Mutations in the cytoplasmic domain of P0 reveal a role for PKC-mediated phosphorylation in adhesion and myelination

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Mutations in P0 (MPZ), the major myelin protein of the peripheral nervous system, cause the inherited demyelinating neuropathy Charcot-Marie-Tooth disease type 1B. P0 is a member of the immunoglobulin superfamily and functions as a homophilic adhesion molecule. We now show that point mutations in the cytoplasmic domain that modify a PKC target motif (RSTK) or an adjacent serine residue abolish P0 adhesion function and can cause peripheral neuropathy in humans. Consistent with these data, PKCα along with the PKC binding protein RACK1 are immunoprecipitated with wild-type P0, and inhibition of PKC activity abolishes P0-mediated adhesion. Point mutations in the RSTK target site that abolish adhesion do not alter the association of PKC with P0; however, deletion of a 14 amino acid region, which includes the RSTK motif, does abolish the association. Thus, the interaction of PKCα with the cytoplasmic domain of P0 is independent of specific target residues but is dependent on a nearby sequence. We conclude that PKC-mediated phosphorylation of specific residues within the cytoplasmic domain of P0 is necessary for P0-mediated adhesion, and alteration of this process can cause demyelinating neuropathy in humans.

Introduction

Myelin is a multilamellar structure that surrounds axons in both the central nervous system and the peripheral nervous system (PNS),* facilitating nerve conduction. P0, a transmembrane protein of the Ig superfamily, is the major myelin structural protein in the PNS, expressed exclusively by myelinating Schwann cells and necessary for normal myelin structure and function. In mice, the absence of P0 (Giese et al., 1992; Martini et al., 1995) or overexpression of P0 (Wrabetz et al., 2000) results in hypomyelination and peripheral neuropathy. In addition, mutations in the human P0 gene cause the demyelinating peripheral neuropathy Charcot-Marie-Tooth disease 1B, the more severe Dejerine-Sottas syndrome, and congenital hypomyelination, all associated with muscle weakness, atrophy, and sensory loss (for review see Nelis et al., 1999; Shy et al., 2001).

P0 functions as a homophilic adhesion molecule in vitro (Filbin et al., 1990; Filbin and Tennekoon, 1993), and this activity may mediate compaction of adjacent leaflets in peripheral nerve myelin. Analysis of the crystal structure of the P0 extracellular domain indicates that P0 molecules have the potential to interact in cis to form homotetramers, and these interact in trans to mediate homophilic adhesion (Shapiro et al., 1996). Consistent with this model, mutation of several of the amino acid residues critical for these putative cis and trans interactions can cause the inherited demyelinating peripheral neuropathy, CMT1B (Warner et al., 1996; Nelis et al., 1999).

The cytoplasmic domain of P0 is also important for its function, and mutations in this region result in loss of P0-mediated homophilic adhesion in vitro (Wong and Filbin, 1994). Coexpression of both wild-type and cytoplasmically truncated P0 causes loss of wild-type function, presumably due to a dominant negative effect of the truncated molecule (Wong and Filbin, 1996). Mutations within the P0 cytoplasmic domain are also found in patients with inherited demyelinating neuropathies, some of which have very severe clinical phenotypes (Nelis et al., 1999; Shy et al., 2001).
that alteration of this process can cause demyelinating neuropathies. We now demonstrate that deletion of a 14 amino acid sequence in the cytoplasmic domain of P0 abolishes adhesion function. Point mutations in this region that alter a PKC substrate motif (RSTK) and mutation of an adjacent serine residue (S204) are critical to P0 adhesive function. Moreover, PKCα and the receptor for activated C kinase (RACK1) are coimmunoprecipitated from cells expressing wild-type P0 but not P0 bearing a deletion of 14 amino acid sequence. In addition, inhibition of PKC activity results in loss of P0-mediated adhesion. We have also identified a mutation of the initial arginine of the PKC substrate motif to a serine residue (R198S) in a patient with CMT1B, and we find that cells expressing P0 bearing this mutation fail to form homophilic adhesions. These data indicate that PKC-mediated phosphorylation is an important component of the regulation of P0-mediated adhesion and demonstrate that alteration of this process can cause demyelinating neuropathy in humans. This is the first glimpse of the molecular machinery that regulates the function of P0 in myelination.

