Myelin protein zero/P0 phosphorylation and function require an adaptor protein linking it to RACK1 and PKCα

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Point mutations in the cytoplasmic domain of myelin protein zero (P0; the major myelin protein in the peripheral nervous system) that alter a protein kinase Cα (PKCα) substrate motif (198HRSTK201) or alter serines 199 and/or 204 eliminate P0-mediated adhesion. Mutation in the PKCα substrate motif (R198S) also causes a form of inherited peripheral neuropathy (Charcot Marie Tooth disease [CMT] 1B), indicating that PKCα-mediated phosphorylation of P0 is important for myelination. We have now identified a 65-kD adaptor protein that links P0 with the receptor for activated C kinase 1 (RACK1). The interaction of p65 with P0 maps to residues 179–197 within the cytoplasmic tail of P0. Mutations or deletions that abolish p65 binding reduce P0 phosphorylation and adhesion, which can be rescued by the substitution of serines 199 and 204 with glutamic acid. A mutation in the p65-binding sequence G184R occurs in two families with CMT, and mutation of this residue results in the loss of both p65 binding and adhesion function.

Introduction

The ensheathment of axons by myelin is essential for the efficient propagation of nerve impulses both in the central nervous system and peripheral nervous system (PNS). In the PNS, myelin is made by Schwann cells, each cell wrapping around a single axon, creating concentric layers. The compaction of these layers results in the formation of the dense and interperiod lines typical of mature myelin (Scherer and Arroyo, 2002). The wrappings created by each Schwann cell are distinct and separated by the Nodes of Ranvier, structures specialized for Na and K ion exchange (Girault and Peles, 2002; Sherman and Brophy, 2005). The major protein component of myelin in the PNS is myelin protein zero (P0), a single-pass transmembrane molecule containing one Ig-like loop in the extracellular domain and a 69–amino acid highly basic cytoplasmic domain (Lemke and Axel, 1985). P0 is found throughout peripheral myelin and is essential for normal myelination.

Mutations in the *P0* gene cause peripheral neuropathy with either prominent demyelination, slowed nerve conductions and onset in childhood, or mainly axonal dysfunction, essentially normal nerve conductions, and onset as an adult (Warner et al., 1996; Nelis et al., 1999; Shy et al., 2001, 2004). There are currently >95 different mutations in P0 correlating with human neuropathies (http://www.molgen.ua.ac.be/CMTMutations/default.cfm). Mice null for the *P0* gene have uncompacted myelin in the PNS and develop a severe, early onset demyelinating neuropathy, whereas heterozygotes have a later onset neuropathy with substantial amounts of inflammation (Giese et al., 1992; Martini et al., 1995; Shy et al., 1997; Pareyson et al., 1999). Mutations in P0 are dominant, suggesting that the mutant protein interferes with the function of wild-type protein (Warner et al., 1996; Shy et al., 2004). Tissue culture and knock-in experiments have borne this out; cotransfection with wild-type and mutant P0 results in the loss of adhesion function (Wong and Filbin, 1996), and transgenic mice expressing a mutant P0 in a wild-type background develop demyelinating peripheral neuropathy (Runker et al., 2004).

The role of P0 in maintaining compact myelin may, in part, be caused by its ability to mediate homotypic interactions (Filbin et al., 1990; Schneider-Schaulies et al., 1990; Filbin and Tennekoon, 1993; Doyle et al., 1995), and decreases in adhesion have been correlated with the severity of disease (Ekici et al., 1998). Crystallographic studies of the extracellular domain of P0 suggest that it interacts in cis to form homotetramers, which, in turn, interact with similar tetramers in an apposing

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Abbreviations used in this paper: CBP, CaM-binding peptide; CMT, Charcot Marie Tooth disease; MBP, maltose-binding protein; PNS, peripheral nervous system; RACK1, receptor for activated C kinase 1.

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fold of the myelin membrane, thus contributing to the formation of compact myelin (Shapiro et al., 1996). Supporting this model, mutations in several of the residues likely to participate in cis- and trans-interactions of the homotetramers can cause inherited neuropathy and reduced cell–cell adhesion in vitro (Shy et al., 2004).

