A Dual Tyrosine-Leucine Motif Mediates Myelin Protein P₀ Targeting in MDCK Cells

GRAHAME J. KIDD,¹* VIJAY K. YADAV,¹ PING HUANG,¹ STACEY L. BRAND,¹ SENG HUI LOW,² THOMAS WEIMBS,² AND BRUCE D. TRAPP¹

¹Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio
²Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, California

KEY WORDS
myelin; protein targeting; protein sorting; membrane assembly; Schwann cells

ABSTRACT
Differential targeting of myelin proteins to multiple, biochemically and functionally distinct Schwann cell plasma membrane domains is essential for myelin formation. In this study, we investigated whether the myelin protein P₀ contains targeting signals using Madin-Darby canine kidney (MDCK) cells. By confocal microscopy, P₀ was localized to MDCK cell basolateral membranes. C-terminal deletion resulted in apical accumulation, and stepwise deletions defined a 15-mer region that was required for basolateral targeting. Alanine substitutions within this region identified a functional tyrosine-based target-
all Schwann cell membranes and resulted in arrestment of myelination through P₀-P₀ interactions preventing mesaxon spiral elongation (Yin et al., 2000). Mistargeting of P₀ and MAG to the same mesaxonal membranes is observed in the trembler mouse (Heath et al., 1991), an animal model for Charcot-Marie-Tooth disease, suggesting that protein mistargeting may be a component of human pathology.

At a molecular level, it is unknown by what basis P₀ is recognized for sorting in the TGN or targeted to compact myelin, although several possibilities have been proposed. Compact myelin is enriched for cholesterol and glycosphingolipids (Norton and Cammer, 1984), characteristic of lipid rafts. Association of P₀ with lipid rafts in TGN membranes could thus promote targeting to compact myelin. Raft-association could potentially occur through P₀ acylation, which occurs on C₁₅₃ (Gao et al., 2000; Zhang and Filbin, 1998), or through transmembrane domain interactions. Self-association of P₀ tetramers (Inouye et al., 1999; Shapiro et al., 1996) might potentially generate P₀-enriched membrane microdomains that spontaneously segregate P₀ from other transmembrane proteins. Homophilic adhesion in trans (between membranes) may also stabilize and concentrate P₀ in apposing target membranes, as observed in some cultured cells (D'Urso et al., 1990; Filbin et al., 1990). In other polarized cell systems, peptide motifs within the cytoplasmic domain frequently direct protein targeting to particular cell surfaces (Matter and Mellman, 1994; Mostov et al., 2005; Nelson and Yeaman, 2001), and extracellular glycosylation can also contribute to protein targeting.

Identifying P₀ targeting motifs in myelinating Schwann cells is difficult, because mutations that disrupt P₀ adhesion may produce similar phenotypes to those affecting targeting. As P₀ is the principal compact myelin protein, alterations in its distribution during initial myelination can also block Schwann cell membrane polarization and prevent other myelin membrane domains from forming (Yin et al., 2000). Targeting mechanisms are often conserved among otherwise highly divergent cell types, as shown for epithelial cells and neurons (de Hoop and Dotti, 1993; Silverman et al., 2005). In this study, we have investigated whether P₀ contains targeting signals using Madin-Darby canine kidney (MDCK) cells as a model system. MDCK cells are ideally suited for this purpose because they are an easily transfected cell line that polarizes into two membrane domains. Proteins are targeted through a variety of mechanisms involving peptide motifs, post-translation modifications, and lipid raft associations (Mostov et al., 2000, 2005; Weimbs et al., 1997; Yeaman et al., 1999). This approach has been used to study other myelin proteins (Kroepfl and Gardinier, 2001; Minuk and Braun, 1996), as it obviates practical problems of cell-specific protein interactions and interpretation of complex membrane morphologies of myelinating cells. Using this approach, we have identified several candidate targeting signals in P₀, including a novel motif that includes active tyrosine-based and leucine-based signals.

