

# Dominant-Negative Effect on Adhesion by Myelin Po Protein Truncated in Its Cytoplasmic Domain

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**Abstract.** The myelin Po protein is believed to hold myelin together via interactions of both its extracellular and cytoplasmic domains. We have already shown that the extracellular domains of Po can interact in a homophilic manner (Filbin, M.T., F.S. Walsh, B.D. Trapp, J.A. Pizzey, and G.I. Tennekoon. 1990. *Nature (Lond.)*. 344:871–872). In addition, we have shown that for this homophilic adhesion to take place, the cytoplasmic domain of Po must be intact and most likely interacting with the cytoskeleton; Po proteins truncated in their cytoplasmic domains are not adhesive (Wong, M.H., and M.T. Filbin. 1994. *J. Cell Biol.* 126:1089–1097). To determine if the presence of these truncated forms of Po could have an effect on the functioning of the full-length Po, we coexpressed both molecules in CHO cells. The adhesiveness of CHO cells expressing both full-length Po and truncated Po was then compared to cells expressing only full-length Po. In these coexpressors, both the full-length and the truncated Po proteins were glycosylated. They reached the surface of the cell in approximately equal amounts as shown by an ELISA and surface labeling, followed by immunoprecipitation. Furthermore, the amount of full-length Po at the cell

surface was equivalent to other cell lines expressing only full-length Po that we had already shown to be adhesive. Therefore, there should be sufficient levels of full-length Po at the surface of these coexpressors to measure adhesion of Po. However, as assessed by an aggregation assay, the coexpressors were not adhesive. By 60 min they had not formed large aggregates and were indistinguishable from the control transfected cells not expressing Po. In contrast, in the same time, the cells expressing only the full-length Po had formed large aggregates. This indicates that the truncated forms of Po have a dominant-negative effect on the adhesiveness of the full-length Po. Furthermore, from cross-linking studies, full-length Po, when expressed alone but not when coexpressed with truncated Po, appears to cluster in the membrane. We suggest that truncated Po exerts its dominant-negative effect by preventing clustering of full-length Po. We also show that colchicine, which disrupts microtubules, prevents adhesion of cells expressing only the full-length Po. This strengthens our suggestion that an interaction of Po with the cytoskeleton, either directly or indirectly, is required for adhesion to take place.

**T**HE most abundant protein of PNS myelin, a single-domain Ig-like molecule termed Po, is responsible for compaction at both the extracellular and cytoplasmic membrane surfaces (for review see Filbin and Tennekoon, 1992). Evidence supporting this notion derives from studies both in vitro (D'Urso et al., 1990; Filbin et al., 1990; Schneider-Schaulies et al., 1990) and in vivo (Giese et al., 1992; Hayasaka et al., 1993; Kulkens et al., 1993). In mice that do not express Po as a result of gene disruption by homologous recombination, the majority of axons are surrounded by loose whorls of uncompacted membranes (Giese et al., 1992). Lack of compaction in these Po  $-/-$  mice at both the extracellular and the cyto-

plasmic surfaces provides strong evidence that Po is indeed a "double" adhesion molecule, involved in interactions through both its extracellular and cytoplasmic domains. The importance of Po in the compaction and hence the physiological function of PNS myelin is further demonstrated in patients suffering from the peripheral neuropathy Charcot-Marie-Tooth type 1B (CMT1B)<sup>1</sup>. In all pedigrees of this subclassification of CMT, abnormalities in Po protein have been reported, either mutations or deletions of particular amino acids (Hayasaka et al., 1993; Kulkens et al., 1993; Su et al., 1993; Blanquet-Grossard et al., 1996).

Consistent with observations in vivo, the ability of Po to behave as a homophilic adhesion molecule through inter-

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1. *Abbreviations used in this paper:* CMT, Charcot-Marie-Tooth; Endo F, Endoglycosidase F; TPo52, Po truncated by 52 amino acids; TPo59, Po truncated by 59 amino acids.

actions of its extracellular domains has been clearly shown using *in vitro* adhesion assays (Filbin et al., 1990; D'Urso et al., 1990; Schneider-Schaulies et al., 1990). In these assays, CHO cells or other fibroblast cell lines, induced to express the Po protein by transfection of the Po cDNA, are at least two orders of magnitude more adhesive than the control cells not expressing Po (Filbin et al., 1990). In addition, where two Po-expressing cells meet, the protein has been shown to accumulate at the interface of the cells, consistent with a homophilic interaction (D'Urso et al., 1990). These results demonstrate directly that because of its homophilic adhesion, the extracellular domain of Po has the capacity to maintain compaction at the intraperiod line of myelin.

Although the cytoplasmic domain of Po is believed to hold myelin membranes together at the cytoplasmic surfaces, the adhesion of this domain is more difficult to monitor directly in intact cells. However, Ding and Brunden (1994) have shown that the isolated, purified cytoplasmic domain of Po can induce the clustering of lipid vesicles composed of acidic lipids. Furthermore, clustering is influenced by the phosphorylation of Po cytoplasmic domain (Ding and Brunden, 1994). This binding characteristic is in keeping with the suggestion that the cytoplasmic domain of Po interacts with acidic lipids in the opposing membrane to maintain compaction at the major dense line of myelin (Braun, 1984; Lemke et al., 1988).

In addition to its inherent adhesive capabilities, we have recently shown that the cytoplasmic domain of Po can influence the adhesive interactions of the extracellular domain. We showed that although they reach the surface when expressed in CHO cells, Po molecules with either 52 or 59 amino acids deleted from the 69-amino acid-long cytoplasmic domain were not adhesive (Wong and Filbin, 1994). Furthermore, since a portion of the full-length Po expressed in Schwann or CHO cells is insoluble in the nonionic detergent NP-40, an interaction with the cytoskeleton is suggested (Wong and Filbin, 1994). In contrast, only very little of the Po missing 52 amino acids and none of the Po missing the last 59 amino acids is insoluble in this detergent, consistent with a decreased interaction with the cytoskeleton, concomitant with a loss of adhesion. Based on these observations, we suggested a model whereby the cytoplasmic domain of Po influences the adhesion of the extracellular domain by both inducing a conformational change in the extracellular domain and also allowing Po to cluster within the membrane, thereby strengthening the adhesive interactions (Wong and Filbin, 1994). It is proposed that the clustering is brought about by interactions with the cytoskeleton at the early stages of myelination, before cytoplasm is extruded and cytoplasmic surfaces are brought into close apposition. If this model is correct, then the presence in the same membranes of nonadhesive Po proteins with truncated cytoplasmic domains could adversely affect the functioning of the full-length Po. Here we test this hypothesis by coexpressing full-length Po with Po missing either 52 or 59 amino acids from the cytoplasmic domain. We report that in these transfected cell lines, both the full-length and the truncated Po proteins reach the cell surface in quantities that should be sufficient for adhesion to occur. The presence, however, of either of the truncated Po proteins prevents the full-length Po protein

from behaving as an adhesion molecule. Because full-length Po appears to cluster in the membrane when expressed alone but not when coexpressed with truncated Po, we suggest that the dominant-negative effect is brought about by truncated Po, preventing clustering of full-length Po. This shows for the first time that mutated forms of Po can have a dominant-negative effect on the adhesiveness of the wild-type protein. In addition, we show that the microtubule cytoskeleton must be intact for adhesion of the full-length Po to take place.