The cytoplasmic domain of P0 is also important for myelin compaction and adhesion. Several different nonsense or point mutations in the intracellular domain have been found in patients presenting with different neuropathies (Shy et al., 2004), and truncation of the cytoplasmic domain eliminates adhesion function (Wong and Filbin, 1994; Xu et al., 2001). Our own results implicate PKCα-mediated phosphorylation of the cytoplasmic domain of P0 in the regulation of P0-mediated adhesion and potentially formation/maintenance of compact myelin. First, we showed that deletion of a 14–amino acid sequence that eliminates a putative PKCα target site (198RSTK201) as well as point mutations within this domain eliminate P0-mediated adhesion. A patient presenting with late onset Charcot Marie Tooth disease (CMT) 1B was found to have a mutation in the PKCα target site R198S. In vitro analysis of the P0 function bearing the R198S mutation revealed a deficit in P0-mediated adhesion. We further demonstrated that PKCα and the receptor for activated C kinase 1 (RACK1) are associated with the cytoplasmic domain of P0 and that the inhibition of PKCα activity also inhibits P0-mediated adhesion. Point mutations that eliminate potential phosphorylation target sites (S199 or 204A) also result in the loss of adhesion, and deletions eliminating these serine residues also result in CMT (Shy et al., 2004).

We have now identified a protein, p65, that interacts directly with P0 and RACK1, bringing PKCα in close proximity to its target sites in the cytoplasmic domain of P0. Deletion of the P0 domain responsible for p65 binding results in the loss of P0-associated RACK1 and PKCα and the loss of P0-mediated cell adhesion. Importantly, two CMT patients carrying a point mutation in this domain, G184R, have been identified. Recombinant P0 with this mutation does not interact with p65, and cells transfected with this mutant P0 are unable to form adhesions. These data strongly suggest that the interaction of p65 with the cytoplasmic domain of P0 provides the foundation for the attachment of RACK1 and PKCα, resulting in the phosphorylation of P0 at serines 199 and/or 204. This is substantiated by functional rescue of the G184R mutant P0 by mutation of serines 199 and 204 to glutamic acid. Collectively, these data suggest that regulation of P0 phosphorylation and adhesion, which is mediated, in part, by the binding of p65, RACK1, and PKCα to a specific sequence in the cytoplasmic domain of P0, plays an important role in myelination.

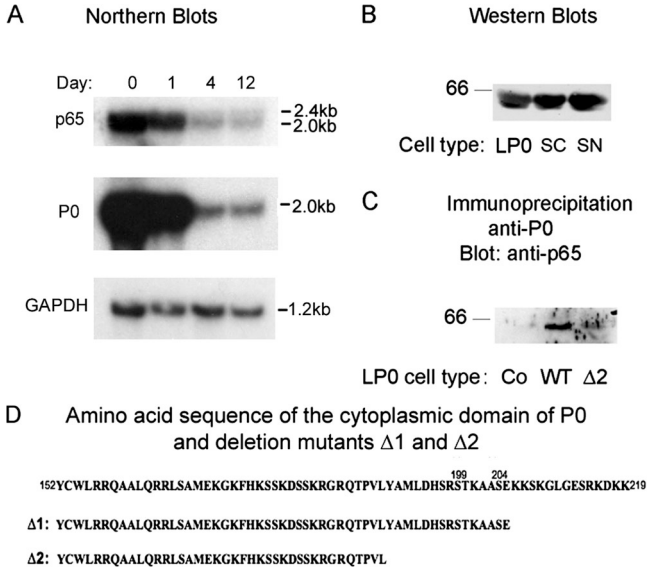

Figure 1. **Expression of p65 message in sciatic nerve and culture cells.** (A) Northern blot analysis of sciatic nerve. mRNA extracted from sciatic nerve of adult rats (10 μg/lane) before or after transection of the nerve was hybridized with a radiolabeled probe to p65 (top) or P0 (bottom). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a loading control. Notice that by 4 d after transection, both the p65 transcript and the P0 message are greatly reduced. (B) Western blot analysis. Extracts from rat sciatic nerve (SN), cultured Schwann cells (SC), or L cells expressing wild-type P0 were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted with an antibody raised to a peptide in p65. (C) Extracts of L cells (LCo), L cells expressing wild-type P0 (LP0WT), or L cells expressing a P0 deletion mutant (LP0Δ2) were immunoprecipitated with anti-P0 antibody. The resulting immunoprecipitates were assayed for the presence of associated p65 by Western blotting. (D) Amino acid sequence of the cytoplasmic domain of P0. Deletion mutant Δ2 lacks the C-terminal 28 amino acids, including the HRSTK domain and serine residue 204, which was previously identified as being crucial for P0 adhesion activity.

