The Cytoplasmic Domain of the Myelin Po Protein Influences The Adhesive Interactions of Its Extracellular Domain

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Abstract. The extracellular domain of the myelin Po protein is believed to engage in adhesive interactions and thus hold the myelin membrane compact. We have previously shown that Po can behave as a homophilic adhesion molecule through interactions of its extracellular domains (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 cytoplasmic domain of Po must be intact for the extracellular domains to adhere, we compared the adhesive capabilities of Po proteins truncated at the COOH-terminal to the full-length Po protein. Po cDNAs lacking nucleotides coding for the last 52 or 59 amino acids were transfected into CHO cells, and surface expression of the truncated proteins was assessed by immunofluorescence, surface labeling followed by immunoprecipitation, and an ELISA. Cell lines were chosen that expressed at least equivalent amounts of the truncated Po proteins at the surface as did a cell line expressing the full-length Po. The adhesive properties of these three cell lines were compared. It was found that when a suspension of single cells was allowed to aggregate for a period of 60 min, only the cells expressing the full-length Po had formed large aggregates, while the cells expressing the truncated Po molecules were still mostly single cells indistinguishable from the control cells. Furthermore, 25-30% of the full-length Po was insoluble in NP40, indicative of an interaction with the cytoskeleton, whereas only 5-10% of Po lacking 52 amino acids and none of Po lacking 59 amino acids were insoluble. These results suggest that for the extracellular domain of Po to behave as a homophilic adhesion molecule, its cytoplasmic domain must be intact, and most probably, it is interacting with the cytoskeleton.

I T is generally accepted that the multilamellar membranes of myelin are held together in a compact form by the interactions of adhesive proteins (Kirschner and Ganser, 1980; Braun, 1984; Lemke et al., 1988). In the peripheral nervous system, the most abundant protein is a single-domain member of the Ig gene superfamily, which is termed Po protein (Kitamura et al., 1976; Ishaque et al., 1980; Lemke and Axel, 1985; Lai et al., 1987; Filbin and Tennekoon, 1992). The importance of Po to the correct and efficient functioning of the nervous system has recently been demonstrated in that patients suffering from the hereditary dysmyelinating disease Charcot-Marie-Tooth type 1B (CMT1B) have been shown to have point mutations in the gene coding for Po (Kulkens et al., 1993; Hayasaka et al., 1993). The Po protein represents >50% of the total peripheral nervous system myelin protein, and it has long been proposed to hold together the myelin membranes at both the intraperiod line, via interactions of its extracellular domains, and at the major dense line, via interactions of its cytoplasmic domains (Kirschner and Ganser, 1980; Braun, 1984;

Lemke et al., 1988). We (Filbin et al., 1990) and others (D'Urso et al., 1990; Schneider-Schaulies et al., 1990) have shown that indeed Po does behave as an adhesion molecule, interacting homophilically via its extracellular domains and, thus, is capable of holding the extracellular leaflets of myelin together. In contrast, the interactions of the basically charged cytoplasmic domain of Po are proposed to be heterophilic, involving acidic lipids in the opposing membrane (Braun, 1984; Lemke et al., 1988). Recently, it has been shown that the isolated cytoplasmic domain of Po can cause lipid vesicles to aggregate, which supports the notion of a lipophilic interaction for Po in vivo (Ding and Brunden, 1994). Confirmation that Po is required for 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). The vast majority of axons in these Po-deficient mice are surrounded by uncompacted membrane layers. However, a little compact myelin was detected; this was attributed to the over-expression of other adhesion molecules in these mutant mice.

To demonstrate that the extracellular domains of Po undergo homophilic adhesive interactions, we developed a transfection/adhesion assay system (Filbin et al., 1990). By comparing the adhesive/aggregation properties of trans-

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fected CHO cells expressing an abundance of Po with those of control-transfected cells (not expressing Po), we showed, both qualitatively and quantitatively, that Po can behave as a homophilic adhesion molecule (Filbin et al., 1990). This type of assay system, however, cannot be used to address the adhesiveness of the cytoplasmic domain directly because the domain of interest must be expressed at the cell surface. On the other hand, it is possible that the cytoplasmic domain, in addition to, but not necessarily exclusive from, its adhesive interactions that contribute to compaction, can influence the interactions of the extracellular domains. This has been shown to occur in another family of adhesion molecules, the cadherins, because cadherin molecules lacking portions of their cytoplasmic sequences do not interact via their extracellular domains (Nagafuchi and Takeichi, 1988). Furthermore, it has been demonstrated that this influence is dependent on the interaction of the cadherin cytoplasmic domain with the cytoskeleton via another protein, α-catenin (Nagafuchi et al., 1991; Hirano et al., 1992). In the present study, therefore, we used our transfection/adhesion assay to determine whether the cytoplasmic domain of Po influences the interactions of the extracellular domains.