## Materials and Methods

### Cell Maintenance

CHO cells deficient in the *dhfr* gene (Urlaub and Chasin, 1980) were maintained in MEM (supplemented with 10% FCS and proline 40 mg/liter) at 37°C in 5% CO<sub>2</sub>. 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<sub>2</sub> was added.

### Truncation of Po cDNA

Po cDNAs missing the nucleotides coding for the last 52 or 59 amino acids were prepared and characterized as previously described (Wong and Filbin, 1994). Briefly, the 1.08-kb ECoRI to XbaI fragment of the Po cDNA (Lemke and Axel, 1985) in bluescriptII (pBSP) was cut at base pairs 599 and 620 by restriction enzymes PstI and NcoI, respectively. The protruding end created by PstI in Po cDNA was filled to create a blunt end with dNTPs and T4 polymerase, whereas the NcoI protruding end was partially filled in by dATP, dCTP, and T4 polymerase, after which the remaining protruding sequence was cut by Mungbean nuclease to make it blunt ended. The truncated, blunt-ended Po cDNA was religated into pBSP vector that had been cut by SmaI restriction enzyme, which results in a blunt end. A new stop codon and four new amino acids (Arg, Gly, Ile, and His) were brought in at the end of each truncated Po cDNA as a result of a frame shift. This was confirmed by sequencing the 3' end of each truncated Po cDNA (Sanger et al., 1977). For the PstI and NcoI truncated Po cDNA, nucleotides coding for the last 59 and 52 amino acids, respectively, of Po's cytoplasmic domain were missing.

### Ligation of Truncated Po cDNAs into a Suitable Plasmid

The plasmid used for the expression of truncated Po cDNAs has been described previously (Lee and Nathans, 1988; Filbin and Tennekoon, 1990). Briefly, after attachment of the appropriate linkers, the truncated Po cDNAs were ligated into the pSJL plasmid at a unique XhoI cloning site downstream from the mouse metallothionein promoter and upstream from the poly (A) tail of the SV-40 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). A molar ratio of 1:1 of pSJL containing full-length Po cDNA/pSJL containing TPo52 or TPo59 cDNAs was used. 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 shown by Western blot analysis to be positive for expression of full-length Po, as well as either of the truncated Po proteins, were 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  $\mu\text{M}$ ) of methotrexate (MTX). Cells were plated at  $5 \times 10^5$  cells per 10-cm dish, 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%  $\beta$ -mercaptoethanol, and the following antiproteases: 1  $\mu\text{g}/\text{ml}$  leupeptin; 2  $\mu\text{g}/\text{ml}$  antipain; 10  $\mu\text{g}/\text{ml}$  benzamide; 1  $\mu\text{g}/\text{ml}$  chymotrypsin; 1  $\mu\text{g}/\text{ml}$  pepstatin; and 1  $\mu\text{g}/\text{ml}$  phenylmethylsulfonylfluoride. 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 recovered and the protein concentration was measured with a Bio-Rad kit (Bio-Rad Labs, Hercules, CA) before the addition of  $\beta$ -mercaptoethanol. The lysates were incubated at 95°C for 3 min, after which they were subjected to SDS-PAGE in a 12% acrylamide gel (Laemmli, 1970). The proteins were transferred to nitrocellulose and immunostained (Filbin and Poduslo, 1986) with a polyclonal antibody raised to the intact Po molecule at 1:2,000 by incubating overnight at 4°C; second antibody was alkaline phosphatase-conjugated goat anti-rabbit, 1:20,000 (Sigma Chemical Co., St. Louis, MO). The substrate was 5-bromo-4-chloro-3-indolylphosphate and the chromogen was nitro blue tetrazolium (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD) used according to the manufacturer's instructions.

### **Deglycosylation of Po with Endoglycosidase F (Endo F)**

Cells, 80–90% confluent, were lysated in 50 mM Tris, pH 7.2, containing 1% NP-40 and the following antiproteases: 1  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, 10  $\mu\text{g}/\text{ml}$  benzamide, 10  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  chemostatin, 1  $\mu\text{g}/\text{ml}$  pepstatin, and 0.1 mM PMSF. The lysate from one 10-cm dish was divided in half. To one half, 0.4 U of Endo F was added (Boehringer-Mannheim Corp., Indianapolis, IN). Samples, with and without Endo F, were incubated at 37°C for 16 h, after which the proteins were precipitated with 8 vol of ice-cold acetone. Samples were then subjected to SDS-PAGE, followed by transfer to nitrocellulose and immunodetection for Po.

### **Biotinylation of Cell Surface Proteins**

Cells at  $\sim 90\%$  confluency in 10-cm culture dishes were washed with PBS three times and then incubated with 0.5 mg/ml of sulfo-NHS-biotin for 30 min at room temperature, according to the manufacturer's instructions (Pierce, Rockford, IL). The labeling solution was removed and the cells were incubated one more time with the same concentration of sulfo-NHS-biotin for another 30 min. The cells were then washed with PBS three times and Po was immunoprecipitated from the surface biotin-labeled cells.

### **Immunoprecipitation of Po**

Sulfo-NHS-biotin-labeled cells in a 10-cm dish were lysed with 300  $\mu\text{l}$  of 250 mM Tris, pH 7.4, containing 2% SDS, 4 mM EDTA, and 150 mM NaCl. The cell lysate was collected by centrifugation at 14,000 rpm for 10 min. The supernatant fraction was removed to a fresh tube. A 200- $\mu\text{l}$  aliquot of this fraction was diluted to 1 ml in 250 mM Tris, pH 7.5, containing 4 mM EDTA, 150 mM NaCl, and 2.5% Triton. Then 15  $\mu\text{l}$  of Po polyclonal antibody was added and the mixture was incubated at 4°C overnight, with gentle rocking. 100  $\mu\text{l}$  of 10% protein A-Sepharose was added to the immunoprecipitated sample, and the mixture was incubated for an additional 2 h at 4°C with gentle rocking. The incubation mixture was centrifuged at 14,000 rpm for 10 min and the pellet was washed three times with the dilution buffer described above. Where indicated, the samples were deglycosylated with Endo F, as described above, before being eluted from the protein A-Sepharose. After the final wash, 50  $\mu\text{l}$  of  $1\times$  SDS sample buffer and 4  $\mu\text{l}$  of  $\beta$ -mercaptoethanol were added to the pellet, which was incubated for 3 min at 98°C and then centrifuged for 10 min at 14,000 rpm. The supernatant fraction was collected, subjected to SDS-PAGE (Laemmli, 1970), and biotinylated Po was detected by immunodetection for biotin after Western blotting using streptavidin-HRP (1:7,000) and enhanced chemiluminescence (ECL), according to the manufacturers instructions (Amersham, Corp., Arlington Heights, IL).

To detect Po that may be shed from transfected cells, cells were grown in 3 ml of media containing 2% serum per 10-cm dish. After 24 h, media

from  $3 \times 10$ -cm dishes, each with cells coexpressing full-length and truncated Po at  $\sim 80\%$  confluency, was collected and combined. The media was immunoprecipitated for Po as described above except that the initial volume of Po antibody used was increased to 90  $\mu\text{l}$ . As controls, media from cells expressing only Po or control-transfected cells was used.