**MATERIALS AND METHODS**

**Materials and Antibodies**

P₀ was detected by immunostaining using rabbit polyclonal antibodies (Trapp and Quarles, 1982). MDCK cell surfaces were labeled with mouse monoclonal antibodies against GP135 (apical) or p58 (antibody 6.23;23; basolateral), as previously described (Low et al., 1998). Internal organelles were labeled using mouse monoclonal antibodies against LAMP-2 (lysosomes; AC17 antibody), EEA1 (endosomes; BD Biosciences, San Jose, CA), transferrin receptor (endosomes; CHEMICON, Temecula, CA), and Golgin97 protein (Molecular Probes, Eugene, OR). Mouse monoclonal antibodies (CHEMICON) were used to stain MAG, both L- and S-isoforms; rabbit L-MAG specific antibodies (Bö et al., 1995) were used for Western blots. Secondary antibodies raised in donkey and directed against rabbit, mouse, and rat immunoglobulins (Jackson Immunobiologicals, Bar Harbor, MN) were directly conjugated to either FITC or TexasRed. Unless specified, all other reagents were purchased from Sigma-Aldrich (St Louis, MO).

**Generation of P₀ Constructs**

Full length rat P₀ cDNA was provided by Dr. David Colman (accession number NM_017027.1). Constructs based on this cDNA were generated by PCR using Pfu DNA polymerase (Stratagene, La Jolla CA) and the resulting PCR products were gel purified, digested, and ligated into the multiple cloning site of pcDNA4/T0 (Invitrogen, Carlesbad, CA). This vector allows tetracycline-regulated expression of the cloned gene in mammalian host cells co-transfected with pcDNA6/TR (Invitrogen). Cloning was carried out in super-competent XL1-Blue MRF cells (Stratagene), and plasmid DNA harvested using QIAGEN endofree maxiprep kit (QIAGEN, Valencia CA). Constructs were confirmed by sequencing by the Cleveland Clinic Foundation DNA Sequencing Core Facility.

Figure 1 shows the constructs generated in this study, using amino acid numbering based on the mature peptide (i.e. not including the signal peptide). For full length P₀, a PCR fragment including the P₀ coding region, 5’ untranslated region (UTR), and most of the 3’ UTR was amplified, and 5’ HindIII and 3’ BamHI restriction sites were introduced, using the primers shown in Table 1. Truncations in which the C-terminal was progressively removed were generated using the same forward primer, and reverse primers for P₀-TMD (i.e. R₁₅₁-stop), P₀Δ15 (i.e. S₂₀₄-stop), P₀Δ30 (i.e. V₁₈₉-stop), P₀Δ45 (i.e. F₁₇₃-stop) and P₀Δ60 (i.e. L₁₅₅-stop) as listed in Table 1.

P₀ constructs with alanine substitutions for Y₁₅₂, Y₁₉₁, M₁₆₅, L₁₃₄, singly and in combinations, were generated initially as two overlapping PCR fragments. The 3’ end of fragment 1 (upstream sequence) and 5’ end of fragment 2 (downstream sequence) were complementary and contained the engineered base changes; primers used are shown in Table 1. Fragments 1 and 2 were purified, annealed together, and the final construct produced by PCR using the forward primer for fragment 1 and the reverse primer for fragment 2.
Two constructs were generated in which amino acids between L155 and either L190 (construct P0-TMD+30) or E205 (construct P0-TMD+15) were removed. Two PCR fragments were initially generated for each construct in which the 3' end of fragment 1 and the 5' end of fragment 2 were complementary and included codons upstream and including L155, and downstream and including L190 or E205 (see Table 1 for primer pairs). The final construct was generated by annealing fragments 1 and 2, then performing PCR using the forward primer for fragment 1 and the reverse primer for fragment 2.