In this paper, we describe the adhesive properties of Po molecules truncated to various extents in their cytoplasmic sequences. We found that although Po molecules lacking either 52 or 59 amino acids from their carboxy terminal can reach the cell surface after transfection into CHO cells of the appropriate truncated Po cDNA, they do not engage in homophilic adhesion. Furthermore, whereas the full-length Po molecule appears to interact with the cytoskeleton, this interaction is either abolished or greatly reduced for the truncated Po proteins. These results strongly suggest that for Po to behave as a homophilic adhesion molecule through its extracellular domains, its cytoplasmic sequences must be intact and that, most likely, they interact with some component of the cytoskeleton.

Materials and Methods

Cell Maintenance

CHO cells deficient in the dihydrofolate reductase (*dhfr*)[1] gene (Urlaub and Chasin, 1980) were maintained in MEM supplemented with 10% FCS and proline (40 mg/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.

Truncation of Po cDNA

The 1.08-kb ECoRI to XbaI fragment of the Po cDNA (Lemke and Axel, 1985) was subcloned into the plasmid, Bluescript II (pBSP) at the XhoI restriction enzyme site by use of T4 DNA ligase. The full-length Po-cDNA in pBSP was cut at basepairs 599 and 620 by restriction enzymes PstI and NcoI, respectively. Neither of these enzymes cuts pBSP. The protruding end created by PstI in Po-cDNA was filled to create a blunt end with deoxy-NTPs 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

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1. *Abbreviations used in this paper: dhfr,* dihydrofolate reductase; MTX, methotrexate; pBSP, Bluescript II plasmid; TPo52, Po protein lacking 52 amino acids from the cytoplasmic domain; TPo59, Po protein lacking 59 amino acids from the cytoplasmic domain.

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 *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/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 next 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 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 μM) of methotrexate (MTX). Cells were plated at 5×10^5 cells/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 were 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; chymotrypsin, 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 recovered, and the protein concentration was measured with a Bio Rad kit (Bio Rad Laboratories, Richmond, CA) before the addition of β-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 either a polyclonal antibody raised to the intact Po molecule or a polyclonal antibody raised to the last 20 amino acids of the Po cytoplasmic domain, each at 1:2,000, by incubating overnight at 4°C; the second antibody was alkaline phosphatase-conjugated goat anti-rabbit, 1:20,000 (Sigma Immunochemicals, St. Louis, MO). The substrate was 5-bromo-4-chloro-3-indolyl-phosphate and the chromogen was nitro blue tetrazolium (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) used according to the manufacturer's instructions.

Indirect Immunofluorescence of Intact Cells

Cells were grown on glass coverslips coated with poly-L-lysine (Sigma Immunochemicals) and fixed in 4% paraformaldehyde for 10 min at 4°C. Nonspecific binding sites were blocked with 3% normal goat serum in MEM for 1 h at room temperature, after which the cells were incubated with a Po polyclonal antibody, 1:100 (gift of Dr. David Colman, Mount Sinai Medical School, New York) for 3 h at 4°C. The cells were then washed three times with MEM, incubated with phycoprobe-conjugated goat anti-rabbit IgG, 1:50 (Biomeda Corp., Foster City, CA) for 1 h at room temperature, washed twice with MEM, and washed once with distilled H_2O before being mounted with Gel-mount and viewed with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Biotinylation of Cell Surface Proteins

Cells at ~90% confluency in 10-cm culture dishes were washed with PBS three times and then incubated with 0.5 mg/ml of sulfo-N-hydroxysulfosuccinimide-biotin for 30 min at room temperature, according to the manufacturer's instructions (Pierce Chemical Co., 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

Biotin-labeled cells in a 10-cm dish were lysed with 300 μl 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-μl 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 μl of Po polyclonal antibody (from Dr. David Colman) was added and the mixture was incubated at 4°C overnight with gentle rocking. 100 μl of 10% protein A–Sepharose was added to the immunoprecipitated sample, 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. After the final wash, 50 μl of 1× SDS sample buffer and 4 μl of β-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, according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Extraction of Po with NP-40

Cells, 90% confluent, in a 10-cm dish, were collected mechanically with use of a rubber policeman and were then washed with PBS. The cells were centrifuged at 14,000 rpm for 5 min, and the pellet was resuspended in 100 μl of 2.5% NP-40, incubated for 15 min at room temperature with gentle pipetting, and then centrifuged at 10,000 rpm for 30 min. To the supernatant, (considered the detergent-soluble fraction) an equal volume of 2× SDS sample buffer was added. The pellet, (considered the detergent-insoluble fraction) was taken up in 250 μl of 1× SDS sample buffer. The detergent-soluble and -insoluble fractions were analyzed by immunodetection of a Western blotting using a Po polyclonal antibody.

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 were then incubated overnight at 4°C with a rabbit Po-peptide antibody directed against sequences in the extracellular domain of Po (1:200) 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-mouse IgG (1:250) (Sigma Immunochemicals). The cells were rinsed with PBS and then water. Color was developed by the addition of 50 μl of 0.2% (wt/vol) o-phenylenediamine (Sigma Immunochemicals) and 0.02% 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 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 Laboratories, Grand Island, NY) 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/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.