Results

A subterminal 13 amino acid sequence in the cytoplasmic domain of P0 is essential for adhesion

To define the regions of the cytoplasmic domain of P0 that are essential for homophilic adhesion, we stably transfected mouse L cells and HeLa cells with cDNAs encoding wild-type P0 or P0 deletion mutants lacking the COOH-terminal 13 (Δ13), 28 (Δ28), 43 (Δ43), or 59 amino acids (Δ59). Expression of the transfected P0 constructs was determined by reverse transcription (RT)-PCR using specific primers for each construct and by Western blotting. RT-PCR reveals that all constructs were expressed in relatively equal amounts in both cell types (Fig. 1 B shows L cells). To ensure that protein was expressed and inserted in the plasma membrane, we labeled live cells with a membrane impermeable biotinylated reagent followed by immunoprecipitation with anti-P0 antibody. The immunoprecipitated material was fractionated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with HRP-streptavidin. Each of the P0 constructs is expressed at the cell surface (Fig. 1 C shows L cells).

Cells expressing each of the deletion mutants were assayed for their ability to form homophilic adhesions as measured by binding of single cells expressing P0 constructs to a confluent layer of cells expressing wild-type P0. Both L cells and HeLa cells expressing Δ13 were comparable to the same cell types expressing wild-type P0 (Fig. 2, A and B). However, P0-mediated adhesion was reduced dramatically in all cell lines with deletions of 28 amino acids or greater (Fig. 2, A and B). This suggests that the sequence between amino acids 193 and 206 is essential for P0-mediated cell–cell adhesion. This region contains three serine residues that are phosphorylated in mouse sciatic nerve: S197, S199, and S204 (Hilmi et al., 1995). These residues are conserved in the human P0 sequence. Furthermore, serine 199 is part of a PKC recognition motif (198-RSTK-201) (Kishimoto et al., 1985; Woodgett et al., 1986), suggesting that phosphorylation mediated by PKC is critical to P0 adhesive function.

Since it has been reported that expression of P0 in HeLa cells results in upregulation of cadherin expression (Doyle et al., 1995), we were careful to perform all P0 adhesion assays in the absence of Ca²⁺, which is necessary for cadherin-mediated adhesion. Additionally, we examined both HeLa and L cells bearing each of the P0 deletion constructs for cadherin expression using a pan cadherin antibody to plot equivalent amounts of cell lysate. In agreement with the previous observations, cadherin and the cadherin-associated protein β-catenin are upregulated in HeLa cells stably transfected with pCMV Tag4 containing the full-length wild-type P0 but not in L cells similarly transfected (unpublished data). Furthermore, among HeLa cells upregulation requires the same region of the molecule that is essential for adhesion function, amino acids 193–206 (unpublished data). The two cell lines do differ in their basal level of cadherin expression: HeLa cells express a small amount of cadherin, whereas L cells do not express detectable levels of cadherin. Thus, upregulation of cadherin expression
PKC and P0 adhesion

A PKC target motif and PKC activity are essential for P0-mediated adhesion

To investigate the importance of the RSTK motif and the adjacent serine, we assayed L cells transfected with P0 containing mutations in the RSTK motif (S199A and T200A) and in serine 204 (S204A) for their ability to form homophilic P0 adhesions. In addition, we assayed cells bearing a mutation at serine 197 (S197A), a site of low level in vivo phosphorylation (Hilmi et al., 1995), aspartic acid 195 (D195A), and tyrosine 191 (Y191A) as controls. Stable cell lines were created and tested for P0 expression by RT-PCR and immunoprecipitation of cell surface labeled P0 as above. All constructs were expressed at the cell surface (unpublished data). Cells expressing P0 mutated at serine sites 199 or 204 or threonine 200 failed to form P0-mediated adhesions (Fig. 3 A), indicating that both the PKC motif and serine residue 204 are functionally important. In contrast to these mutations, substitution of serine 197 or aspartic acid 195 have little or no effect (Fig. 3 A). Furthermore, replacement of tyrosine 191, a site shown recently to be phosphorylated in vivo (Xu et al., 2000a) with alanine, also has little or no effect on P0-mediated adhesion (Fig. 3 A).

To further examine the importance of the PKC function in P0-mediated adhesion, we tested the effect of the PKC inhibitor, calphostin C (Tamaoki and Nakano, 1990; Svetlov and Nigam, 1993). Calphostin C inhibits P0-mediated adhesion in a dose-dependent manner with maximum inhibition at 60 nM (Fig. 3 B). At this concentration (60 nM), calphostin has no detectable effect on cell viability as measured by Sytox green (Molecular Probes).

