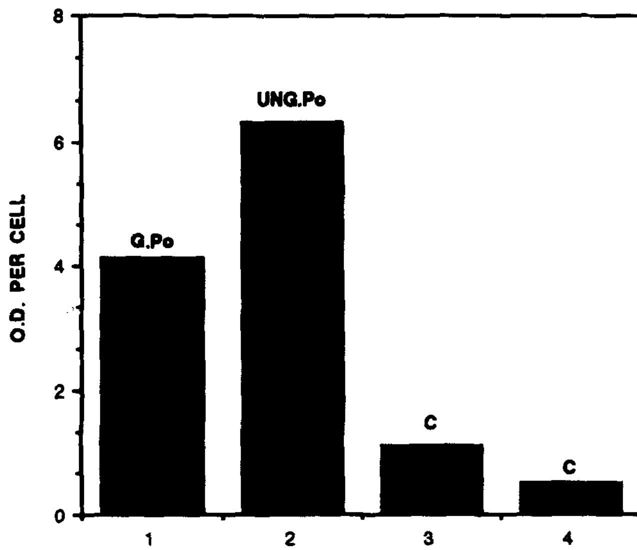

Figure 4. Quantitation of Po expressed at the surface of transfected CHO cells. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected CHO cells expressing GPo (column *1*), UNGPo (column *2*), and control transfected cells (column *3*), using a Po-peptide antibody directed against sequences in Po's extracellular domain. An ELISA was also conducted using a Po-peptide antibody directed against sequences in the cytoplasmic domain and the results for UNGPo cells are shown in column *4*. Results are expressed in relative absorbance units per cell and are the mean of three experiments, 40 samples per experiment. The SEM was <10%.

surface Po-protein is being measured in the ELISA. These two cell lines were used to compare the adhesive properties of glycosylated and unglycosylated Po protein.

Adhesion of Cells Expressing Unglycosylated Po

To determine whether unglycosylated Po protein behaves like a homophilic adhesion molecule, similar to glycosylated Po, a reaggregation adhesion assay was carried out. After 60-min incubation of a single cell suspension, the cells expressing the unglycosylated Po protein had not formed large aggregates and indeed were indistinguishable from the control transfected cells after the same incubation time (Fig. 5, *a* and *c*, respectively). On the other hand, by 60 min the CHO cells expressing equivalent amounts of glycosylated Po protein had, as we previously reported (Filbin et al., 1990; Filbin and Tennekoon, 1991), formed large aggregates consisting of up to 100–200 cells (Fig. 5 *b*).

The adhesive properties can be quantitated by counting the total particle number of a cell suspension as an incubation proceeds; a drop in total particle number indicates aggregate formation. Fig. 5 *d* shows the change in total particle number with time for CHO cells expressing glycosylated Po, cells expressing unglycosylated Po, and control transfected cells. In agreement with our previous report, (Filbin et al., 1990; Filbin and Tennekoon, 1991) and with the microscopic observations (Fig. 5 *b*), the total particle number for the cells expressing the glycosylated Po (Fig. 5 *d*, GPo) had dropped to ~25% by 60 min. The total particle number of both control transfected cells and the cells expressing unglycosylated Po (Fig. 5 *d*, UNG Po) dropped to only 55–60% in the same time. Thus, both the microscopic observations and the quantitative analysis strongly suggest that, unlike glycosylated Po protein, expression of unglycosylated Po protein by CHO cells does not increase the adhesiveness of these cells. These results in turn imply that Po protein must be glycosylated to behave like a homophilic adhesion molecule.

To Determine if Both Po Molecules Must Be Glycosylated for Adhesion to Take Place

It is possible that only one Po molecule in the homophilic Po:Po pair must be glycosylated for adhesion to take place. To determine if this is the case, two types of mixed adhesion