Results

Expression of Truncated Po Proteins in CHO Cells

We have already shown that the Po protein of myelin behaves as a homophilic adhesion molecule via interactions of its extracellular domains (Filbin et al., 1990). To determine if all 69 amino acids of the cytoplasmic domain of this molecule must be present for adhesion of the extracellular domains to take place, mutated Po proteins lacking either the last 52 or the last 59 amino acids from the cytoplasmic sequences were expressed in CHO cells (Fig. 1). The strategy used to create these truncated Po molecules resulted in the introduction of four novel amino acids at the COOH-terminal of each truncated protein (Fig. 1). The Po protein missing 52 amino acids is designated TPo52, and the Po missing 59 amino acids, is designated TPo59. Fig. 2 shows expression of the full-length Po, the TPo52, and the TPo59 proteins by single cell clones. The molecular masses of the truncated Po proteins were those predicted for molecules missing 52 or 59 amino acids but which are still glycosylated; the TPo52 protein is 23.8 kD and the TPo59 protein is 23 kD (Fig. 2, lanes c and e, respectively). Neither of these proteins was recognized by an antibody against the last 20 amino acids of Po's cytoplasmic domain, which confirms that they are indeed Po proteins truncated in their cytoplasmic domains (not shown).

The Po protein carries sugars attached via a single N-linkage at asparagine 93, which represents ~6%, by weight, of the molecule (Everly et al., 1973; Kitamura et al., 1979; Lemke and Axel, 1985; Sakamoto et al., 1987). Although the apparent molecular weights of TPo52 and TPo59 suggest that they are glycosylated, we confirmed this by removing all of the N-linked sugar attachments by digestion with the enzyme Endo F and monitoring the concomitant decrease in molecular weight. As was expected, the molecular weight of the full-length Po molecule drops by ~6% when treated with this enzyme (Fig. 2, lanes a and b). The same is true for both of the truncated Po molecules (Fig. 2, lanes c–f), confirming that these molecules are glycosylated to the same extent as the full-length Po molecule.

It can also be seen from Fig. 2 that the levels of expression of full-length or truncated Po protein per 30 μg of total protein for these particular cell lines are comparable. Indeed, the cells expressing the truncated Po molecules appear to be expressing slightly more Po protein (Fig. 2, lanes c and e, respectively) than those expressing the full-length Po protein (Fig. 2, lane a). We have already shown that the level of expression of full-length Po by the cell line represented in Fig. 2, lane a, is sufficient to measure the homophilic adhesion of this molecule (Filbin et al., 1990; Filbin and Tennekoon 1991, 1993). Therefore, because the cells transfected with the truncated Po cDNA express at least equivalent amounts

a


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                          ED                    TD    CD
WT    ▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭

                                                              52aa
TPo52 ▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭              <─────────>

                                                              59aa
TPo59 ▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭▭             <──────────>
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b
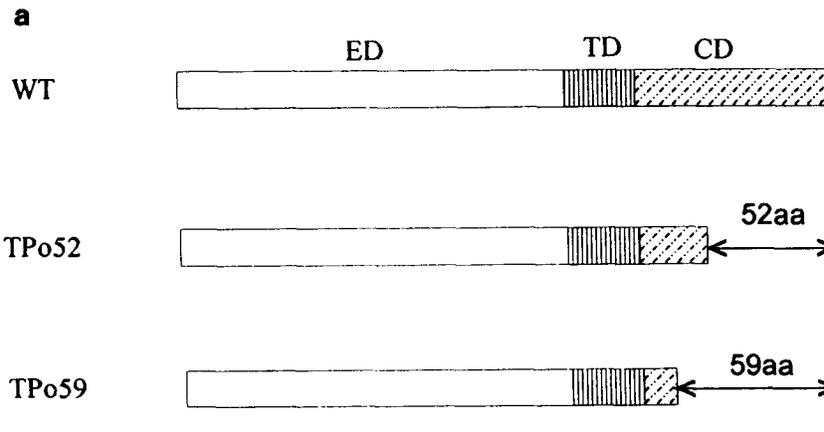
WT: [151]RYCWLRRQAALQRRLSAMEKGKFHKSSKDSSKRGRQTPVLYAMLDHSRSTKAASEKKSKGLGESRKDKK[219]

TPo52:[151]RYCWLRRQAALQRRLSA[167](RGIH)<-------------- 52 aa ------------------->

TPo59:[151]RYCWLRRQAA[160](RGIH)<------------------ 59 aa -------------------->

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.

of Po, these three cell lines are appropriate for comparing the adhesive properties of the three Po molecules. To verify this cell surface Po protein was first visualized by (a) immunofluorescence of intact cells, then estimated by (b) labeling of surface proteins followed by immunoprecipitation of Po, and finally quantitated by (c) an ELISA assay, as described below.
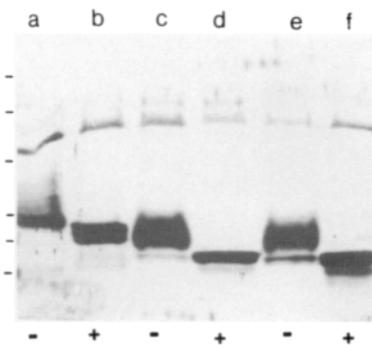
Figure 2. Effect of treatment of transfected CHO cell lysates with Endo F. Proteins (30 μg/lane) from cell lysates with (+) and without (−) treatment with Endo F, transfected with the full-length Po cDNA (lanes *a* and *b*), the TPo52 Po cDNA (lanes *c* and *d*), and the TPo59 Po cDNA (lanes *e* and *f*) were separated by SDS-PAGE and immunostained for Po. +, Treated with Endo F; −, not treated with Endo F. Bars indicate molecular mass standards from top to bottom as follows: 106, 80, 49.5, 32.5, 27.5, and 18.5 kD.