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Mutation within the RSTK domain (R198S) causes the demyelinating peripheral neuropathy CMT1B and loss of P0 homophilic adhesion

We have identified an individual with CMT1B who is heterozygous for a P0 mutation producing a substitution of arginine for serine at amino acid 198 (R198S) within the PKC binding motif. The patient had normal growth, development, and motor function until 5 yr ago, in his late 30s, when he noted the onset of lower extremity clumsiness; however, these symptoms have not progressed notably since that time. On neurological examination, he had minimal symmetrical foot dorsiflexor weakness, a mild decrease in both large and small fiber sensory modalities (vibration and pin prick) in his feet, and diminished Achilles and patellar deep tendon reflexes. His gait appeared normal, but he was unable to walk on his heels, confirming the mild weakness of the foot dorsiflexors. In addition, he had bilateral pes cavus, a common finding in patients with inherited neuropathy, probably caused by weakness of the intrinsic muscles of the feet. On electrophysiological testing, the nerve conduction velocities of peroneal and tibial motor nerves were slowed bilaterally to ~25 m/s, whereas his right sural sensory NCV was slowed to 28 m/s. The left sural potential was unobtainable. These neurological signs and symptoms and electrophysiological findings are consistent with the presence of a mild demyelinating sensory and motor peripheral neuropathy.

To determine if this mutation affected P0 homophilic adhesion, we transfected L cells with P0 bearing this mutation. Expression was assayed as above and was found approximately equivalent to other P0 constructs in L cells (unpublished data). These cells showed a much reduced ability to form P0 homophilic adhesions (Fig. 3 C). Thus, elimination of PKC target residues or alteration of the PKC target motif not only abolishes P0 adhesive function in vitro but also causes demyelinating disease in humans.
PKC and the PKC binding protein RACK1 are associated with P0 in situ

The functional requirement for the PKC target motif in P0 and its importance to human disease led us to determine which of the many isoforms of PKC is present in sciatic nerve. We find that two conventional PKCs, γ1 and γ2, are detected, as well as the novel isoforms δ, ε, and η (Table I). All of these isoforms are also detected in P0-transfected L cells (Fig. 4, none). However, P0 immunoprecipitates from L cells lysed in neutral detergent reveal that only PKCγ1 is present in association with P0 (Fig. 4). Furthermore, PKCγ1 is present and associated with P0 in cultured Schwann cells (Fig. 4, SC).

We further probed P0 immunoprecipitates derived from each of our deletion mutants for the association of PKC and the PKC binding protein RACK1. RACKs bind activated PKC at a site distinct from the substrate-binding site (Mochly-Rosen et al., 1991) and are thought to target the enzyme to specific cellular sites independent of PKC substrate recognition (Sim and Scott, 1999; for reviewed see Jaken and Parker, 2000). As can be seen in Fig. 5, deletion of the same 14 amino acid sequence containing the RSTK motif that is essential for P0-mediated adhesion eliminates the association of PKCγ1 and RACK1 with P0 (Fig. 5, compare Δ13 with Δ28 and Δ59). However, point mutations altering the RSTK substrate recognition motif or the adjacent serine 204, mutations that do eliminate P0-mediated adhesion, do not alter PKC or RACK1 binding. Thus, substrate recognition by PKC and binding, presumably mediated by RACK1, are two independent events occurring through distinct sites within a 14 amino acid domain of P0.

Discussion

In this study, we present data correlating specific amino acid residues in the cytoplasmic domain of P0 with adhesion function and ultimately with human disease. By creating a series of deletions in the cytoplasmic domain, we find that the region between amino acids 193 and 206 is essential for P0-mediated adhesion.

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**Table 1. PKC expression in mouse sciatic nerve**

adhesion. L cells expressing full-length P0 were assayed for adhesion in the presence of increasing concentrations of calphostin. Adhesion in the absence of the inhibitor was considered 100%. Each cell type was assayed in triplicate. (C) A point mutation in the PKC target motif results in human disease and loss of P0-mediated adhesion in transfected L cells. Stable clones of L cells expressing the R198S P0 mutant were assayed for P0-mediated adhesion as above. Adhesion is represented as percent of control. Bars represent the standard deviation from the mean (p < 0.0001).
serine residues (serine 199) (Hilmi et al., 1995). Using site-directed mutagenesis, we demonstrate that this motif is important for adhesive function. A second serine residue at position 204, closely juxtaposed to the RSTK site, is also critical for adhesion function, and this residue is also phosphorylated in the sciatic nerve (Hilmi et al., 1995). Another serine (197) that is phosphorylated in situ (Hilmi et al., 1995) does not appear to be necessary for adhesion function, nor is a tyrosine residue in the cytoplasmic domain, Y191, which is also phosphorylated in sciatic nerve (Iyer et al., 1996).