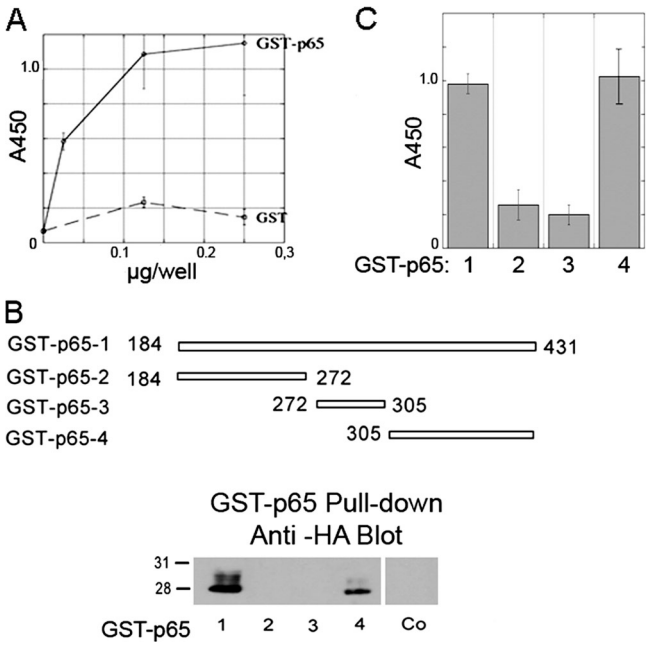

Figure 2. **In vitro interaction between p65 and P0.** (A) CBP-P0 immobilized on CaM-coated wells was incubated with increasing concentrations of GST alone or GST-p65. After extensive washing, the wells were incubated with anti-GST antibody, and binding was determined by ELISA. (B, top) The indicated fragments of p65 cDNA were prepared as GST fusion peptides. (bottom) GST-p65 pull-down assay. Lysates of L cells stably expressing HA-tagged P0 were incubated with the indicated GST-p65 constructs immobilized on glutathione beads. After extensive washing, material bound to beads was fractionated by SDS-PAGE and immunoblotted with anti-HA antibody. (C) Immobilized CBP-P0 was incubated with equimolar amounts of the different GST-p65 peptides as described in A. P0 preferentially binds to the C-terminal domain of p65. Error bars represent SD.

Results

P65 is a novel P0-interacting protein identified by a yeast two-hybrid assay

To identify potential proteins that interact with the cytoplasmic tail of P0, we screened a cDNA library from P30 rat sciatic nerve, a time of peak myelin expression, using the P0 cytoplasmic domain as bait in a yeast two-hybrid assay (Finley and Brent, 1994). 13 cDNA sequences encoding peptides that interact with the P0 bait were sequenced; 11 contained an identical 1.1-kb cDNA fragment. The complete cDNA codes for a 65-kD protein (p65) previously identified in the nucleus of cells undergoing meiosis (GenBank/EMBL/DDBJ accession no. Q64375; Chen et al., 1992) and as a 55-kD nucleolar protein (GenBank/EMBL/DDBJ accession no. NM_006455; Ochs et al., 1996). The selectivity of the interaction of p65 and P0 in the yeast two-hybrid system used was determined by testing other bait proteins as well as a C-terminal 28–amino acid deletion of P0 that fails to mediate cell adhesion (Xu et al., 2001). Only the full-length P0 or P0 lacking the 13 C-terminal amino acids (Fig. 1 D, Δ1) that do not affect P0-mediated adhesion were able to interact (not depicted). The 1.1-kb p65 cDNA reacts with two distinct mRNA bands from rat sciatic nerve of ~2.3 and 2 kb (Fig. 1 A). Transection of the sciatic nerve results in a progressive loss of p65 message: 4 d after transection, the transcripts are greatly reduced and are barely detectable by day 12 (Fig. 1 A). This loss parallels the loss of P0 message (Fig. 1 A, bottom), suggesting that the levels of expression of P0 and p65 are coordinately regulated. An antibody developed to a peptide corresponding to a sequence in p65 recognizes an ~65-kD protein band in sciatic nerve, cultured mouse Schwann cells, and L cells expressing full-length P0 (Fig. 1 B). In addition, full-length P0 but not P0 lacking the 28 C-terminal amino acids (Fig. 1 D, Δ2) is associated with p65, as determined by co-immunoprecipitation (Fig. 1 C), further validating the results of the two-hybrid screen.