Surface Expression of Po Protein

(a) Expression of the truncated Po proteins at the cell surface was detected by immunostaining the intact transfected cells with a rabbit polyclonal Po antibody. Fig. 3 shows immunofluorescent detection of Po antiserum bound to fixed unpermeabilized CHO cells expressing truncated or full-length Po proteins. The intensity of staining is comparable for all three cell lines, indicating that equivalent amounts of Po, full length (Fig. 3 *c*), lacking 52 amino acids (Fig. 3 *a*), or lacking 59 amino acids (Fig. 3 *b*), are reaching the surface for each cell line. Under the same conditions, no staining was apparent in the control transfected cells that did not express Po (Fig. 3 *d*). 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 (not shown), adding further support to the notion that under the fixation conditions used, the cells were intact and, therefore, only surface expression of Po was being monitored.

(b) To confirm that Po protein was reaching the cell surface in cell lines expressing the truncated or the full-length protein, surface proteins were first labeled with biotin, Po was then immunoprecipitated from the total cell lysate, and the biotinylated proteins in the precipitate were identified on Western blots with streptavidin-HRP and a chemiluminescence detection system. Fig. 4 shows that at least a fraction of the Po immunoprecipitated from all three cell lines is biotinylated, confirming that Po is at the cell surface. Moreover,
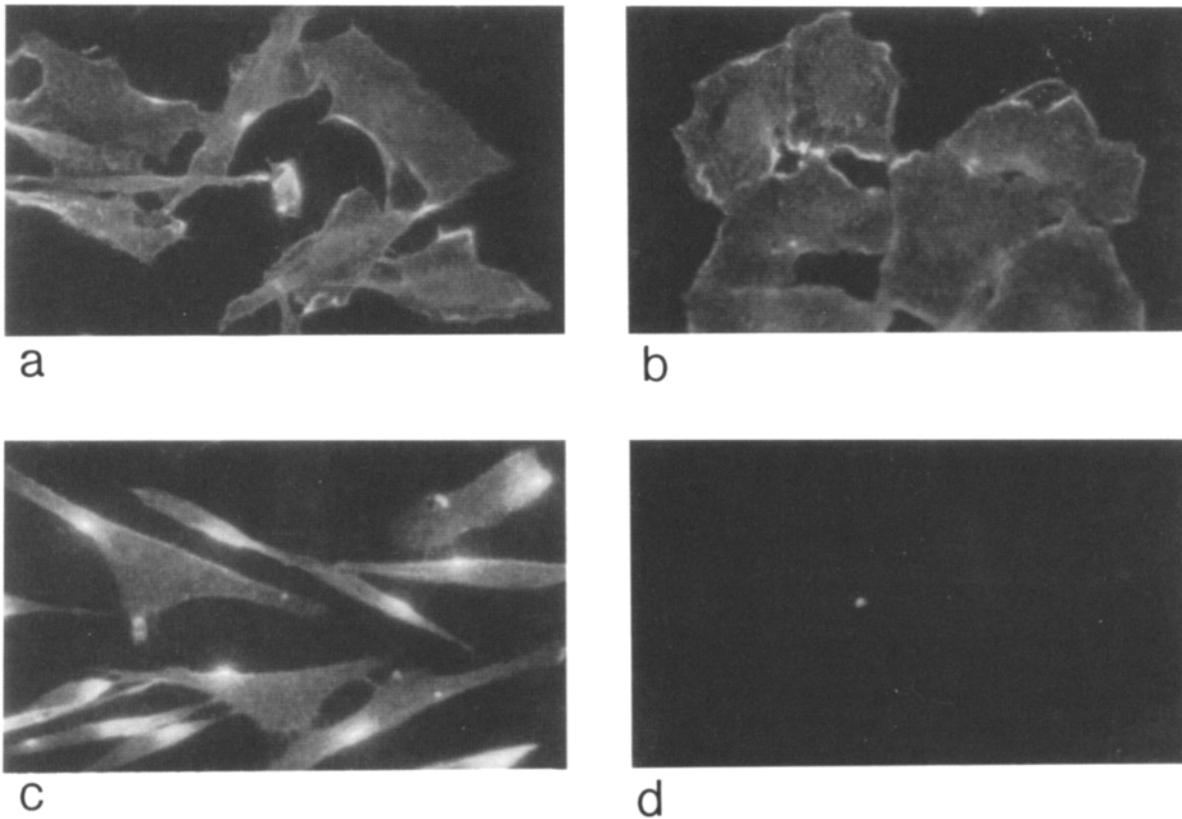
Figure 3. Surface immunofluorescence staining for Po of fixed CHO cells. (*a*) Cells expressing TPo52 protein; (*b*) cells expressing TPo59 protein; (*c*) cells expressing full-length Po; (*d*) control-transfected cells.