Of the PKC isoforms present in sciatic nerve and our L cell model, PKCα is specifically immunoprecipitated with P0, indicating an in situ association between the two molecules. This interaction may be mediated by RACK1, the receptor for activated C kinase, since we find that under our experimental conditions the association of these two proteins is absolutely correlated. Furthermore, the association of both proteins with P0 is dependent on a 14 amino acid sequence in the cytoplasmic domain, amino acids 193–206. This is the same region containing the PKC substrate motif; however, the substrate site and the binding site are distinct, since point mutations in the substrate site that eliminate adhesion function do not eliminate binding of PKC or RACK1. RACK1 is one member of a group of proteins that bind PKC at a site distinct from the substrate-binding site (Mochly-Rosen et al., 1991; Sim and Scott, 1999; for review see Jaken and Parker, 2000). We suggest that RACK1 binds activated PKC and interacts with P0 either directly or through an additional adaptor to bring activated PKCα into substrate proximity.

PKC has been implicated previously in phosphorylation of P0 in vitro (Suzuki et al., 1990; Rowe-Rendleman and Eichberg, 1994) and tumor promoters that activate PKC accentuate phosphorylation in situ (Agrawal and Agrawal, 1989). Most pertinent to our identification of PKCα as the critical isoform, abolition of phospholipid-dependent–induced up-regulation of PKC in sciatic nerve is accompanied by a reduction in PKCα (Rowe-Rendleman and Eichberg, 1994). However, these prior studies were not able to correlate the PKC-dependent phosphorylation of P0 with a specific function. Our data indicate for the first time that PKC-mediated phosphorylation on serine residues is important for P0 adhesion function: P0-mediated adhesion is abolished by mutations of a PKC target motif, PKCα and the adaptor RACK1 are immunoprecipitated with wild-type P0, and P0-mediated adhesion is decreased dramatically by calphostin C, a kinase inhibitor that preferentially blocks PKC activity (IC50 for PKC = 50 nM).

There are two possible mechanisms by which phosphorylation of the cytoplasmic domain of P0 may regulate adhesion and/or myelination. One is a direct mechanism in which phosphorylation of P0 alters its conformation, allowing the cis or trans interactions required for adhesion (Shapiro et al., 1996). The second is an indirect mechanism in which phosphorylation of P0 allows or facilitates the binding of effector or adaptor molecules essential for adhesion. In either case, dynamic changes in the phosphorylation state...
of P0 would have significant effects on P0-mediated adhesion and/or myelination. Further experiments will clearly be required to distinguish between these two possibilities.

Our deletion and mutational analysis are consistent with some of the mutations known to give rise to human neuropathies. There are no known human mutations in the COOH-terminal 13 amino acids that give rise to pathology (Fig. 6); the single amino acid change identified in this region (R215L) appears to have no physiological ramifications (Neelis et al., 1999). Accordingly, we find that deletion of this entire region has no effect on adhesion. Five human mutations causing neuropathy produce a shift in the reading frame, and two result in introduction of a stop codon, all altering or deleting the PKC target site and/or serine 204. One of the frame shifts leaves the RSTK motif intact but deletes serine 204. This mutation gives rise to a severe form of CMT1, emphasizing the importance of this residue. In addition, we have identified a patient with CMT1B in which an arginine residue has been changed to a serine within this PKC target motif (RSTK→SSSTK), potentially eliminating the ability of PKC to phosphorylate residues critical to adhesive function (Fig. 6). Indeed, mimicking this mutation in our in vitro assay abolishes P0 homophilic adhesion. Thus, these data implicate PKC-mediated phosphorylation of the cytoplasmic domain of P0 in both the regulation of homophilic adhesion and myelination. Consistent with this possibility, mutations in a dual specificity phosphatase, myotubularin-related protein-2, cause an autosomal recessive form of demyelinating CMT, called CMT4B (Bolino et al., 2000). Although it is not yet known whether myotubularin-related protein-2 cleaves phosphate groups on P0, these results clearly demonstrate that a phosphorylation/dephosphorylation cascade plays a role in the regulation of normal PNS myelination.

Given the central role that P0 plays in myelination, it is likely that other components interact with the cytoplasmic domain to further regulate the process of myelination. Thus, P0 mutations that may have profound effects on the process of myelination independent of its adhesive function will render incomplete any correlation between adhesion itself and severity of disease. Recent work from our group emphasizes this point, since the expression of P0 has dramatic effects on the presence of 1 mg/ml and maintained in the presence of 200 μg/ml of G418 (GIBCO BRL). Single clones were isolated and expanded. Clones of cells expressing high levels of transfected proteins as determined by immuno blotting with anti-P0 serum were chosen for adhesion assays.