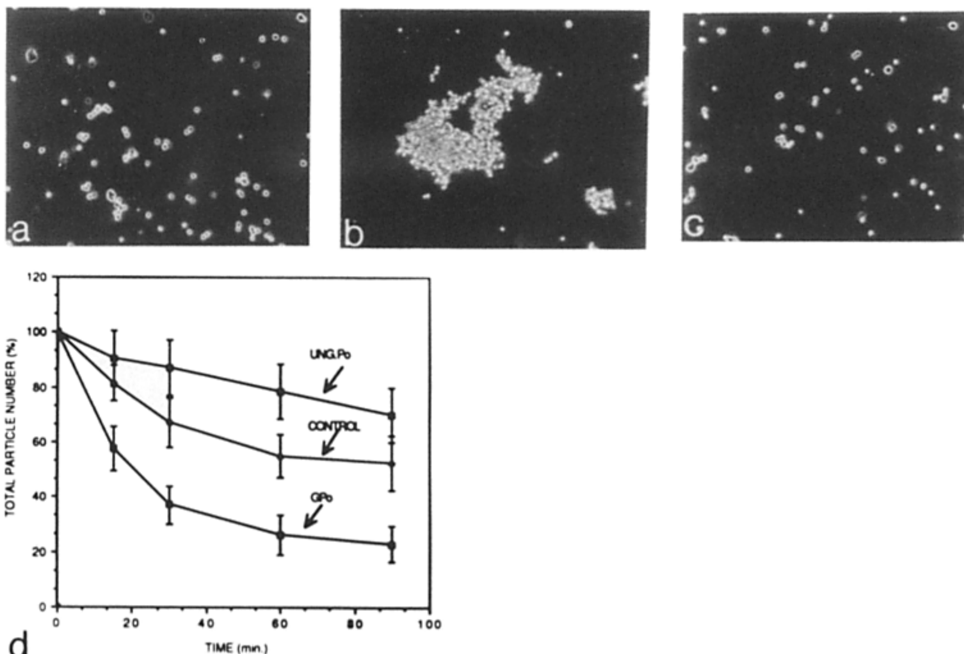

Figure 5. Aggregation properties of Po-expressing cells. A single cell suspension of CHO cells expressing (*a*) UNGPo, (*b*) GPo, or (*c*) control transfected cells were allowed to aggregate. Samples were withdrawn at various intervals and examined under the microscope (*a–c* represent results after 60 min aggregation) and counted in a Coulter counter for total particle number. The total particle number ± SEM was plotted against time (*d*).

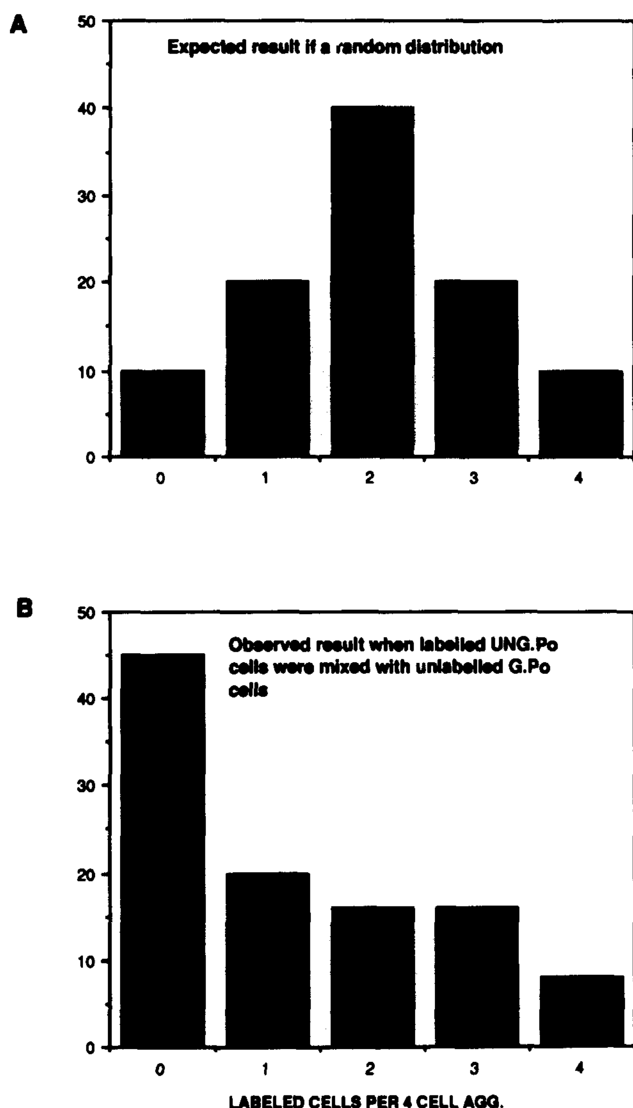

Figure 6. Statistical analysis of quantitative mixed adhesion assay to determine if UNGPo cells will adhere to GPo cells. Equal numbers of UNGPo-expressing cells were mixed with GPo-expressing cells and allowed to aggregate, one population was pre-labeled with CFDA. After 20 min, four-cell aggregates were scored for UNGPo-expressing and GPo-expressing cells using phase and fluorescence microscopy and statistically evaluated. If the cell types have no adhesive preference, aggregation will result from random interactions, as presented in *A*. The observed results of mixing labeled UNGPo cells with unlabeled GPo cells are represented in B and are indicative of a nonrandom distribution.