P0 interacts with p65 in vitro

The results of the yeast two-hybrid assay suggest that the interaction between P0 and p65 is direct. To definitively demonstrate this, we engineered a fusion peptide containing the cytoplasmic domain of P0 linked to the CaM-binding peptide (CBP) and the fragment of p65 identified in the yeast two-hybrid assay as a GST fusion. GST-p65 binds directly to CBP-P0 immobilized on CaM-coated wells in a dose-dependent manner, whereas GST alone does not (Fig. 2 A).

The 1.1-kb message identified by the two-hybrid screen corresponds to a fragment of p65 lacking the N terminus (amino acids 184–431). An analysis of the primary structure of p65 shows that the fragment obtained by the yeast two-hybrid screen contains three possible tetratricopeptide repeat (TPR) motifs; these are amino acid stretches likely involved in protein–protein interactions and, therefore, are good candidates for the interaction between P0 and p65. Thus, we created three different p65 fragments, each containing one of the TPR motifs (Fig. 2 B). Using these constructs both in an in vitro binding assay (Fig. 2 C) or pull-down assay (Fig. 2 B, bottom), we were able to determine that the domain responsible for interaction with P0 is located within the C-terminal 126 amino acids of p65.

Mapping of the p65-binding site on P0

To more specifically define the p65-binding site on the cytoplasmic tail of P0, we created a series of P0 deletion mutants (Fig. 3 A) and generated cell lines stably expressing these mutants. Lysates of the cell lines expressing P0 were then used in pull-down experiments with GST-p65. Full-length wild-type P0 specifically interacts with GST-p65, and deletion of the first 22 amino acids in the N terminus of the cytoplasmic tail (P0Δ7 and P0Δ6; see Fig. 3 A) does not prevent the interaction (Fig. 3 B). However, P0 carrying deletion Δ5, corresponding to amino acids 173–184, is not pulled down by GST-p65 beads (Fig. 3 B), and the association of p65 with P0Δ4 (amino acids