Transfection of L and HeLa cells
All of the constructs and pCMV-Tag4 without inserts (Vect) were transfected into mouse L cells. WTP0,Δ13,Δ28,Δ43, and Δ59, and Vect were also transfected into HeLa cells. Stable cell lines were selected for 2 wk in the presence of 1 mg/ml and maintained in the presence of 200 μg/ml of G418 (GIBCO BRL). Single clones were isolated and expanded. Clones of cells expressing high levels of transfected proteins as determined by immuno blotting with anti-P0 serum were chosen for adhesion assays.

RT-PCR
Expression of transfected constructs was assayed by RT-PCR. In brief, total RNA was isolated from transfected cells using a QIAGEN kit and reverse transcribed with SuperScript II and oligo dT primers (GIBCO BRL). The resulting cDNA was used to amplify the transfected construct using the same primers, which were used for cloning. For RT-PCR, the hypoxanthine phosphoribosyl transferase (HPRT) cDNA was used as an internal control. The two primers for HPRT amplification were: forward primer, 5′-CACAAGACCCACACGCAAA-3′, and reverse primer, 5′-GCTGTCGACCAGCTTCGCTGCT-3′.

Expression of transfected cDNAs
To analyze protein expression of the transfected cDNAs and determine plasma membrane localization, cell surface molecules were labeled by biotinylation of intact cells followed by immunoprecipitation. Confluent cell layers in 100-mm dishes were rinsed free of serum and labeled with 0.1 mg/ml freshly prepared NHS-Lc-biotin (Pierce Chemical Co.) in cold PBS for 30 min. After washing and quenching, cell lysates were prepared using 1 ml of lysis buffer (1.5% NP-40, 0.15 M NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 1 mM PMSF, 100 μg/ml protease inhibitor cocktail [Sigma-Aldrich], and 100
μg/mL DNAse) per 100-mm plate, cleared by centrifugation at 14,000 g for 5 min, and the supernatant immunoprecipitated with anti-P0 antibody. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and biotinylated proteins were detected with HRP-streptavidin.

Immunoprecipitation and Western blotting
To analyze the expression of PKC isoforms in sciatic nerve, equal weights of mouse sciatic nerve were solubilized in 4% SDS, fractionated by SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with the indicated anti-PKC isoform antibody followed by HRP-conjugated anti–mouse IgG and visualized using the extended duration HRP substrate (Pierce Chemical Co.). For L cells transfected with wild-type P0, cultures were grown to confluence on 100-mm plates, washed, and incubated free of serum for 2 h before lysis as indicated above. An aliquot of the lysate was fractionated by SDS-PAGE and immunoblotted with antibodies to the PKC isoforms detected in sciatic nerve. The remaining lysate wasprecipitated with preimmune rabbit serum followed by goat anti–rabbit magnetic beads. The beads were discarded, and the cleared supernatant was incubated with anti-P0 antibody. The immunoprecipitated P0 and associated molecules were then fractionated by SDS-PAGE, transferred to PVDF, and probed with anti-PKC and anti-RACK1 antibodies. The immunoblots were processed as described above.

Adhesion assays
Cell layers were washed free of serum, harvested using 0.1% trypsin (GIBCO BRL) in PBS buffer, and resuspended in serum-free medium (DME) containing 10 mM EDTA at 5 × 10^5 cells/mL. 2 μl of fluorescent dye calcine acetoxyethyl ester (calcine AM) (Molecular Probes) were added to the cell suspensions and incubated at 37°C for 20 min. After two washes, 5 × 10^5 single cell–labeled cells were added to prepared microplate wells containing confluent monolayers of cells expressing wild-type P0 or BSA-coated wells as controls. After 1 h incubation at 37°C, the nonadherent cell–labeled cells were removed by carefully washing several times with DME containing 10 mM EDTA until the BSA-coated wells had no cells left. The number of cells adhering to the monolayer was measured with a molecular device microplate reader equipped with a fluorescein filter set at 494 nm.

To determine the importance of the PKC motif in the P0 sequence, calphostin C (Calbiochem) was used to specifically inhibit PKC. Wild-type P0-transfected cells were preincubated with increasing concentrations of calphostin C for 1 h and then assayed for adhesion as described above.

References