as suggested from the Western blot of the total cell lysates, the cells expressing the truncated Po molecules are expressing more Po (Fig. 4, lanes *3* and *4*) at the surface than the cells expressing the full-length Po (Fig. 4, lane *2*). Furthermore, if the total cell lysates from cells in which the surface proteins have been biotinylated were subjected to West-
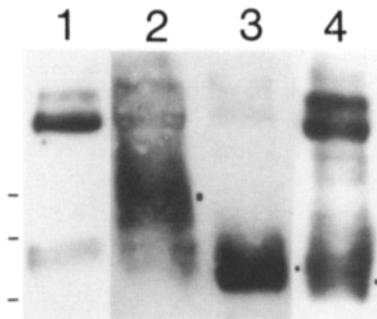
Figure 4. Immunoprecipitation of Po from cells that are surface labeled with biotin. Intact cells were covalently labeled with biotin, then solubilized, and Po was immunoprecipitated with a rabbit anti–rabbit Po polyclonal antibody. Precipitated proteins were separated by SDS-PAGE, and the biotinylated proteins were visualized with a streptavidin-HRP–conjugated second antibody and a chemiluminescence method of detection. (Lane *1*) control cells; (lane *2*) cells expressing full-length Po; (lane *3*) cells expressing TPo52; and (lane *4*) cells expressing TPo59. Dots indicate full-length Po, TPo52, and TPo59. Bars refer to molecular mass as follows: 32.5, 27.5, and 18.5 kD.

ern blotting without previous Po immunoprecipitation and stained for biotinylated proteins, prominent protein bands that corresponded in size to the truncated Po proteins or the full-length Po were seen, depending on the cell line. These proteins were not apparent when the control-transfected cells were treated in a similar fashion (not shown). In contrast, if the total cellular proteins in the lysate, surface, and internal proteins were silver stained, no prominent band corresponding in size to Po was seen (not shown); Po could only be detected in the total cell lysate by immunostaining. This indicates that all three of these cell lines show an enrichment of Po at the cell surface relative to both the total cellular proteins and to other surface proteins.

(*c*) For quantitative comparison of the surface expression of full-length Po and truncated Po proteins in the three cell lines, an ELISA was carried out on fixed, unpermeabilized cells. Cells expressing either TPo52 or TPo59 expressed approximately twice as much Po at the cell surface (Fig. 5, columns *1* and *2*), as do the cells expressing the full-length Po (Fig. 5, column *3*). Only background staining was apparent in the control-transfected cells when the same antibody was used (Fig. 5, column *4*) or when the ELISA was carried out with a Po-peptide antibody directed against sequences in the cytoplasmic domain of Po (not shown). This confirms that the majority of cells were indeed intact, and that under these conditions of fixation, only surface Po protein was being measured in the ELISA. Therefore, these three cell lines were used to compare the adhesive properties of full-length Po and truncated Po proteins, as described below.
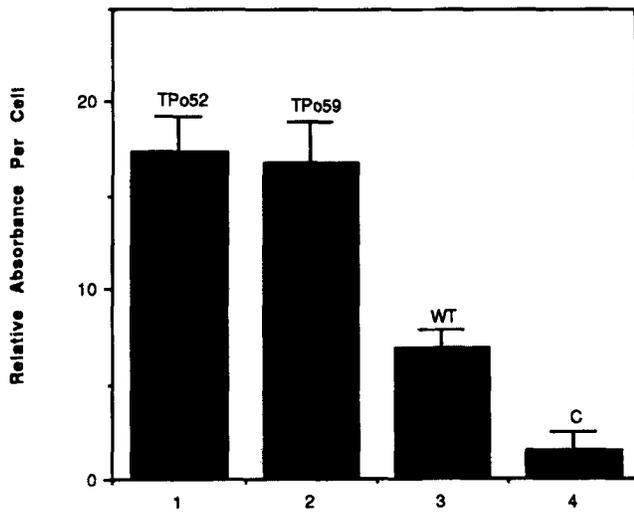
Figure 5. Quantitation of Po expressed at the surface of transfected CHO cells. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected cells expressing TPo52 (column 1), TPo59 (column 2), full-length Po (column 3), and control-transfected cells (column 4), by using a Po peptide antibody directed against sequences in the extracellular domain. Results are expressed (± SEM) in relative absorbance units per cell and are the mean of three experiments, 40 samples per experiment.

Adhesion of Cells Expressing Truncated Po Proteins

To determine whether Po lacking either 52 or 59 amino acids behaves like a homophilic adhesion molecule, similar to the full-length Po protein, a reaggregation/adhesion assay was carried out (Filbin et al., 1990; Filbin and Tennekoon, 1993). After a 60-min incubation of a single-cell suspension, the cells expressing either the TPo52 or the TPo59 proteins had not formed large aggregates (Fig. 6, a and b, respectively), and they indeed were indistinguishable from the control-transfected cells after the same incubation time (Fig. 6 d). On the other hand, although expressing less Po at the cell surface, within 60 min, the CHO cells expressing the full-length Po had, as we previously reported (Filbin et al., 1990; Filbin and Tennekoon, 1991, 1993), formed large aggregates consisting of up to 100-200 cells (Fig. 6 c).