### **Quantitation of Po Expressed at the Cell Surface**

An ELISA was carried out as previously described by Doherty et al., (1990), 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. The cells were blocked for 30 min with 3% normal goat serum in MEM and then incubated overnight at 4°C with a rabbit Po peptide antibody directed against sequences in the extracellular domain of Po (1:1,500) in MEM containing 1% normal goat serum. The cells were rinsed, blocked again, and then incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (1:250) (Sigma Chemical Co.). The cells were rinsed with PBS and then water. Color was developed by the addition of 50  $\mu\text{l}$  of 0.2% (wt/vol) *O*-phenylenediamine (Sigma Chemical Co.) and 0.02%  $\text{H}_2\text{O}_2$  in citrate buffer (pH 5.0) to each well. The reaction was stopped after 15 min by the addition of 50  $\mu\text{l}$  of 4.5 M  $\text{H}_2\text{SO}_4$  and the optical density at 490 nm was determined with a 96-well plate reader. All incubations were at room temperature unless stated otherwise. Controls consisted of control transfected cells incubated with Po antibodies. For each experiment, 40 wells were assayed at least three times for each cell line. The average number of cells per well was estimated by removing them with trypsin and counting them with a Coulter counter. Cells from five separate wells were counted for each 96-well plate. Results were standardized to absorbance units per cell.

### **Adhesion Assay**

The adhesion 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  $1.5\text{--}2 \times 10^6$  cells per ml and allowed to aggregate at 37°C in 5%  $\text{CO}_2$  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.

### **Mixed Adhesion Assay**

The mixed adhesion assay was carried out as previously described (Filbin and Tennekoon, 1993) with the following modifications. A single-cell suspension of cells expressing only full-length Po was mixed at a 50:50 ratio with cells coexpressing Po and Po truncated by 59 amino acids (TPo59) to give a final cell concentration of  $2\text{--}3 \times 10^6$  cells per ml. Aggregation was then allowed to proceed as described above. To distinguish the two different populations of cells, in each experiment one cell population or other was vitally labeled by incubation with 10  $\mu\text{M}$  of the dye calcein AM (Molecular Probes, Eugene, OR), for 15 min at 37°C and washed with PBS before mixing. Samples were withdrawn at different times and examined under both phase and fluorescent microscopes. At least three separate incubations were performed for each experiment, and duplicate samples were withdrawn at each time point.

### **Chemical Cross-linking of Surface Po**

Cells (80–90% confluent), surface labeled with sulfo-NHS-biotin while as a monolayer in a 10-cm dish, were removed by incubation with ice-cold calcium, magnesium-free PBS (CMF PBS). Cells were collected by centrifugation in a microfuge and resuspended in 2 ml of CMF PBS. The sample was divided in half. To one half, 10  $\mu\text{l}$  of 0.5 mM 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) in DMSO was added, and to the other, 10  $\mu\text{l}$  of DMSO was added. Both samples were rocked gently for 45 min at room temperature. The reaction was stopped by the addition of 20  $\mu\text{l}$  of 1 M Tris, pH 7.5, followed by a further 15 min incubation while rocking. The cells were then washed three times with CMF PBS and lysed in immuno-

precipitation buffer. Samples were immunoprecipitated with Po antibody and deglycosylated as described above. Samples were separated by SDS-PAGE, with and without  $\beta$ -mercaptoethanol, as indicated, and biotinylated proteins were detected by chemiluminescence as described above.

### Treatment of Cells with Agents That Disrupt the Cytoskeleton

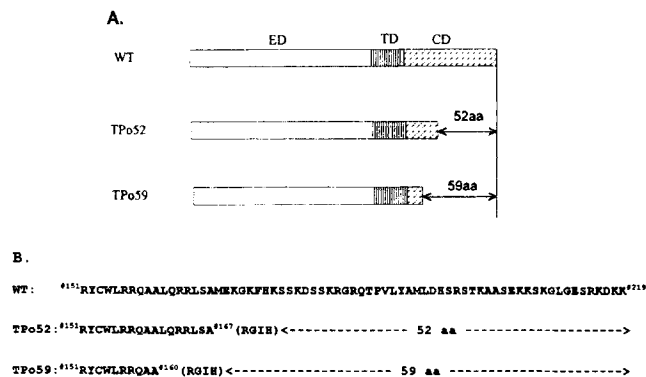
A single cell suspension of cells expressing full-length Po or control cells was incubated with either 100  $\mu$ M colchicine or 35  $\mu$ g/ml cytochalasin for 30 min at 37°C while gently rocking. The cells were then washed and resuspended in MEM with or without either colchicine or cytochalasin. The ability of the cells to exclude 0.4% trypan blue was used as assessment of their viability. The adhesion assay was carried out in the presence or absence of either colchicine or cytochalasin.

## Results

### Coexpression of Full-Length Po and Truncated Po Proteins in CHO Cells

We have already established (a) that full-length Po can behave as a homophilic adhesion molecule via interactions of its extracellular domain (Filbin et al., 1990) and (b) that the cytoplasmic domain must be intact for extracellular domain adhesion to take place. We demonstrated that Po proteins missing either 52 or 59 amino acids from the cytoplasmic sequences are not adhesive (Wong and Filbin, 1994). To test if the presence of the cytoplasmic domain-truncated Po proteins could influence the adhesive functioning of the full-length Po molecules, full-length Po (designated Po) and Po missing either 52 (TPo52) or 59 amino acids (TPo59) from their cytoplasmic sequences were coexpressed in CHO cells. The adhesiveness of these coexpressors was then compared to the adhesiveness of the cells expressing only the full-length Po.

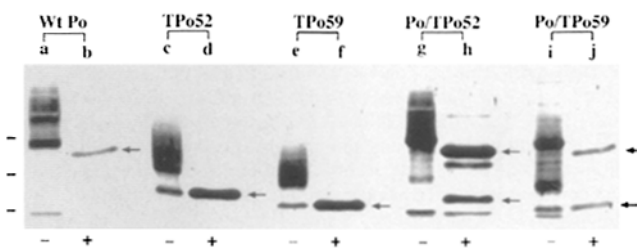
Plasmids containing the cDNA for Po were transfected into CHO cells at a 1:1 molar ratio with plasmids containing the cDNA for either TPo52 or TPo59 (Fig. 1). After selection in G418, amplification in methotrexate (Filbin and Tennekoon, 1990), and single cell cloning by limiting dilution, a number of clones were shown by Western blotting to express both the full-length and the truncated Po proteins. Fig. 2 shows the results from two such clones, one coexpressing Po and TPo52, designated Po/TPo52 (Fig. 2, lanes g and h) and the other coexpressing Po and TPo59, designated Po/TPo59 (Fig. 2, lanes i and j). Sugar residues, which represent approximately 6% by weight of the molecule, are attached to Po protein via a single N-linkage at asparagine 93 (Everly et al., 1973; Kitamura et al., 1976; Lemke and Axel, 1985; Sakamoto et al., 1987). The Po proteins expressed by Po/TPo52 and Po/TPo59 cell lines are of the same approximate molecular weights as when they are expressed individually in CHO cells, indicating that they are all glycosylated (Fig. 2, compare lanes g and i with a for the Po, lane c with g for the TPo52, and lane e with i for the TPo59). This is confirmed by the decrease in size for all Po species after treatment with Endo F, which removes all N-linked sugars. As the gels represented in Fig. 2 are of low percentage acrylamide, the glycosylation pattern appears as multiple minor components. This can be attributed to microheterogeneity of the glycosylation pattern, as only sharp bands are observed after deglycosylation with Endo F. Occasionally a band of about 20 kD is



**Figure 1.** (A) Diagrammatic representation of the construction of the truncated Po cDNAs. WT, wild-type Po; ED, extracellular domain; TD, transmembrane domain; CD, cytoplasmic domain. (B) The amino acids in the cytoplasmic domain of Po showing the 52 and the 59 amino acids removed from TPo52 and TPo59 and also showing the four new amino acids introduced into the truncated proteins (taken from Wong, M., and M. Filbin, 1994. *J. Cell Biol.* 126:1089–1097).

apparent. It is well documented that Po is very susceptible to proteolysis (Cammer et al., 1981) despite the presence of antiproteases when the cells are lysed. As this proteolysis is likely to occur only when the cells are lysed, it would not affect the adhesion of the intact cells. This view is supported by the fact that this proteolytic form of Po is sometimes apparent in lysates from cells expressing only the full-length Po protein which are adhesive (Fig. 2, lane a).