assay were carried out. Previously, we used these types of analysis to establish that Po interacts homophilically and not with some ubiquitous component of the opposing cell's membrane (Filbin et al., 1990). The first assay provides a quantitative assessment (Sieber and Roseman, 1981; Pizzey et al., 1988) of the aggregation of cells expressing glycosylated Po and those expressing unglycosylated Po mixed in a 50:50 ratio. One population of cells is prelabeled with the vital fluorescein dye CFDA and after a 20-min incubation, the components of four-cell aggregates are scored for cells expressing glycosylated Po and unglycosylated Po. If after scoring of aggregates, a normal distribution of glycosylated Po and unglycosylated Po cells in the four-cell aggregates is observed (Fig. 6 *A*), this would suggest a completely random adhesion of cells, implying that cells expressing glycosylated Po adhere equally well to either glycosylated Po cells or unglycosylated Po cells and therefore, that only one molecule in the Po:Po homophilic pair must be glycosylated for adhesion to occur. On the other hand, if a nonrandom distribution of glycosylated Po and unglycosylated Po expressing cells is observed, then it is probable that glycosylated Po expressing cells adhere preferentially to glycosylated Po expressing cells, and would therefore imply that both molecules in the homophilic pair must be glycosylated for adhesion to occur. We found that distribution of unglycosylated Po and glycosylated Po expressing cells in the four-cell aggregates, regardless of which population was fluorescein-labeled, was completely nonrandom, ($P < 0.0001$ Fig. 6 *B*). These results strongly suggest that for Po to behave like a homophilic adhesion molecule, both molecules in the homophilic pair must be glycosylated.

To confirm this result, the preformed aggregate mixed adhesion assay was carried out (Snow et al., 1989). In this assay, unlabeled CHO cells expressing glycosylated Po were allowed to aggregate, after which a single cell suspension of fluorescein-labeled CHO cells expressing either glycosylated Po or unglycosylated Po was added, and the incubation was continued for a further 20 min. The ability of the added cells to join the preformed aggregates indicates the binding requirements of Po:Po interaction (see Fig. 7 *A*). That is to say, if the unglycosylated Po expressing cells were found to join the aggregates of glycosylated Po expressing cells, this would suggest that only one Po molecule must be glycosylated for adhesion to occur. Alternatively, if the unglycosylated Po expressing cells did not join the aggregates, this would suggest that both molecules in the homophilic pair must be glycosylated for adhesion to take place. It was found that glycosylated Po expressing, single cells joined the aggregates (Fig. 7 *B*) as would be expected and as we previously reported (Filbin et al., 1990). On the other hand, a single cell suspension of the fluorescein-labeled cells expressing unglycosylated Po did not join the aggregates in any significant number but remained as single cells (Fig. 7 *C*). These results indicate that glycosylated Po will only adhere to glycosylated Po. Thus, from both types of analysis, the four-cell aggregate and the pre-formed aggregate adhesion assays, we conclude that both molecules in the Po:Po homophilic pair must be glycosylated to adhere.

Discussion

The Ig-domain of Po-protein, like all members of this family of molecules, is believed to be essential to its functioning as an adhesion molecule. As the single glycosylation site of Po protein is contained within this domain, it is possible that the sugar residues are involved in the adhesive process. In the present study, we provide strong evidence that, whereas glycosylated Po-protein, expressed in abundance at the surface of CHO cells, increases the adhesiveness of these cells, the expression of equivalent amounts of the unglycosylated Po molecule does not enhance adhesion, indicating that the unglycosylated Po molecule does not appear to be adhesive. Furthermore, through adhesion assays with mixed populations of cells, i.e., expressing either glycosylated Po or un-