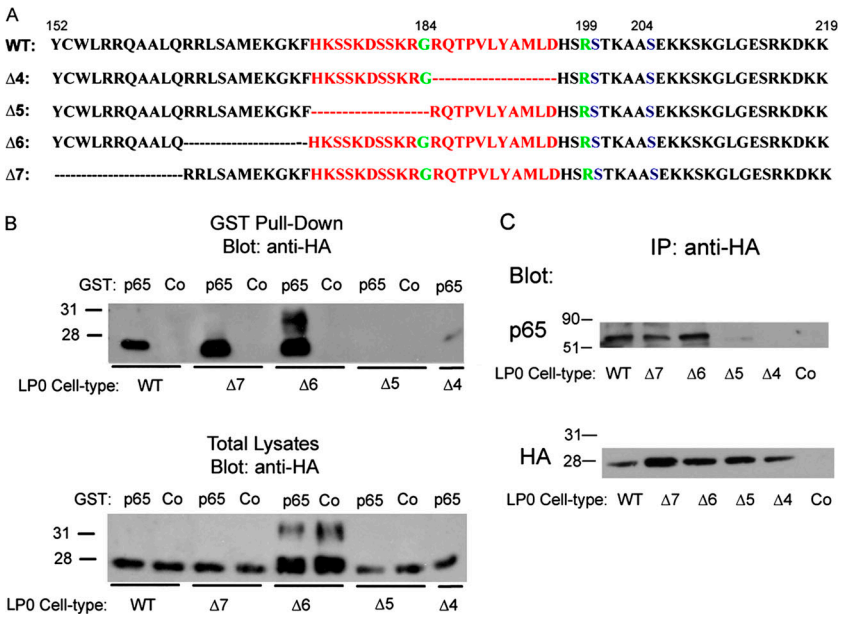

Figure 3. **Mapping of the p65 interaction domain on P0.** (A) Diagram of the cytoplasmic domain of P0 showing the deletion mutations. Serine residues 199 and 204 are shown in blue. Amino acids 184 G and 198 R are shown in green. The domain responsible for p65 interaction is highlighted in red based on the results shown in B and C. (B) GST-p65 pull-down assay. Lysates of L cells stably transfected with the indicated P0 constructs were incubated with GST-p65 immobilized on glutathione beads. After extensive washing, the material bound to beads was fractionated by SDS-PAGE and immunoblotted with anti-HA. Aliquots of the lysates were also blotted with anti-HA to determine the expression of P0 by the different cell types. (C) Co-immunoprecipitation of p65 and P0. Lysates of the indicated cell types were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-p65 antiserum. The amount of P0 immunoprecipitated was determined by immunoblotting with anti-HA antibody.

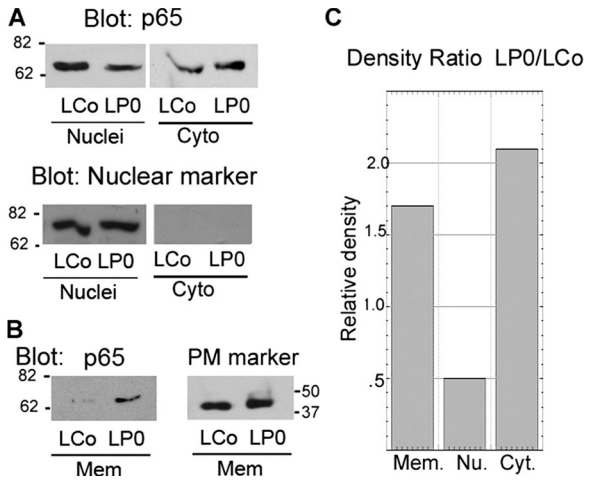

Figure 4. **Subcellular distribution of p65.** (A) L cells (LCo) or cells expressing wild-type P0 (LP0) were fractionated into a nuclear and cytoplasmic fraction (nuclei and cyto). Equal amounts of protein were immunoblotted with anti-p65 antiserum. An antibody against a nucleolar antigen was used as a nuclear marker. (B) Plasma membrane–enriched fractions of LCo and LP0 cells were immunoblotted with anti-p65. The same immunoblot was probed with antiflotillin as a plasma membrane marker. (C) The density ratio of the p65 band in LP0 and LCo cells was calculated for each cellular fraction. The values shown are representative of several experiments.

185–194) is severely reduced (Fig. 3 B). These results suggest that p65 interacts with the region between amino acids 173 and 194.

We also verified the association between endogenous p65 and P0 by coimmunoprecipitation. Immunoprecipitates of P0 bearing an HA tag from cell lysates using anti-HA antibody followed by immunoblotting with anti-p65 show association between the two proteins in cells expressing wild-type P0 or P0 mutants Δ6 and Δ7 but not P0 mutants Δ4 and Δ5 (Fig. 3 C).

p65 was originally identified as a nuclear (SC65) or nucleolar protein (No55); however, the predicted localization of SC65 using the pSort program (ExPASy Tools) is cytoplasmic. The 55-kD nucleolar protein No55 has an additional 34 amino acids at the N terminus, and the first 18 amino acids have the property of a signal peptide. No55 lacks C-terminal amino acids present in SC65 and has no nuclear localization signal. The predicted localization for No55 is 97% cytoplasmic, suggesting that it may be transported to its final destination via association with another molecule. The association of p65 with P0 suggests a nonnuclear localization. We used two different cell fractionation procedures to analyze the relative localization of p65 in control L (LCo) cells and L cells expressing P0 (LP0; Fig. 4, A and B). p65 is found in the nuclear, cytoplasmic, and membrane fractions in both LCo and LP0 cells; however, more p65 is detected in the membrane and cytoplasmic fraction of LP0 than LCo cells (Fig. 4, A–C). In contrast, the amount of p65 detected in the nuclear fraction of LCo cells is about double that of LP0 cells (Fig. 4, A and C). These results clearly indicate that the presence of P0 affects a redistribution of p65,