It can also be seen from Fig. 2, and more clearly when the proteins are deglycosylated, that each of the two cell lines, Po/TPo52 and Po/TPo59, express amounts of full-length Po, per 30  $\mu$ g of total protein, at least equivalent to that expressed by the CHO cell line expressing only full-length Po, already shown by us to be adhesive (Fig. 2, lanes a and b; Filbin et al., 1990; Filbin and Tennekoon, 1991, 1993; Wong and Filbin, 1994). Hence, these cell lines are expressing sufficient full-length protein to measure the adhesiveness of Po. In addition, it is apparent from Fig. 2 that these coexpressors are expressing approximately equal amounts of the full-length Po and the truncated Po,

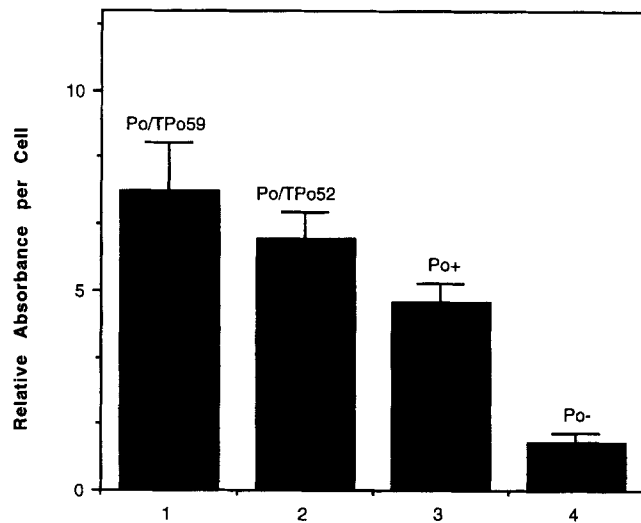


**Figure 2.** Effect of treatment of Po expressed by transfected CHO cells with Endo F. Proteins (30  $\mu$ g/lane) from cell lysates with (+) and without (-) treatment with Endo F, transfected with the full-length Po cDNA (lanes a and b), the TPo52 cDNA (lanes c and d), the TPo59 cDNA (lanes e and f), the full-length and the TPo52 cDNAs (lanes g and h), and the full-length and the TPo59 cDNAs (lanes i and j) were separated by SDS-PAGE and immunostained for Po. Arrows refer to deglycosylated full-length and truncated Po. Bars indicate molecular weight standards from top to bottom as follows: 32.5, 29, and 21 kD.

either TPo52 or TPo59. Therefore, these two cell lines, Po/TPo52 and Po/TPo59, are suitable to assess if the truncated Po proteins affect the adhesiveness of the full-length protein. However, before these experiments were carried out, we first ensured not only that Po was reaching the cell surface in these coexpressors but that both the full-length and the truncated Po proteins are reaching the cell surface in approximately equal amounts. Surface expression of Po in the coexpressors was (a) quantitated by an ELISA and compared to the surface expression of cells expressing only full-length Po, and (b) surface expression of Po and TPO in each coexpressor was compared by labeling of surface proteins, followed by immunoprecipitation of Po, as described below.

### Surface Expression of Po Protein

To quantitate the level of expression of Po at the cell surface in the coexpressors, an ELISA assay was carried out on fixed, unpermeabilized cells. Cells expressing either Po/TPo59 or Po/TPo52 (Fig. 3, columns 1 and 2) expressed at least equivalent levels of Po at the cell surface as cells expressing only full-length Po (Fig. 3, column 3), which we had already shown to be adhesive (Filbin et al., 1990; Filbin and Tennekoon, 1991; 1993; Wong and Filbin, 1994). In fact, the Po/TPo59 cells are expressing significantly more Po at the surface than the cells expressing only full-length Po. Only background staining was apparent for control-transfected cells when the Po antibody was used (Fig. 3, column 4) and when the ELISA was carried out with an antibody raised to the cytoplasmic domain of Po (results not shown). This demonstrates that the majority of



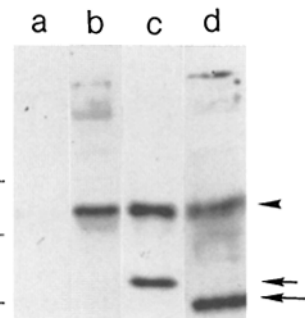
**Figure 3.** Quantitation of Po expressed at the surface of transfected CHO cells. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected cells expressing full-length Po and TPo59 (column 1, *Po/TPo59*), full-length Po and TPo52 (column 2, *Po/TPo52*), only full-length Po (column 3, *Po+*), and control-transfected cells (column 4, *Po-*), by using a Po peptide antibody directed against sequences in the extracellular domain. Results are expressed ( $\pm$ SE) in relative absorbance units per cell and are the mean of three experiments with 40 samples per experiment.

cells are intact and that under these conditions of staining, only surface Po is being measured.

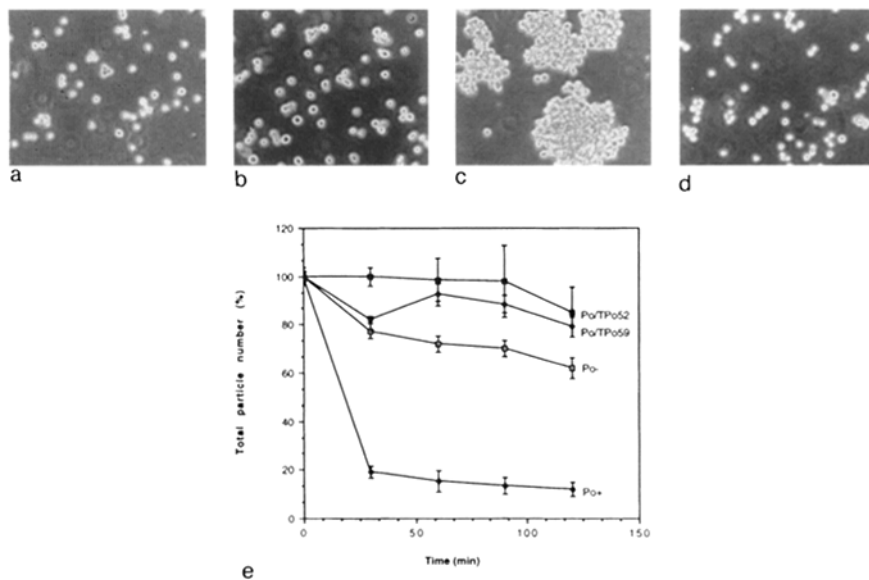
To confirm that both Po proteins, the full-length and the truncated proteins, were reaching the cell surface in the coexpressors, surface proteins were first labeled with biotin, and Po was then immunoprecipitated from the total cell lysate and deglycosylated to aid in detection and quantitation. The biotinylated proteins in the precipitate were identified on Western blots with streptavidin-HRP and a chemiluminescence detection system. Fig. 4 shows that for the cotransfected cells, both full-length and truncated Po are biotinylated for both the Po/TPo52 and Po/TPo59 cell lines, demonstrating that for each cell line, both proteins are reaching the cell surface (Fig. 4, lanes *c* and *d*). For each of the coexpressors, approximately equal amounts of the TPo52 or TPo59 and Po appear to be biotinylated, suggesting that they appear at the surface in equal amounts (Fig. 4, lane *c* and *d*). Furthermore, in both Po/TPo52 and Po/TPo59 there appears to be approximately the same amount of the full-length Po as when it is expressed alone (Fig. 4, lane *b*, *c*, and *d*), again suggesting that there is sufficient full-length Po at the surface in these coexpressors to be adhesive. These three lines were then used to compare the effect of coexpression of Po proteins truncated in their cytoplasmic domains on the adhesiveness of the full-length Po proteins.