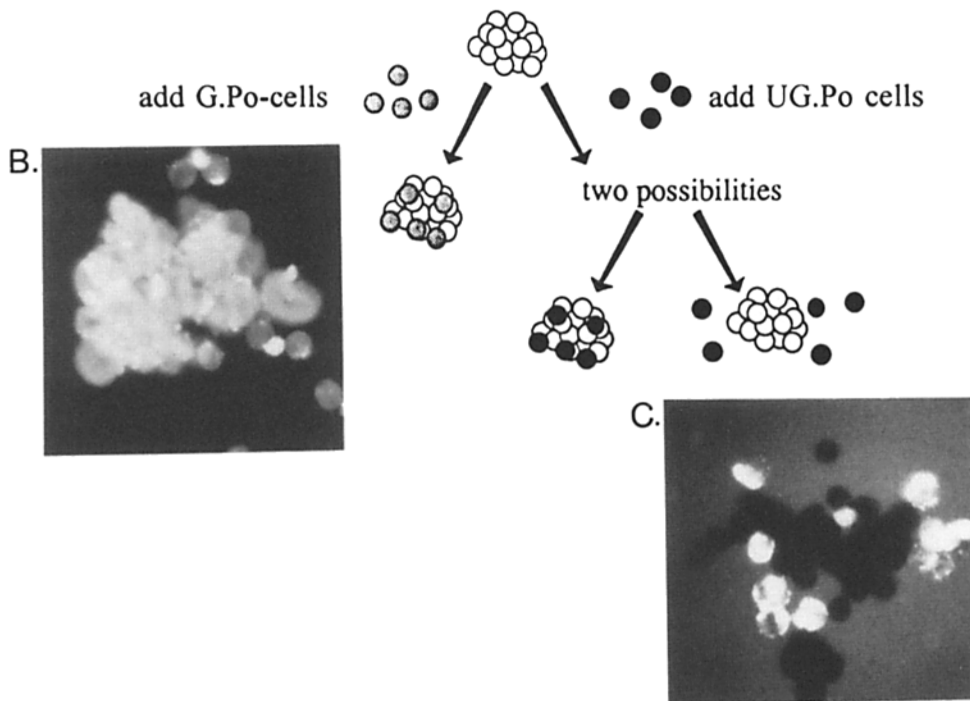

Figure 7. Fluorescein-labeled GPo-expressing or UNGPo-expressing cells adhering to unlabeled preformed aggregates of GPo-expressing cells. (*A*) Diagrammatic representation of the assay. Cells expressing GPo are allowed to form aggregates. A single cell suspension of CFDA labeled GPo (*gray*) or UNGPo cells (*black*) are then added to the preformed aggregates and incubated for a further 45 min after which aliquots are examined by both phase and fluorescence microscopy. (*B*) Addition of labeled GPo cells to the aggregates. These cells joined the aggregates. (*C*) Addition of labeled UNGPo cells to the aggregates. Very few of these cells joined the aggregates.

glycosylated Po protein, we have demonstrated both qualitatively (preformed aggregate mixed adhesion assay) and quantitatively (four cell aggregate mixed adhesion assay), that both molecules in the Po:Po interactive pair must be glycosylated to adhere.

This finding is supported by data reported by Yazaki et al. (1992) in which a glycosylated Po-peptide from amino acids 90–98 was much more effective at blocking the aggregation of Po expressing cells than was the same peptide without the sugar residues (80% vs 50% inhibition). The ability of the unglycosylated Po-peptide to inhibit adhesion by as much as 50% suggests that it is unlikely that the sugar residues interact directly with each other on opposing molecules, in which case only glycosylated peptide would inhibit adhesion. We have found a similar inhibition with both the peptide corresponding to amino acids 91–95 of Po's extracellular domain and also with antibodies raised against this peptide sequence (Filbin, 1992). Furthermore, a recombinant form of the unglycosylated extracellular domain of Po, expressed in and purified from bacteria, was found to bind to itself and to fully glycosylated Po protein, either purified from human sciatic nerve or expressed in transfected CV-1 cells (Schneider-Schaules et al., 1990; Griffith et al., 1992). From these results the authors suggest that Po is capable of both direct protein:protein interaction and direct protein: carbohydrate interaction and therefore that only one molecule in the Po:Po pair must be glycosylated for adhesion to occur. Alternatively, it is possible that the sugar residues of Po are only required for adhesion when the molecule is in the confines of the membrane. That is to say, the presence of Po's carbohydrates may hold the Ig domain of this molecule in a position whereby it can interact with a Po molecule in the opposing membrane. If the sugars are removed, then the Ig domain would come into closer contact with the membrane from which it extends and in so doing, mask the interactive regions of the Ig domain. Such restrictions on the positioning of the interactive regions of the Ig domain would not exist if the extracellular domain were free in solution, as is the case for the unglycosylated Po used in the studies of Schneider-Schaulies et al. (1992), and Griffiths et al. (1992) then positioning relative to the membrane is not required.