In several constructs, the extracellular domain of P0 was deleted between Y4 and E119, and the entire sequence of enhanced green fluorescent protein (EGFP) inserted. These constructs were generated as three fragments. One fragment encompassed the P0 5'UTR and codons for the signal sequence through to D5. A second DNA encompassed all of the EGFP sequence using pEGFP-N1 (BD Biosciences) as a template. Fragments 1 (300 bp) and 2 (700 bp) contained overlapping 3' and 5' (respectively) regions, and PCR of annealed fragments 1 and 2 using forward primer 19 (Table 1) and reverse primer 20 produced a cDNA encoding P0 5'UTR signal sequence and first 5 amino acids spliced to the EGFP protein. A third set of PCR products was generated that encoded P0 from L155 into the 3'UTR (primers, Table 1), with a 5' overhang that complemented the EGFP 3' end. Control and mutated forms of this segment were generated by using the mutated P0 constructs (above) as PCR templates for this step. When annealed to fragments from steps 1 and 2, these produced a DNA encoding the P0 5'UTR, signal sequence to D5, EGFP, the P0 transmembrane domain, and the P0 cytoplasmic domain with several mutations or truncations (Fig. 1). All of these P0-EGFP constructs were ligated into the pcDNA4/T0 vector, maxipreped, and confirmed by sequencing, as described above.

**Cell Culture and Transfection**

Untreated Madin Darby Canine kidney (MDCK II) cells were grown from stocks maintained in liquid nitrogen, and expanded at 37°C in a 5% CO2 atmosphere in MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin.

For transfection, the cells were plated at high density onto 6-well Falcon tissue culture plates and grown to 60–80% confluence in overnight. They were transfected using ExGen500 (Fermantas, Hannover MD). For one well, 6.6 μL of ExGen500 was combined with 2 μL of the construct DNA in 100 μL of 0.15 M NaCl and incubated at room temperature for 10 min. The DNA/ExGen mixture was then combined with MDCK Cells in serum-reduced opti-MEM medium (Invitrogen) were incubated for 2 h at 37°C. After growing for 6 h, the cells were plated onto Transwell polycarbonate filters (12 mm, 0.4 μm pore size, Corning Costar, Cambridge, MA) and allowed to grow and polarize on the membrane filters for up to 60 h in the incubator. In experiments using the Tet repressor-expressing cells, gene expression was derepressed by addition of doxycyclin (Invitrogen) after 24 h.

For initial experiments using full length P0, P0-TMD, and L-MAG or S-MAG (constructs generously provided by Dr Peter Braun), stably transfected cell lines were generated from MDCK II stock cells, as previously described (Low et al., 1996). Briefly, the cells were transfected, and allowed to grow in medium for 2 days. Cells were then switched to kanamycin-containing medium (Invitrogen), and grown for a further 2 days. Resistant cells were then dissociated, diluted, and allowed to grow up as single clones in large culture dishes. Twenty clones were selected from each experiment, grown to high density and Western blotted to detect those cells expressing the transfected protein. High expressing clones were then propagated and stored in liquid nitrogen until required.

**Immunostaining and Confocal Imaging**

All steps were carried at room temperature. PBS contained 100 μM each of CaCl2 and MgCl2 unless stated.
otherwise. Cells on membranes in transwell inserts were washed with chilled PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, they were quenched with 75 mM NH₄Cl and 20 mM glycerine in PBS for 10 min. Cells were then washed three times, and incubated with secondary antibodies applied for 1 h at 37°C. The cells transfected with P0 constructs were immunostained with primary antibodies for P0 (rabbit polyclonal, (Trapp et al., 1981)) and mouse monoclonal antibodies for markers of apical (GP135) or basolateral (p58, a component of the basolateral membrane domain, but did colocalize with p58, a component of the basolateral membrane domain (Fig. 2B). Western blotting with P0 antiserum detected an abundant 28 kDa protein (Fig. 2C) in P0-transfected cells. In several lines of stably transfected MDCK cells (Fig. 2), and in transiently transfected cultures (Fig. 3), full length myelin P0 protein was consistently detected by confocal microscopy at the basolateral surfaces of polarized cells, but not at the apical surface. P0 immunostaining did not overlap with GP135 (Fig. 2A), a marker of the apical membrane domain, but did colocalize with p58, a component of the basolateral membrane domain (Fig. 2B). Western blotting with P0 antiserum detected an abundant ~28 kDa protein (Fig. 2C) in P0-transfected cell lines that were not present in untransfected cells, confirming that the full-length P0 protein was expressed.