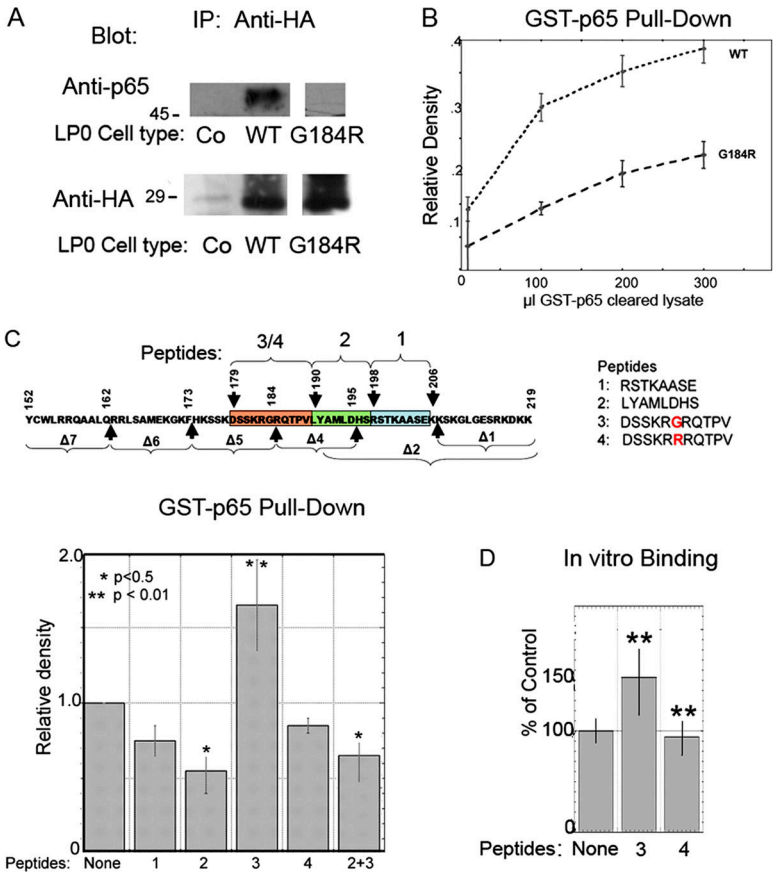

Figure 5. **Effect of a G184R point mutation on p65–P0 interaction.** (A) Lysates of cells expressing wild-type P0 or P0 carrying a point mutation on residue G184 were immunoprecipitated with anti-HA antibody, and the resulting precipitates were immunoblotted with anti-p65 antiserum. The amount of P0 precipitated was determined by immunoblotting with anti-HA antibody. (B) Lysates of cells expressing equivalent amounts of wild-type or G184R mutant P0 were incubated with increasing amounts of immobilized GST-p65. After extensive washing, the amount of bound P0 was determined by immunoblotting with anti-HA antibody. The density of the scanned P0 bands was compared with the amount present in the total lysate and expressed as a function of the GST-p65 concentration. (C) Synthetic peptides mimicking sequences in the P0–p65-binding region were used as competitors in a GST-p65 pull-down assay. Equal aliquots of wild-type P0 were incubated with GST-p65 in the presence of increasing concentrations of the indicated peptides. The amounts of P0 pulled down at plateau peptide concentrations (20 μM) were compared with the amount of P0 pulled down by p65 in the absence of any peptide competitor. The results of multiple experiments were analyzed using Kaleidagraph. The statistics software GB-Stat (Dynamic Microsystems) was used to obtain p-values (Fisher's lest significant difference–protected *t* tests). (D) GST-p65 immobilized on glutathione-coated wells was incubated with the cytoplasmic domain of P0 expressed as a CBP fusion in the presence of increasing concentrations of peptides 3 or 4. The amount of P0 bound was determined by ELISA using anti-Flag antibody. CBP–cytoplasmic cadherin was used as a negative control. The specific binding of CBP-P0 to GST-p65 in the presence of peptides is presented as the percentage of control (binding in the absence of peptides). Results of multiple experiments were pooled and analyzed as described in C. Error bars represent SD.

reducing its nuclear presence and increasing its cytoplasmic and membrane localization. This is consistent with a dual function for this molecule.