### Adhesion of Cells Coexpressing Full-Length and Truncated Po Proteins

To assess the adhesiveness of the CHO cells simultaneously expressing full-length and truncated Po proteins, relative to cells expressing only the full-length Po protein, we carried out a reaggregation assay as we have used previously to monitor the adhesiveness of Po protein under various conditions (Filbin et al., 1990; Filbin and Tennekoon, 1991, 1993; Wong and Filbin, 1994; Zhang and Filbin, 1994). In this assay, a single cell suspension is incubated and aggregation is monitored both by microscopic examination and by counting the total particle number at various times. This permits both a qualitative (microscopic examination) and quantitative (counting) analysis since a



**Figure 4.** Immunoprecipitation of Po from cells that are surface labeled with biotin. Intact cells were covalently labeled with biotin and then solubilized, and Po was immunoprecipitated with a rabbit anti-bovine Po polyclonal antibody. Precipitated proteins were deglycosylated and separated by SDS-PAGE, and the biotinylated proteins were visualized with a streptavidin-HRP-conjugated second antibody and a chemiluminescence method of detection. (Lane *a*) Control cells, (lane *b*) cells expressing only full-length Po, (lane *c*) cells expressing full-length Po and TPo52, and (lane *d*) cells expressing full-length Po and TPo59. The arrowhead refers to full-length Po, the short arrow refers to TPo52, and the long arrow refers to TPo59. Bars refer to molecular weight standards from top to bottom as follows: 32.5, 29, and 21 kD.



**Figure 5.** Aggregation properties of Po-expressing cells. Single-cell suspensions of CHO cells expressing (a) full-length Po and TPo52, (b) full-length Po and TPo59, (c) full-length Po only, or (d) control-transfected cells were allowed to aggregate. Samples were withdrawn at intervals and examined under the microscope (a–d results after 60 min aggregation), and the total particle number was counted in a Coulter counter. The total particle number  $\pm$  SE was plotted against time (e).

decrease in total particle number correlates with aggregate formation, and the rate of aggregate formation is an indication of the strength of adhesion.

By 60 min, only the cells expressing the full-length Po alone had formed large aggregates (Fig. 5 c). As we have previously reported (Filbin et al., 1990; Filbin and Tennekoon, 1991; 1993; Wong and Filbin, 1994; Zhang and Filbin, 1994), these aggregates consist of up to 300 cells per aggregate. In sharp contrast, the cells coexpressing truncated Po proteins with the full-length Po had not formed aggregates within the same time (Fig. 5, a and b). They were predominantly single cells, doublets, and triplets and were indistinguishable from the appearance of the control-transfected cells (Fig. 5 d). Consistent with the microscopic observations, the total particle number for the cells expressing only the full-length Po had dropped to  $\sim$ 20% of the starting number by 60 min. By contrast, the total particle number for the cells coexpressing full-length and truncated Po proteins (Po/TPo52 and Po/TPo59) dropped to about only 80% in the same time, a value similar to that for the control cells (Fig. 5 e).

It is possible that, because of the short cytoplasmic domain, truncated Po is being shed from the coexpressing cells and, in a soluble form, blocking Po–Po interactions between cells, consequently preventing aggregation. This does not appear to be the case for two reasons. First, after immunoprecipitation with Po antibody of conditioned media from Po-expressing cells (combined media from  $3 \times 10$ -cm dishes of semiconfluent cells, conditioned 24 h), no Po was detected in the precipitate. Second, when a single-cell suspension of a 50:50 mix of cells expressing only full-length Po and cells coexpressing full-length and truncated Po were allowed to aggregate, with one cell type vitally labeled with a fluorescent dye, only the cells expressing full-length Po alone formed aggregates. The cells coexpressing full-length and truncated Po remained as mostly single cells with very few joining the aggregates. The same result was obtained regardless of which cell population was vitally labeled (Fig. 6, A and B). Therefore, the presence of

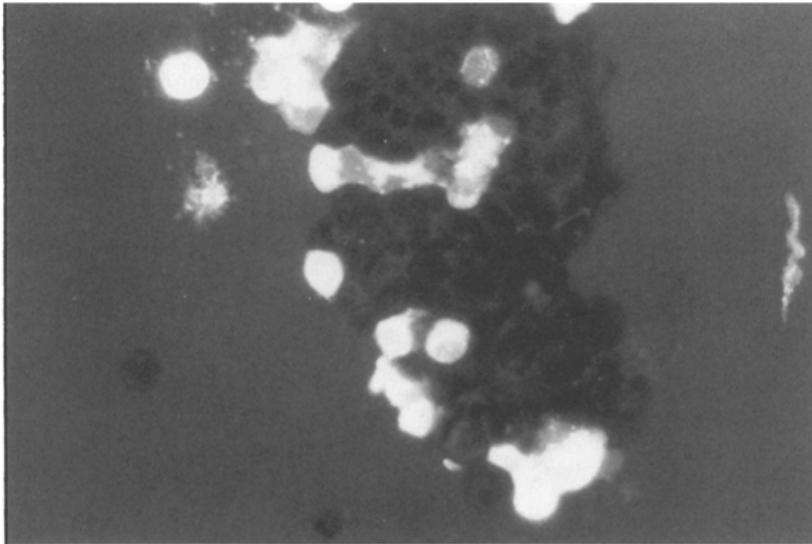
cells coexpressing full-length and truncated Po has no effect on the aggregation properties of cells expressing only full-length Po.

Taken together, these results strongly suggest that, even though expression of the full-length Po protein should be sufficient to increase the adhesiveness of these cells, adhesion of full-length Po is prevented by the presence of truncated Po in the same membrane. Truncated Po, therefore, has a dominant-negative effect on adhesion.