In addition to basolateral plasma membrane labeling, P0 was also detected in organelles in the apical cytoplasm. In myelinating Schwann cells, P0 is a marker for the Golgi apparatus (Kidd et al., 1994; Trapp et al., 1981), but in MDCK cells, only a small amount of the intracellular P0 staining colocalized with the Golgin97 protein (Fig. 2D). The majority of the intracellular P0 was in LAMP-2-positive organelles (Figs. 2E,F), indicating that some P0 was delivered to an internal lysosomal compartment. Little P0 colocalized with transferrin-receptor or EEA1 staining for endosomes (data not shown). These data indicate that P0 contains targeting information that is interpretable by the targeting mechanisms of MDCK cells. As P0 accumulates in the basolateral membrane, targeting

### RESULTS

**P0 is Localized to the Basolateral Surface of MDCK Cells**

To determine whether P0 contains targeting signals that can be interpreted by other polarized cells, full-length rat P0 was expressed by transfection in MDCK cells. In several lines of stably transfected MDCK cells (Fig. 2), and in transiently transfected cultures (Fig. 3), full length myelin P0 protein was consistently detected by confocal microscopy at the basolateral surfaces of polarized cells, but not at the apical surface. P0 immunostaining did not overlap with GP135 (Fig. 2A), a marker of the apical membrane domain, but did colocalize with p58, a component of the basolateral membrane domain (Fig. 2B). Western blotting with P0 antiserum detected an abundant ~28 kDa protein (Fig. 2C) in P0-transfected cell lines that was not present in untransfected cells, confirming that the full-length P0 protein was expressed.

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**TABLE 1. Primer Pairs Used in PCR Generation of P0 Constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Pr no.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<tr>
<td>Full P0</td>
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<td>GCCCAAGCTTCTACCCCAAGCTATGCTGCTCT</td>
<td>GCCGCGGATCCCTATTCTTATCTCTGCGAG</td>
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<tr>
<td>EGFP</td>
<td>2</td>
<td>GCCCAAGCTTCTACCCCAAGCTATGCTGCTCT</td>
<td>GCCGCGGATCCCTATTCTTATCTCTGCGAG</td>
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<tr>
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<td>3</td>
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<tr>
<td>P0Δ30</td>
<td>4</td>
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<tr>
<td>P0Δ45</td>
<td>5</td>
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</tr>
<tr>
<td>P0Δ60</td>
<td>6</td>
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<tr>
<td>P0 Y152A</td>
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<tr>
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<tr>
<td>P0 AAAA</td>
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<td>GCCGCGGATCCCTATTCTTATCTCTGCGAG</td>
</tr>
<tr>
<td>P0 TMD+15</td>
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<tr>
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<tr>
<td>Frag 2</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>GCCCAAGCTTCTACCCCAAGCTATGCTGCTCT</td>
<td>GCCGCGGATCCCTATTCTTATCTCTGCGAG</td>
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</table>

See Materials and Methods for detailed explanation of construct assembly.
is not predominantly mediated by lipid rafts association, as raft-associated proteins are apically targeted in MDCK cells.

Homophilic trans-interactions between adjacent P0-containing membranes occur in some P0-transfected tissue culture lines (Filbin et al., 1990; Spiryda and Colman, 1998; Xu et al., 2001). In contrast, where P0-expressing and nonexpressing MDCK cells apposed one another in transiently transfected cultures (Fig. 3A) or in mixed cultures of stably transfected and nontransfected cells, there was no obvious enrichment between the two P0-expressing cells. No evidence of compact-myelin-like adhesion was observed between MDCK cell lateral membranes by electron microscopy (not shown), unlike CHO, HeLa, and L1 cells (Filbin et al., 1990; Spiryda and Colman, 1998; Xu et al., 2001). MDCK cell lateral membranes are normally separated by large intercellular gaps (~100 nm) except at junctional complexes, and this may prevent trans-interactions forming between P0 molecules.