A point mutation found associated with CMT substantially reduces the interaction between P0 and p65

A missense mutation found in the p65-binding region of P0, G184R, correlates with a mild form of CMT with variable penetrance (unpublished data). G184 is at the interface of the two deletions that affect p65 binding to P0, prompting us to investigate the possibility that the G184R point mutation may result in a reduced or weakened interaction between P0 and p65. Thus, we generated cell lines expressing P0 carrying this mutation; coimmunoprecipitation assays using anti-p65 antibody show that the interaction between P0 and p65 is much reduced in these mutants as compared with wild-type P0 (Fig. 5 A). The same results are seen in pull-down assays using GST-p65 (Fig. 5 B).

The results presented so far suggest that p65 interacts with an extended domain in the cytoplasmic tail of P0 and that residue G184 is necessary but not sufficient for the interaction. However, the limits of this domain remain ill defined. In particular, the C-terminal extent of this region overlaps deletion Δ2, a region of the cytoplasmic domain we previously showed was critical for interaction with RACK1 and PKCα. Deletion of this domain also abrogates p65 binding (Fig. 1 C). Thus, to better define the p65–P0 interaction domain, we designed a series of peptides to use as competitors in the GST-p65 pull-down assay. The sequence and position of the peptides are shown in Fig. 5 C. Peptide 1 mimics the functionally important RSTKAAS motif. Peptide 2 contains the C-terminal fragment of Δ4, and peptides 3 and 4 include the important G184 residue; however, peptide 4 contains the human G184R mutation. GST-p65 pull-down assays were performed in the presence of increasing concentrations of each peptide. The amount of P0 interacting with p65 was determined by immunoblotting with anti-HA antibody. The immunoblots were scanned, and the density of the immunoprecipitated bands was compared with that of the total amount of P0 in the lysate.

The histogram in Fig. 5 C shows comparisons for each peptide at plateau values. Peptide 1 has no effect; thus, the functionally important RSTKAAS motif is not critical for p65 binding. Peptide 2 reduces the interaction between p65 and P0 by ∼50%, which is consistent with the attenuation of p65 binding in the Δ4 deletion and in the previously analyzed deletion that included the RSTKAAS motif (Xu et al., 2001) but extended into the Δ4 region. Surprisingly, peptide 3 containing G184 results in an increased interaction between p65 and P0 (Fig. 5 C). The effect of this peptide is, in fact, dependent on G184, as peptide 4, which is identical to peptide 3 except that it contains the mutation G184R, neither promotes nor inhibits the interaction between P0 and p65 in pull-down assays (Fig. 5, A and B). The same results are obtained when peptides 3 and 4 are used to perturb the binding of GST-p65 fusion protein to the cytoplasmic domain of P0 in an in vitro binding assay: peptide 3 results in an ∼50% increase in binding over control, whereas peptide 4 has no effect (Fig. 5 D). One possible interpretation of these results is that the region represented in peptides 3 and 4 stabilizes the interaction of P0 and p65 and that G184 is essential for this stabilization effect. The region of P0 defined by peptide 2 is dominant, as a combination of peptides 2 and 3 used in optimal doses is an effective competitor (Fig. 5 C).

p65 mediates the binding of RACK1 to P0

Although the interaction of RACK1 and PKCα and the phosphorylation of the PKCα target motif are essential for P0 adhesion function (Xu et al., 2001), RACK1 and PKCα do not interact directly with P0 (unpublished data). Thus, we considered the possibility that p65 acts as an adaptor, linking P0 with RACK1 and, consequently, PKCα. GST-p65 is able to pull down RACK1 independently of P0 expression (Fig. 6 A, Co cells)