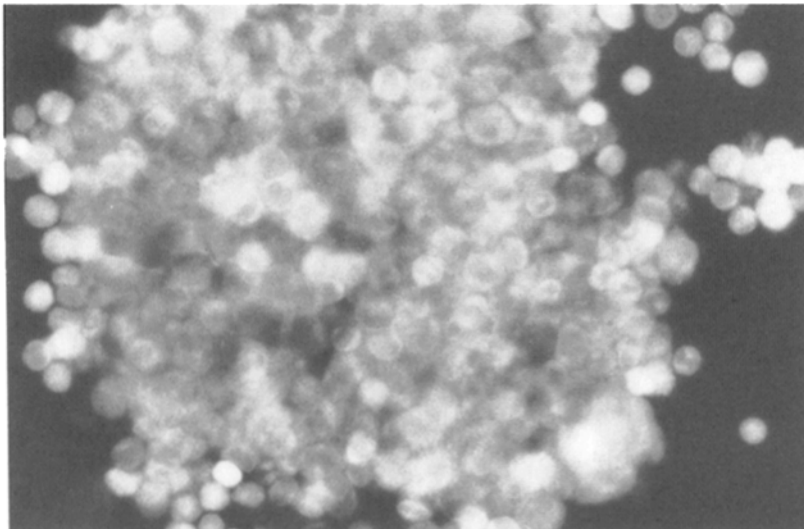
### Clustering of Po and Truncated Po Proteins

To determine if Po clusters in the membrane before interacting with a Po molecule in an opposing cell, a single-cell suspension of cells expressing only full-length Po was treated with the reducible cross-linking agent DTSSP. Before exposure to cross-linker, the cells were surface biotinylated when growing as a monolayer. After incubation with cross-linking agent, the cells were lysated, immunoprecipitated with Po antibody, and, to aid in detection, deglycosylated. The biotinylated proteins in the precipitate were identified on Western blots with streptavidin-HRP and a chemiluminescence detection system. It was found that if cells expressing only full-length Po are treated with cross-linking agent and not reduced, little or no Po is detected in the immunoprecipitates (Fig. 7, lane b). Under the same conditions, but without cross-linker, an abundance of Po is precipitated under nonreducing conditions, apparent at a molecular weight consistent with unreduced Po (Fig. 7, lane a). Similarly, if the cells are exposed to cross-linker and the lysate immunoprecipitated in the absence of reducing agent, but  $\beta$ -mercaptoethanol is added to the sample immediately before separation by SDS-PAGE, an abundance of Po is apparent, equivalent to samples that were not cross-linked (Fig. 7, lanes c and d). These results suggest that when cross-linked, full-length Po expressed alone by CHO cells, after immunoprecipitation, is in very large aggregates that do not enter a 12% polyacrylamide gel and are therefore not detected; Po in these samples is

A



B



*Figure 6.* Aggregation properties of cells expressing only full-length Po in the presence of cells coexpressing full-length Po and truncated Po. A single-cell suspension of CHO cells expressing only full-length Po and coexpressing full-length Po and TPo59 were mixed in a 50:50 ratio. One population or other was labeled with the vital fluorescent dye, Calcein AM. Samples were withdrawn at intervals and examined under a fluorescent microscope. (A) Cells coexpressing Po and TPo59 are labeled with the fluorescent dye; cells expressing only full-length Po are unlabeled. (B) Cells expressing only full-length Po are labeled with the fluorescent dye; cells coexpressing Po and TPo59 are unlabeled. Results shown are after 40 min aggregation.

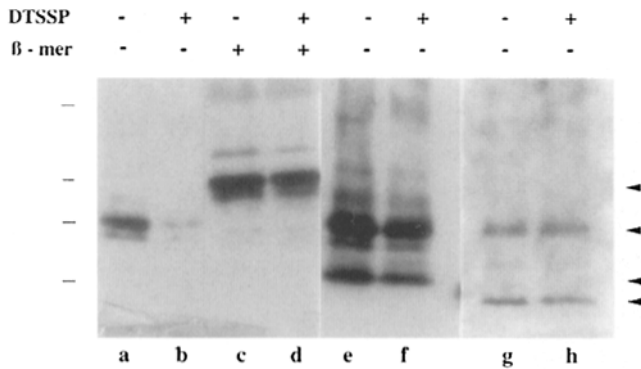
only detected if the aggregates are disrupted by a reducing agent before loading on the gel. In contrast, if truncated Po is coexpressed with full-length Po in the same cell, approximately the same amount of each Po protein is apparent under nonreducing conditions, regardless of whether or not the samples were treated with cross-linking agent, suggesting that there are no large aggregates (Fig. 7, lanes *e-h*). It should be noted that because of the differences in degree of biotinylation for each cell preparation, only samples in lanes *a-d*, *e* and *f*, and *g* and *h*, can be compared quantitatively with each other. However, although the overall level of biotinylation appears less for the cells coexpressing Po and TP59 (Fig. 7, lanes *g* and *h*) than for the other cells, it is still obvious that there is approximately the same amount of Po with and without cross-linking; there are no large aggregates. Together, these results suggest that when expressed alone, full-length Po clusters within the membrane even before coming in contact with a Po

protein in an opposing cell. However, the presence of truncated Po protein not only does not cluster itself but also prevents clustering of the full-length Po protein.

#### ***The Effect of Colchicine and Cytochalasin on the Adhesiveness of Full-Length Po Protein***

We have already shown that a percentage of full-length Po is insoluble in the detergent NP-40, while only very little TPo52 is insoluble and TPo59 is completely soluble (Wong and Filbin, 1994). Insolubility in this nonionic detergent suggests that a protein is interacting with the cytoskeleton. To determine if indeed disruption of the cytoskeleton affects the adhesion of Po expressed in CHO cells, two drugs were used: cytochalasin, which disrupts microfilaments, and colchicine, which disrupts microtubules. Drug concentrations and conditions of incubation were as used by others to successfully disrupt the cytoskeleton (Cheung and





**Figure 7.** Chemical cross-linking of surface Po. A single-cell suspension of surface biotinylated Po-expressing cells were treated with the reducible cross-linking agent DTSSP for 45 min. Cells were then solubilized, and Po was immunoprecipitated with a rabbit anti-bovine Po polyclonal antibody. Precipitated proteins were deglycosylated and separated by SDS-PAGE, and the biotinylated proteins were visualized with a streptavidin-HRP-conjugated second antibody and a chemiluminescence method of detection. Where indicated, DTSSP was omitted (-) from the reaction and  $\beta$ -mercaptoethanol was added (+) immediately before separation by SDS-PAGE. (Lanes a-d) Cells expressing only full-length Po, (lanes e and f) cells coexpressing Po and TPO52, (lanes g and h) cells coexpressing Po and TPO59. Arrows refer to various forms of Po, from top to bottom as follows: reduced full-length Po, unreduced full-length Po, unreduced TPO52, and unreduced TPO59. Bars refer to molecular weight standards, from top to bottom as follows: 35, 29.7, and 21.9 kD.

Juliano, 1984; Murray and Jensen, 1992). Pretreatment of the cells and inclusion of cytochalasin in the incubation had no effect on the adhesiveness of the Po-expressing cells (results not shown). On the other hand, treatment of the cells with 100  $\mu$ M colchicine prevented aggregate formation completely; by 60 min the total particle number for the colchicine treated cells expressing full-length Po and the control cells dropped to only 80% (Fig. 8a). There was no difference in the surface expression of Po on cells treated or not treated with colchicine, as assessed by an ELISA (Fig. 8b). Colchicine had no effect on the behavior of the control transfected cells (Fig. 8, a and b). These results suggest that the adhesiveness of the extracellular domain of full-length Po is dependent on an interaction of the cytoplasmic domain, either directly or indirectly, with microtubules.