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 that aggregate formation and the rate of aggregation is indicative of the strength of adhesion. Fig. 7 e shows the change in total particle number with time for CHO cells expressing full-length Po, cells expressing TPo52, TPo59, and control-transfected cells. In agreement with our previous reports (Filbin et al., 1990; Filbin and Tennekoon 1991, 1993) and with the microscopic observations described above (Fig. 6 c), the total particle number for the cells expressing the full-length Po (Fig. 6 e, Po+) had dropped to ~25% by 60 min. In contrast, the total particle number of control transfected cells and those expressing the truncated Po (Fig. 6 e, Po−, TPo52, and TPo59) dropped to only 80-85% in the same time. Thus, both the microscopic observations and the quantitative analysis strongly suggest that, unlike the full-length Po protein, expression of the truncated Po proteins by CHO cells does not increase the adhesiveness of these cells. These

results imply that the cytoplasmic domain of Po must be intact for it to behave as a homophilic adhesion molecule via interactions of its extracellular domain.

Solubility of Po Protein in the Nonionic Detergent NP-40

It is not known how the cytoplasmic domain of Po influences the interactions of its extracellular sequences. As shown for other adhesion molecules (Nagafuchi et al., 1991; Hirano et al., 1992), this may occur through an interaction of Po with the cytoskeleton. A strong indication that a protein interacts with a component of the cytoskeleton is its insolubility in the nonionic detergent NP-40, which will solubilize membrane proteins, but not proteins of the cytoskeleton or proteins interacting with the cytoskeleton. After incubation of cells with 2.5% NP-40 for 15 min at room temperature, we found that ~25-30% of the full-length Po molecules were insoluble in this detergent (Fig. 7, lanes 1 and 2). When a similar extraction was carried out on a Schwann cell line (Peden et al., 1990), again 25-30% of the Po expressed by these cells was insoluble in NP-40 (Fig. 7, lanes 3 and 4). This demonstrates that the extraction profile of Po with NP-40 is not an exclusive phenomenon of CHO cells, and it indicates that whatever interactions are exerted on Po in the membrane in CHO cells also occur in Schwann cells. Incubation with NP-40 of cells expressing the truncated Po protein lacking 52 amino acids resulted in only 5-10% appearing in the insoluble fraction (Fig. 7, lanes 5 and 6), whereas the Po protein lacking 59 amino acids was completely soluble (Fig. 7, lanes 7 and 8). These results suggest that by removing 52 amino acids from the cytoplasmic domain of Po, its interaction with the cytoskeleton is weakened and by removing an additional seven amino acids, the interaction is completely lost.

Discussion

Traditionally, the cytoplasmic domain of Po is thought to interact with a component of the opposing membrane in compact myelin, possibly an acidic lipid, and thus holds these membranes together at the major dense line (Braun, 1984; Lemke et al., 1988). Recent studies by Ding and Brunden (1994) demonstrate that Po is indeed capable of such an interaction because the isolated cytoplasmic domain of Po can cause the aggregation of lipid vesicles. In the present study, we show an additional, but not necessarily exclusive, role for the cytoplasmic sequences of Po in that they are required for the extracellular domains to behave as adhesion molecules; Po molecules truncated in their cytoplasmic domains do not behave as adhesion molecules. Similarly, the last 59 amino acids of Po appear to be essential for this molecule to interact with the cytoskeleton, while an absence of 52 amino acids greatly reduces this interaction. On the other hand, the truncated Po proteins reach the cell surface in abundance, which indicates that the last 59 amino acids are not required for this sorting event to take place.