Cis-interactions between P0 molecules have been proposed to result in liquid-crystal-like membrane microdomains that may exclude other proteins (Shapiro et al., 1996). P0 and MAG have mutually exclusive distributions in Schwann cells (Trapp and Quarles, 1982, 1984), raising the possibility that P0-containing membranes may exclude MAG. To test whether this occurred in MDCK cells, we coexpressed P0 and either the large or small alternate splice forms of MAG, L-MAG, or S-MAG, and verified their expression by Western blot (not shown). As previously reported (Minuk and Braun, 1996), L-MAG was concentrated in both apical and basolateral membranes of transiently and stably transfected cells. When cotransfected stably (Fig. 2F) with P0, P0 and MAG distributions overlapped substantially in basolateral membranes, suggesting that P0 did not exclude MAG, at least at the resolution of light microscopy. S-MAG accumulated in the apical membranes of both transiently and stably transfected MDCK cells. Coexpression of P0 and S-MAG did not alter distributions of either protein (Fig. 2F).

**P0 Cytoplasmic Domain Contains Basolateral Targeting Information**

Basolateral targeting signals are commonly peptide motifs in the cytoplasmic domain (Mostov et al., 2000, 2005; Yeaman et al., 1999). In an initial experiment, we investigated whether the P0 cytoplasmic domain contained targeting information by introducing a stop codon to terminate translation immediately following R156, which truncated the protein six amino acids beyond the cytoplasmic face of the transmembrane domain (TMD, Fig. 1). In transient transfections, full length P0 went to the basolateral surface (Fig. 3A), while the truncated protein was consistently located at the apical surface (Fig. 3B).

To better define the location(s) and number of basolateral signals, a series of stepwise truncations was introduced, deleting 15, 30, 45, and 60 amino acids from the C-terminal (Fig. 1; constructs D15, D30, D45, D60). Removal of the 15 C-terminal amino acids did not alter P0 distribution (Figs. 3C,D), but deleting 30 amino acids produced a protein that localized entirely in the apical
membrane (Figs. 3E,F), as did the Δ45 and Δ60 truncations (Figs. 3G,H). This result indicated that essential targeting information lay in the C-terminal 30 amino acids between V189 and S204. Apical organelle staining was also consistently observed for the Δ15 construct, but lost with the longer truncations, suggesting that a signal in the Δ15–Δ30 region also targeted protein to these structures. The possibility of a redundant signal(s) in the Δ15 region was tested by fusing the terminal 15 amino acids to the TMD truncation C-terminal. This construct went to the apical surface (Fig. 3I), suggesting either that there were no signals in that region or they were not recognized in this conformation. A similar approach using the C-terminal 30 amino acids yielded a construct that appeared restricted to the cytoplasm (Figs. 3J,K). This result may be due to misfolding of the construct and subsequent RER retention. Alternatively, moving the C-terminal 30-mer adjacent to the plasma membrane may have altered trafficking of this protein so that surface accumulation did not occur.

Within the region between V189 and S204, a YAML sequence at Y191 (Fig. 4A) conformed to the YxxΦ endocytosis/basolateral targeting consensus motif (where x is any amino acid and Φ is F/V/L/M/I; Bonifacino and Traub, 2003; Marks et al., 1997). The YAML motif is highly conserved in vertebrate evolution (Fig. 4A), being identical in chickens, rodents, and humans, with only a conservative substitution in sharks; as discussed below, the teleosts were an exception. To investigate whether the YAML motif was functional and predominant, alanine was substituted for key amino acids in the sequence. As shown in Fig. 4B, a control protein consisting of full length P0 or P0 with a Y152 to A substitution went to the basolateral surface as expected. Y191 to A substitution yielded a protein found at both apical and basolateral domains (Figs. 4C,D). As tyrosine mutation disrupts the YxxΦ motif, this result suggested that another basolateral targeting motif within this region was also active, although not sufficient to drive basolateral targeting alone. An ML sequence serves as a leucine-based targeting signal in MHC Invariant Chain protein (Bremnes et al., 1994; Odorizzi et al., 1994), and resembles di-leucine-type basolateral targeting motifs (LL/I/M; Bonifacino and Traub, 2003; Marks et al., 1997). Conversion of YAML to AAAA resulted in apical accumulation of the P0 protein (Fig. 4E), indicating that the ML motif was a second signal. Mutation of the M to A (YAAL) resulted in basolateral accumulation (Fig. 4F), indicating that the tyrosine-based motif alone was sufficient for basolateral targeting.