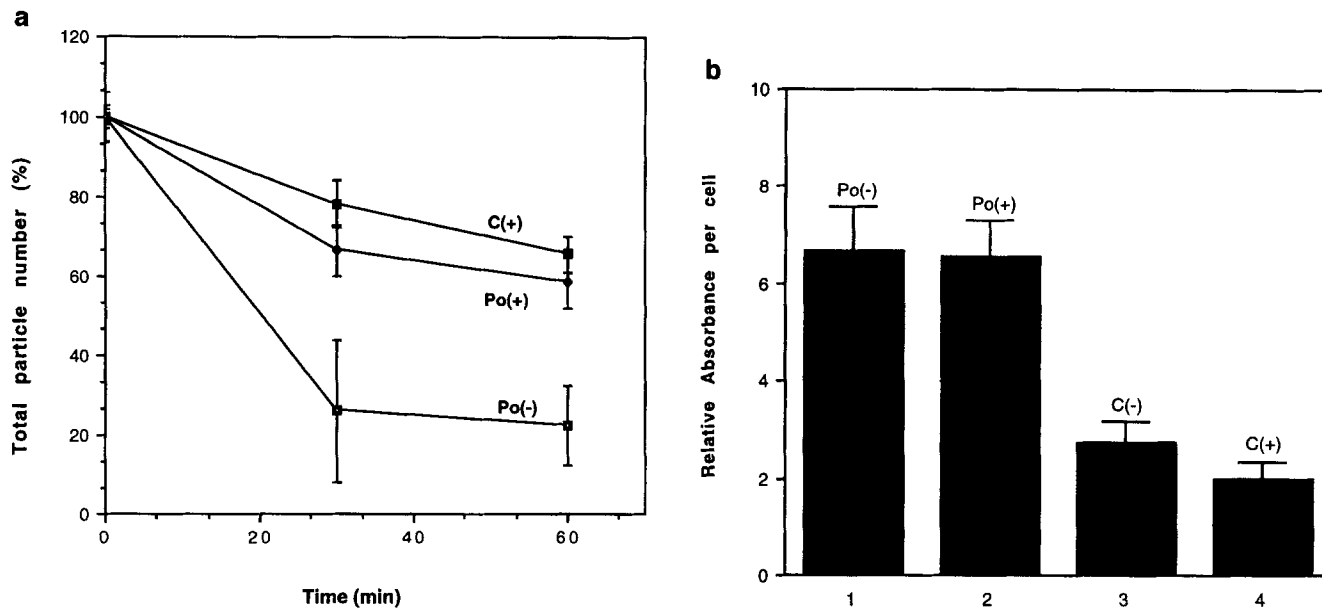
### Discussion

It is widely accepted that the cytoplasmic domain of Po is responsible for compaction at the major dense line of myelin and, most likely, that this is a result of an interaction with acidic lipids in the opposing membrane (for reviews see Braun, 1984; Lemke et al., 1988; Filbin and Tennekoon, 1992; Giese et al., 1992; Ding and Brunden, 1994). In addition, we have demonstrated that an intact cytoplasmic domain is required for the adhesion of the extracellular domain of Po to take place and that full-length, but not truncated Po, is likely to interact with the cytoskeleton (Wong and Filbin, 1994). Based on these observations, we

proposed a model to explain the adhesion of Po whereby the cytoplasmic domain can influence the adhesion of the extracellular domain, perhaps by inducing a conformational change. In addition, we suggest that the molecule must cluster within the membrane, probably after an interaction of the cytoplasmic domain with the cytoskeleton, for stable membrane-membrane adhesion to occur (Wong and Filbin, 1994). Here we strengthen this model. We show that Po truncated in its cytoplasmic sequences can have a dominant-negative effect on the adhesion of the extracellular domain. When the full-length Po is expressed in the same membrane as the truncated Po, adhesion does not occur. Therefore, the model we propose can be extended to suggest that not only must Po cluster in the membrane, but there must also be a critical number of functional Po molecules within the cluster for stable adhesion to take place (Fig. 9). The cross-linking studies indicate that when expressed alone, full-length Po does indeed cluster, but when coexpressed with truncated Po, no clusters form. In keeping with the model, we suggest that the truncated Po molecules are having a dominant-negative effect on adhesion by preventing the clustering of full-length Po (Fig. 9). Furthermore, the ability of colchicine to disrupt the adhesion of the full-length Po strengthens our suggestion that an interaction of Po with the cytoskeleton is crucial for adhesion to take place.

This model contributes to the emerging picture of how Po functions in compact myelin. The idea of clustering and increased adhesion allows for a single, small molecule to result in strong adhesion, as is found in compact myelin. To our knowledge, this is the first time a dominant-negative effect has been reported for an Ig-like adhesion molecule. The ability of Po to function in a dominant-negative manner may have relevance to the phenotype of the demyelinating disease CMT1B. Point mutations or single amino acid deletions in Po protein have been reported for all patients suffering from CMT1B (for reviews see Chance and Pleasure, 1993; Chance and Fischbeck, 1994; Patel and Lupski, 1994). However, these patients are all heterozygotes for the mutated gene and so produce 50% of the usual levels of normal protein. In some instances of CMT1B, a reduction in functional protein could account for the disease, particularly in those patients with a mild phenotype. This is supported by the observation that in mice heterozygote for the Po null mutation, myelination and myelin maintenance is normal until the mice are about 4-mo old. At this later stage of development, demyelination is apparent in a majority of the nerves examined from these older mice (Martini et al., 1995). This suggests that half the amount of Po protein is sufficient for formation of normal myelin initially but not to sustain compact myelin throughout the lifetime of the animal. On the other hand, gene dose cannot alone explain the phenotype of all pedigrees of CMT1B (for reviews see Chance and Pleasure, 1993; Chance and Fischbeck, 1994; Patel and Lupski, 1994). For those patients with a more severe phenotype and an earlier onset of the disease, the presence of the mutated Po protein is likely to contribute directly to the manifestation of the disease. As half the Po protein in these patients is also normal, the presence of the mutated protein, as we report here for truncated Po, could be having a dominant-negative effect on the functioning of the wild-





**Figure 8.** (a) The effect of colchicine on the adhesion of Po-expressing cells. Cells expressing full-length Po (*Po*) or control cells (*C*) were preincubated with 100  $\mu$ M colchicine for 30 min and then allowed to aggregate in the presence of 100  $\mu$ M colchicine, *Po*(+) and *C*(+), or cells were not treated with colchicine, *Po*(-). Samples were withdrawn at intervals and the total particle number counted. The total particle number  $\pm$  SE was plotted against time. (b) Quantitation of Po expressed at the surface of transfected CHO cells after treatment with colchicine. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected cells expressing full-length Po not treated with colchicine (column 1, *Po*(-)), full-length Po treated with colchicine (column 2, *Po*(+)), control cells not treated with colchicine (column 3, *C*(-)), and control cells treated with colchicine (column 4, *C*(+)) by using a Po peptide antibody directed against sequences in the extracellular domain. Results are expressed ( $\pm$ SE) in relative absorbance units per cell and are the mean of three experiments with 40 samples per experiment.