Since we showed previously that the sugar residues of Po are essential to the adhesiveness of its extracellular domain (Filbin and Tennekoon, 1991, 1993), most probably in influencing the orientation of the Ig domain relative to the membrane (Wells et al., 1993), it is possible that the truncated Po molecules are not glycosylated and that this could
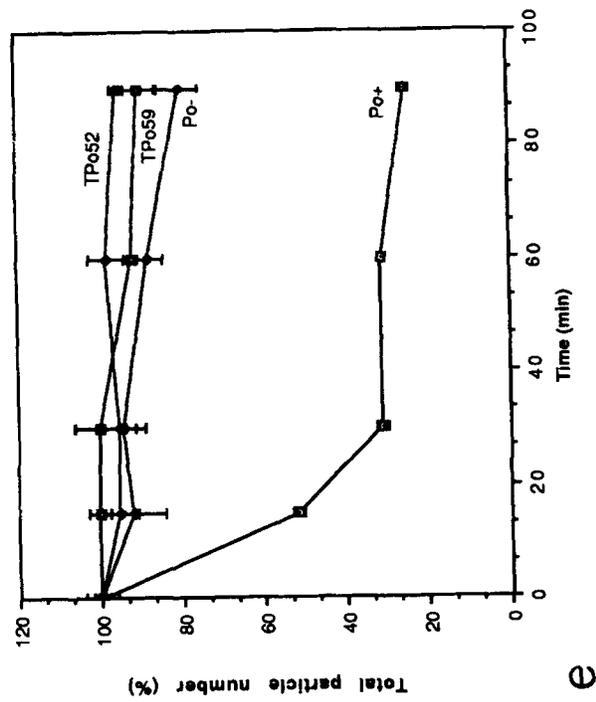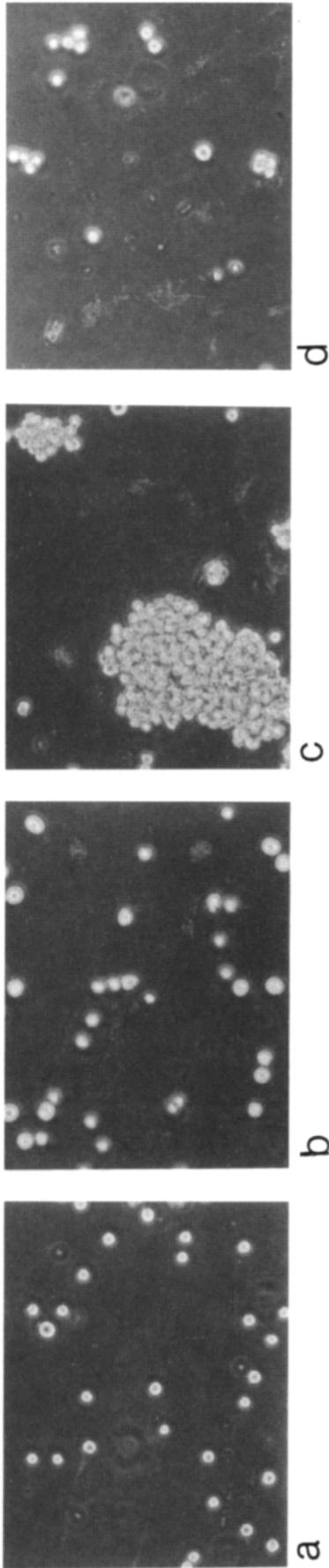
Figure 6. Aggregation properties of Po-expressing cells. Single-cell suspensions of CHO cells expressing (*a*) TPo52, (*b*) TPo59, (*c*) full-length Po, or (*d*) control-transfected cells were allowed to aggregate. Samples were withdrawn at intervals and examined under the microscope (*a–d* represent results after 60 min aggregation) and counted in a Coulter counter for total particle number. The total particle number ± SEM was plotted against time (*e*).
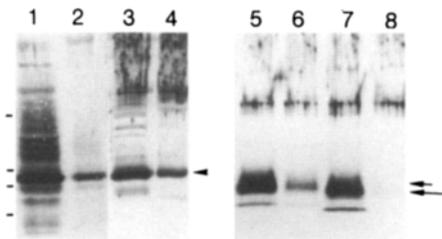
Figure 7. Solubility in NP-40 of Po expressed by transfected CHO cells and Schwann cells. (Lanes *1* and *2*) CHO cells expressing full-length Po; (lanes *3* and *4*) Schwann cell line expressing full-length Po; (lanes *5* and *6*) cells expressing TPo52; and (lanes *7* and *8*) cells expressing TPo59. Each pair of gel lanes represents a starting equivalent of 30 μg of total protein for each cell line separated into NP-40–soluble and –insoluble fractions and loaded onto the gel in separate lanes. The separated proteins were transferred to nitrocellulose and immunostained for Po. Lanes *1, 3, 5,* and *7* are the soluble fractions. Lanes *2, 4, 6,* and *8* are the insoluble fractions. The arrowhead refers to full-length Po, and the arrows refer to the truncated Po molecules. Bars indicate the molecular mass standards as follows: 49.5, 32.5, 27.5, and 18.5 kD.