A role for the carbohydrates in positioning Po relative to the membrane is supported by the predicted three-dimensional structure of Po's Ig domain. As Po has yet to be crystallized, Wells et al., (1993) employed an alternative strategy to model Po's Ig domain based on the coordinates from the crystallized structure of one V-like domain of another Ig family member, i.e., the V-heavy domain of the phosphocholine mouse Ig. In this model, the single carbohydrate chain is, as we suggest, predicted to be responsible for holding the Ig domain of Po in a conformation that allows amino acid sequences of the domain to interact. If the carbohydrates are removed, this model predicts that the Ig domain of Po will collapse onto the membrane from which it extends, such that the Po molecules in opposing membranes would be further away or masked and unable to interact.

It is also possible that if the removal of the sugar residues from Po changes its conformation, this in turn could lower its affinity for the glycosylated Po molecule. This greatly reduced adhesive interaction may not be strong enough to hold two unglycosylated Po expressing cells together, as is required for the reaggregation-whole cell adhesion assay we use, but it may be sufficiently strong to be detected by the immobilized ligand assay used by Schneider-Schaulies et al. (1990). If altering the sugar residues of Po does change the affinity of its homophilic interactions, it is possible that the

homophilic interaction of Po is a two-step reaction: a low affinity first step not requiring the molecule to be glycosylated, followed by a high-affinity second step, which would stabilize the interaction and require a fully glycosylated Po molecule. Such a two-step reaction has been suggested for the homophilic adhesion of cadherins to explain why the isolated extracellular domain of this molecule can block the adhesion of cadherin-expressing cells, yet cadherin molecules truncated in their cytoplasmic domains cannot mediate adhesion when transfected into cells. In this proposed two-step adhesive interaction, the presence of the cadherin cytoplasmic domain stabilizes the interaction, but the cytoplasmic domain is not required for the initial, low affinity interaction which is not strong enough to allow aggregation of cadherin-expressing cells (Nagafuchi and Takeichi, 1988).

The adhesive function of another Ig-superfamily member, the human CD2 molecule, also suggests that the sugar residues play a crucial but not exclusive role in adhesion (Recny et al., 1992). Although the CD2 molecule has two Ig-like domains, the adhesive function has been mapped exclusively to domain 1 (Recny et al., 1990), and elimination of the single glycosylation site within this domain abolishes the adhesive properties of the molecule completely (Recny et al., 1992). As with Po (Filbin, 1992; Yazaki et al., 1992), regions of the CD2 molecule other than the sugar residues have been directly implicated in adhesion. Therefore, the authors suggest that glycosylation on domain 1 of the CD2 molecule is required to stabilize this domain and thus hold it in a conformation optimal for adhesion.

In a previous study we showed that changing the sugar residues from complex type to high-mannose, rather than eliminating them completely, also abolishes the adhesive properties of Po (Filbin and Tennekoon, 1991); this established that even moderate changes in the carbohydrate composition can have profound effects on the functioning of the Po molecule. On the basis of these studies and those presented in this paper, it is possible to envisage a situation in vivo whereby the functioning of Po is modulated via changes in its carbohydrate structure. Recent findings that Po can promote neurite outgrowth (Schneider-Schaulies et al., 1990; Yazaki et al., 1991), which may be important during nerve regeneration after injury, indicate that Po must interact with some component of the plasma membrane of the neuronal cell. As neurons do not express Po, this interaction must be heterophilic, and it is possible that the regions of the Po molecule involved in heterophilic, and homophilic interaction differ. Alteration of the sugar residues of the Ig domain could result in optimal presentation for homophilic or heterophilic interactions. Indeed, changes in the glycosylation pattern of Po have been reported both during development (Brunden, 1992) and after permanent transection of rat sciatic nerve (Poduslo et al., 1985), situations in which a change of Po function may be required.

The Po protein is believed to be the closest relative to the ancestral gene for the whole Ig superfamily and, as such, structure-function studies with this molecule may be pertinent to the whole family of molecules. The results presented in this paper, and those reported by Recny et al. (1992), suggest that sugar residues can modulate the adhesiveness of a single Ig-domain by affecting the conformation, rather than interacting directly. Changes in the sugar composition of other, more complex Ig-family members such as N-CAM (Doherty et al., 1990*a*; Acheson et al., 1991), L1 (Kadmon et al., 1990) and I CAM-1 (Diamond et al., 1991) have been shown to alter their function. This too may be a result of conformational changes in the individual Ig domains of these molecules. Thus, these studies on the role of the sugar residues of Po give a good indication that the carbohydrates are likely to play an important role in the function and perhaps the modulation of function of Ig-like molecules.

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