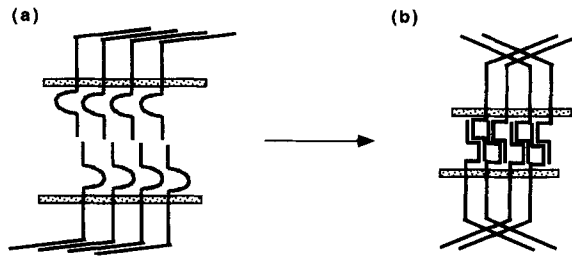
type protein. Although these mutations are for the most part in the extracellular sequences of Po, the model we present and the notion of a critical number of functional Po molecules being necessary for effective membrane-membrane adhesion still applies to these mutations. That is to say, the Po molecules mutated in their extracellular sequences are not adhesive and so dilute the number of functional Po molecules in each cluster, diminishing the sum total of adhesion affinity per cluster to the point where membranes are no longer held together. In those patients with a late onset and mild phenotype, it is possible that some forms of mutated Po never reach myelin and so have no dominant-negative effect. Consequently demyelination in these patients is a result, solely, of gene dose.

As we have suggested before, because there are no cytoskeletal elements in compact myelin, it is probable that the interaction of Po with the cytoskeleton takes place at the early stages of myelination, before cytoplasm is extruded from the leaflets (Fig. 9; Wong and Filbin, 1994). A portion of Po expressed in Schwann cells is also insoluble in NP-40, which shows that the putative cytoskeleton interaction is not an artifact of CHO cells (Filbin and Wong, 1994). The ability of colchicine to abolish adhesion of full-length Po greatly strengthens the idea that an interaction with the cytoskeleton is necessary for adhesion of Po to take place. Drugs that disrupt the cytoskeleton have been shown to prevent the adhesion of other molecules (Cheung and Juliano, 1984; Carpen et al., 1992; Murray and Jensen, 1992; Kinch et al., 1993). Although disruption of the microtubule network has an effect on the functioning of a

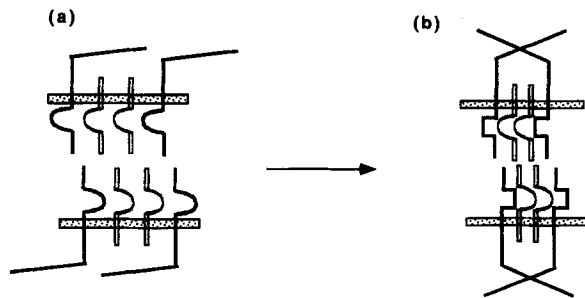
number of adhesion molecules (Cheung and Juliano, 1984; Domnina et al., 1985; Kinch et al., 1993; Timar et al., 1995), more often the actin network has been implicated (for review see Pavalko and Otey, 1994). It should be noted, however, that the precise and perhaps overlapping roles of the different cytoskeletal networks has not been fully characterized. Microtubules are known to be involved in vesicular transport (Hugon et al., 1987; Achler et al., 1989; Gilbert et al., 1991). Indeed in Schwann cells treated with colchicine *in vivo*, Po-containing vesicles have been shown to accumulate in the perinuclear region of the cell. Consequently, Po does not reach myelin (Trapp et al., 1995). In the Po-expressing CHO cells used in the adhesion assay here, there is no difference in the surface expression of Po on cells treated or not treated with colchicine. It is possible that microtubules not only transport the Po-containing vesicles to myelin but also participate in Po clustering before disengaging.

The model we propose for Po to function as an adhesion molecule in membranes/myelin (Fig. 9) is similar to the models proposed for other types of adhesion molecules, namely the cadherins and the integrins. Cadherins are known to interact via their cytoplasmic domains with molecules termed catenins, which in turn interact with the cytoskeleton (Nagafuchi and Takeichi, 1988; Hirano et al., 1992). As we suggest for Po, an interaction of cadherins with the cytoskeleton and concurrent with this, an intact cytoplasmic domain, is necessary for the homophilic adhesion of cadherins to take place (Nagafuchi and Takeichi, 1988; Nagafuchi et al., 1991; Kintner, 1992; Levine et al.,

### A. Full-length Po



### B. Full-length and truncated Po



**Figure 9.** Model of a dominant-negative effect of truncated Po on the adhesion of full-length Po. (A) Interactions of full-length Po when expressed alone. (a) An initial low affinity Po-extracellular domain interaction triggers a change in the interaction of the cytoplasmic domain with the cytoskeleton. (b) The cytoskeleton reorganizes and pulls back, inducing a conformational change in the extracellular domain and clustering. Both clustering and the conformational change strengthen the adhesion of the extracellular domain. (B) Interactions of full-length Po when coexpressed with TPo52 or TPo59. (a) The initial low affinity interaction of the Po extracellular domains takes place, triggering a change in the interaction of the full-length Po molecules with the cytoskeleton. (b) The cytoskeleton reorganizes and pulls back, but the formation of clusters is prevented by the presence of the truncated Po molecules. The overall adhesiveness is decreased because of the presence of the nonadhesive, truncated Po molecules. Adhesion is not strong enough to hold two membranes together.

1994). Integrins have been shown to cluster in the membrane and in so doing cluster other proteins located at the cytoplasmic surface (for reviews see Hynes, 1992; Gumbiner, 1993). Mutated forms of both cadherins (Kintner, 1992; Fujimori and Takeichi, 1993; Levine et al., 1994) and integrins (Balzac et al., 1994; Lukashev et al., 1994; Smilenov et al., 1994), like Po, have been shown to have dominant-negative effects on the adhesion of their respective wild-type counterpart. These mutations consist of truncations of either the cytoplasmic or extracellular domain, suggesting that, as we suggest for Po, there is an interdependence in the functioning of the two regions of the molecule, despite the fact that a lipid membrane separates the two.

Recently, although neither carries a recognizable signaling motif in the cytoplasmic domain, both cadherins and integrins have been implicated in signal transduction (Akiyama et al., 1994; Huang et al., 1993; Heasman et al., 1994;

Funayama et al., 1995). For both types of molecule, this involves an interaction with the cytoskeleton, and for integrins, clustering is required. There are no known signaling motifs in the cytoplasmic domain of Po. However, the possibility that Po plays a role in signal transduction is suggested by two observations. First, in the Po  $-/-$  mice there is a misregulation of other myelin proteins, suggesting that Po can influence, either directly or indirectly, their expression (Giese et al., 1992). Second, when HeLa cells (a cervical carcinoma cell line of epithelial origin) are induced to express Po by transfection, they revert to the morphology of a nontransformed phenotype (Doyle et al., 1995). In addition, these Po-expressing HeLa cells up-regulate the expression of the adhesion molecule N-cadherin and a number of junction-associated proteins. The up-regulation of these molecules and the consequent loss of the transformed morphology could result from direct signaling via Po.

In summary, we have shown that Po missing either 52 or 59 amino acids from the cytoplasmic domain when coexpressed in the same membrane prevents the adhesion of the full-length Po protein. We suggest, therefore, that the truncated Po molecule can have a dominant-negative effect on the adhesion of Po. Furthermore, the ability of colchicine to disrupt adhesion of the full-length Po protein is a strong indication that an interaction with the cytoskeleton is essential for adhesion of the extracellular domain of Po to take place. Finally, the ability of mutated forms of Po to behave in a dominant-negative fashion may have relevance to the manifestation of the phenotype in the demyelinating disease, CMT1B, in which patients have a mutation in the Po gene.

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