account for their lack of adhesion. This is not the case; we found by treatment with the enzyme Endo F that both TPo52 and TPo59 are glycosylated to the same extent as the full-length Po molecule. The question then arises as to how the cytoplasmic domains of Po can influence the interactions of the extracellular domains when they are separated by a lipid bilayer. Very similar results have been reported in studies of another family of adhesion molecules, the cadherins. Like Po, cadherins truncated in their cytoplasmic sequences reached the plasma membrane in transfected cells, but unlike the intact cadherin, they did not behave as adhesion molecules nor did they interact with the cytoskeleton (Nagafuchi and Takeichi, 1988; Nagafuchi et al., 1991; Hirano et al., 1992). It has been suggested for cadherins that the initial interactions of the extracellular domains induce a conformational change in the cytoplasmic domain, which in turn strengthens and stabilizes the extracellular interactions (Nagafuchi and Takeichi, 1988). A similar model could explain the interactions of Po. This would involve a two-step adhesive interaction for both Po and cadherin whereby the first step, the initial interaction of the extracellular domains, is of low affinity and is independent of the cytoplasmic sequences. The second step is of much higher affinity and is dependent on the presence of the cytoplasmic domain. Further similarities between the behavior of Po and cadherins have been reported in that, although expression of the truncated forms of either molecule did not result in adhesion of the cells, their isolated extracellular domains are capable of inhibiting the cell adhesion they mediate (Wheelock et al., 1987; Griffith et al., 1992). This apparent contradiction in adhesive requirements can be explained for both molecules by the two-step adhesive model. It is proposed that the initial, low affinity adhesion is not strong enough to hold two cadherin-expressing (or Po-expressing) cells together, but is of sufficient strength to permit the isolated extracellular domain to act as a competitive inhibitor of the interaction of cadherin (or Po) on opposing cells. This model is extended to propose that the second step in this mechanism requires an interaction of cadherin with the cytoskeleton, most likely through a protein termed α-catenin (Nagafuchi et al., 1991; Hirano et al., 1992). In keeping with this model, our results
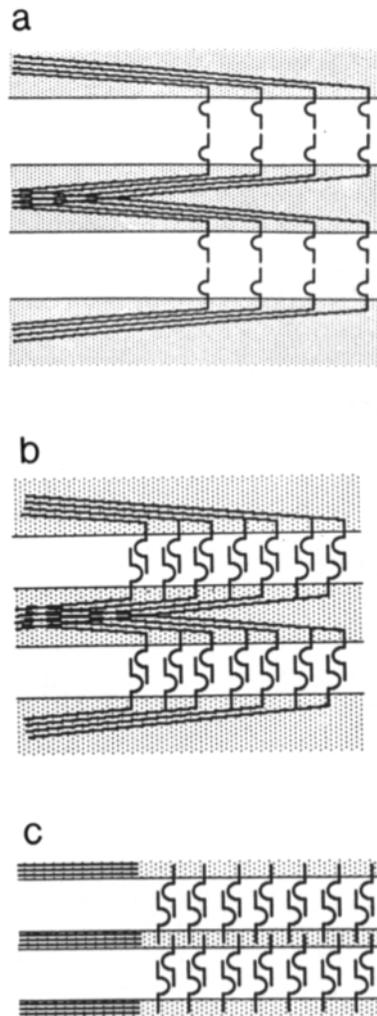
Figure 8. Model of Po's interactions during the compaction of myelin. (*a*) An initial low affinity Po–Po extracellular domain interaction triggers a change in the interaction of the cytoplasmic domain with the cytoskeleton. (*b*) The cytoskeleton reorganizes and pulls back towards the nucleus, inducing Po clustering which in turn strengthens the adhesion of the extracellular domains. (*c*) The cytoskeleton pulls back until the cytoplasmic domains of Po are brought into contact with an opposing membrane. The cytoskeleton disengages. Myelin is compact. Stippled areas represent the cytoplasmic surfaces. Clear areas represent the extracellular surfaces.

also suggest an interaction of Po with the cytoskeleton, which is greatly reduced or lost, coincident with a loss in adhesiveness, after truncation of the cytoplasmic domain. It is not known how Po interacts with the cytoskeleton, but it is possible that the extent of phosphorylation of Po's cytoplasmic domain could influence this putative interaction; Po has been shown, in vitro, to be phosphorylated at a number of amino acids in its cytoplasmic sequences (Brunden and Poduslo, 1987; Suzuki et al., 1990).

For another family of adhesion molecules, the integrins, cell–cell adhesion has been suggested to be strengthened by clustering of the molecules within the plane of the membrane, which in turn would increase the avidity of interacting, opposing molecules. Such clustering is believed to depend on an interaction of integrin cytoplasmic domain with the cytoskeleton (Huang et al., 1993; Gumbiner, 1993). It is possible that the adhesive interactions of the extracellu-

lar domains of Po are also strengthened by cytoskeleton-dependent clustering.

The proposed sequence of Po interactions described above would have to take place in the early stages of myelination, during the time that the membranes are coming together and before cytoplasm and cytoskeleton have been extruded from the leaflets. No cytoskeletal elements have been detected in compact myelin. Upon compaction, the interactions of Po's cytoplasmic domain with the cytoskeleton would be replaced by interactions with a component of the opposing membrane. Consequently, any influence of cytoskeletal interactions on the adhesiveness of Po extracellular domains would be transferred (taken over) by this novel interaction (Fig. 8). Electron microscopic examination of peripheral nerve during the early stages of myelination suggests that indeed that extracellular domains of the myelin membrane come together and adhere before the cytoplasm is extruded and the cytoplasmic sequences of Po meet (Peters et al., 1991). Furthermore, since we have shown that ~30% of the Po expressed by a Schwann cell line in culture is insoluble in NP-40, it is unlikely that an interaction of Po with the cytoskeleton is an artifact of transfected CHO cells, and most likely, it occurs in vivo as well.

In summary, it appears likely that the cytoplasmic domain must be intact for Po to behave as a homophilic adhesion molecule through interactions of its extracellular sequences. In addition, the influence of the cytoplasmic domain on the extracellular domain of Po may be dependent on an interaction of the cytoplasmic domain with the cytoskeleton.

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