Concentration of P0 in the apical LAMP2-positive organelles also depended on the presence of the YAML motif. P0Δ15 mutants were found in these structures (Fig. 3C), but deletion of 30 or more amino acids from the C-terminal abolished targeting there (Figs. 3D–F). YAAL and AAML mutants (Fig. 4F,D respectively) also accumulated in these structures, but AAAA mutants did not (Fig. 4E), indicating that both YxxΦ motif and the ML motif were capable of
directing P0 for inclusion in these structures. Thus accumulation in these structures likely represents a pathway of P0 trafficking and not simply the result of P0 aggregation due to aberrant overexpression.

C-Terminal Tyrosine Motif Targets EGFP-Based P0 Constructs

Proteins lacking any targeting information may be expected to accumulate in both apical and basolateral MDCK cell surfaces, but the P0 constructs lacking the YAML sequence accumulated at the apical surface. This suggests that the P0 extracellular domain may have contained secondary apical signals that were active in the absence of the primary YAML targeting sequences. To test for these effects, we excised the P0 extracellular domain between Y4 and E119, and replaced them with EGFP (Fig. 1). This generated a polypeptide with the P0 signal peptide, the initial 4 amino acids of mature P0, EGFP, the last 6 amino acids of the P0 extracellular domain followed by the P0 transmembrane and cytoplasmic domains.

Expression of this construct provided sufficient EGFP fluorescence for direct confocal imaging (Fig. 5), although the fluorescence signal intensity was reduced compared with cytosolic expression of native EGFP (not shown). Immunostaining for EGFP and P0 negated the possibility that nonfluorescent P0-EGFP was accumulating elsewhere undetected by EGFP fluorescence imaging. Results with P0-EGFP constructs were similar to those obtained with the P0 extracellular domain. The construct with the native P0 cytoplasmic domain accumulated in the basolateral membrane (Fig. 5A), as did the Δ15 deletion (Fig. 5B). The Δ30 and Δ60 constructs were predominantly apical, although unlike those with the P0 extracellular domain, some protein was also observed in the basolateral membranes (Figs. 5C,D). Mutations of the tyrosine motif to AAML resulted in mixed apical and basolateral localization (Fig. 5E), as observed previously for P0. Constructs with the YAML mutated to AAAA accumulated in the apical surface, although minor basolateral fluorescence was also detected (Fig. 5F). YAAL-containing protein was concentrated at the basolateral surface (Fig. 5G). These results confirm that the YAML sequence is the predominant targeting signal, but indicate that the P0 extracellular domain contains signals not present in the P0-EGFP that direct P0 to the apical surface in the absence of the tyrosine motif.

DISCUSSION

We show here for the first time that P0 contains a unique dual-motif targeting signal that contains both tyrosine- and leucine-based elements, as part of a hierarchy of targeting signals that are recognized by the sorting machinery in MDCK cells. The predominant targeting signal is the Yxxφ motif, which is necessary and sufficient for basolateral accumulation of P0. Superim-
posed on the same sequence is a subordinate leucine-based basolateral-targeting motif, ML. The P₀ extracellular domain also contains apical targeting information, which contributes to P₀ localization if the tyrosine-based signal is absent. P₀ localization in MDCK cells was not dependent on raft associations, which direct proteins to the apical surface (Barman and Nayak, 2000; Benting et al., 1999; Weimbs et al., 1997), and P₀ self-association in trans also appeared unnecessary. Possible cis-associations of P₀ did not exclude MAG from membranes. These results provide important candidate signals for studies of P₀ targeting in myelination and in dysmyelinating diseases.

In MDCK cells, the Y₁⁹¹AML region was the primary means of targeting P₀ to the cell surface. Consensus pattern-matching had identified both P₀ tyrosine and leucine motifs as potential targeting components, but in many proteins, potential targeting motifs are buried within folded regions or insufficiently close to the C-terminal to permit interaction with recognition molecules. Our results indicate that the P₀ YAML is accessible to interact with cellular targeting machinery. Tyrosine-based motifs are recognized by μ subunits of the clathrin adapter protein (AP) complexes (Bonifacino and Traub, 2003; Mostov et al., 2000; Owen and Evans, 1998), which incorporate the targeted protein into budding clathrin-coated vesicles on the TGN or during recycling to/from the cell surface (Bonifacino and Traub, 2003; Mostov et al., 2000). We envisage that in Schwann cells, AP-clathrin interactions may be involved in the sorting of P₀ into unique carrier vesicles, which occurs at the TGN (Trapp et al., 1995). To our knowledge, the direct superimposition of tyrosine and leucine motifs has not been described previously, although partial overlaps of tyrosine and leucine motifs occurs in CD1d (Rodionov et al., 2000) and adjacent tyrosine and leucine-based motifs have been described in tyrosinase (Simmen et al., 1999). Leucine-based motifs may be recognized by several adaptor-complex proteins. For stochiometric reasons, it seems unlikely that both motifs are recognized simultaneously, although the possibility that Schwann cells express unusual AP complexes that recognize both cannot be discounted. In other proteins, close proximity of tyrosine and leucine motifs promotes endocytosis and lysosomal delivery as well as basolateral delivery. Although both tyrosine and leucine motifs may direct proteins to endosomal compartments, in Schwann cells, P₀ is not normally found in endosomes, though it does accumulate there in P₀-overexpressing transgenics (Yin et al., 2000), and to a minor extent in dedifferentiated Schwann cells (Poduslo and Windebank, 1995). In MDCK cells, in addition to basolateral targeting, some P₀ was delivered to LAMP-2-positive organelles when either tyrosine or leucine signals were present. Internal accumulation of P₀ is not observed in normal myelinating Schwann cells, but after microtubule disruption (Trapp et al., 1995) and possibly during myelin degradation in Wallerian degeneration, the YAML motif may function as an endocytosis/lysosomal targeting motif.

In humans, point mutations that specifically affect the Y₁⁹¹ in P₀ have not been reported, but several mutations cause C-terminal truncations that delete or disrupt the YAML motif and result in severe, early onset neuropathies (Kamholz et al., 2000; Shy et al., 2004). These include Q¹⁸⁶X (Mandich et al., 1999; Shy et al., 2004), A¹⁹⁵frameshift (Tachi et al., 1998), and A¹⁹²frameshift

Fig. 5. EGFP substitution for the P₀ extracellular domain. Transiently transfected into MDCK cells, P₀-EGFP constructs emitted sufficient fluorescence for imaging by confocal microscopy (green throughout). Full length P₀-EGFP accumulated in the basolateral membrane (A, apical GP135 immunostaining shown in red). Deletion of 15 amino acids from the C-terminal did not alter this (B, red shows p58 in B–G), but deletion of 30 (C) and 60 (D) amino acids resulted in detection of P₀-EGFP at both apical and basolateral surfaces. Alanine substitutions for Y₁⁹¹ (E) also resulted in accumulation at both surfaces. When both Y₁⁹¹ and M₁⁹³ were mutated (F), most of the staining was detected at the apical surface, although minor staining was also detected in the basolateral membrane. M¹⁹³ to A substitution alone resulted in basolateral detection. Confocal XZ images. Scale bar 5 μm.
TARGETING OF MYELIN P0 PROTEIN

(221x466)  

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REFERENCES


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