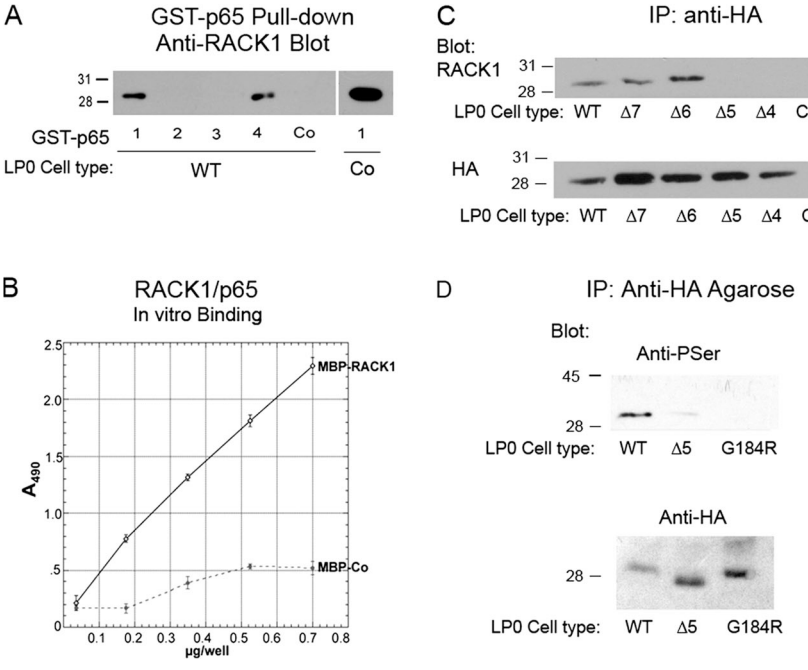

Figure 6. **p65 interaction with RACK1.** (A) GST-p65 pull-down assay. Lysates of L cells expressing wild-type P0 (LP0WT) or L cells (LCo) were incubated with the indicated GST-p65 constructs (see Fig. 2 B, top) immobilized on glutathione beads. After extensive washing, material bound to beads was eluted in SDS sample buffer, fractionated by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted with anti-RACK1 antibody. (B) In vitro interaction between p65 and RACK1. GST-p65 immobilized on glutathione-coated wells was incubated with increasing concentrations of MBP-RACK1 or MBP. After extensive washings, the amount of bound RACK1 was determined by ELISA using an anti-MBP antibody. (C) Interaction of RACK1 with P0. Lysates of the indicated cell types were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-RACK1 antibody. The amount of P0 immunoprecipitated was determined by immunoblotting with anti-HA antibody. Notice that only P0 constructs that are able to bind p65 show an interaction with RACK1 (see Fig. 3 A). (D) P0 lacking the p65 interaction domain is hypophosphorylated on serine residues. Lysates of the indicated cell types were immunoprecipitated with anti-HA antibody covalently attached to agarose. The eluted material was immunoblotted with antiphosphoserine antibody (top) and anti-HA antibody (bottom). Error bars represent SD.

and interacts directly with RACK1 in in vitro binding assays (Fig. 6 B). We next determined whether p65 plays a role in the interaction between RACK1 and P0 in intact cells. The lysates of cells expressing P0 and P0 deletion mutants immunoprecipitated with anti-HA antibody and immunoblotted with anti-p65 antibody shown in Fig. 3 C were also immunoblotted with anti-RACK1 antibodies (Fig. 6 C). RACK1 is detected in the P0 immunoprecipitates whenever p65 is present but not in the absence of p65 binding (compare Figs. 3 C with 6 C). Together, these results indicate that p65 interacts directly with RACK1 and, as p65 interacts directly with P0, further indicate that p65 acts as a bridge between RACK1 and P0, allowing activated PKCα to phosphorylate P0.

To further correlate the loss of p65 binding with the loss of interaction between P0 and PKCα, we compared the phosphorylation of serine residues on P0 wild-type and P0 mutants Δ5 and G184R. Confluent cell layers were lysed and immunoprecipitated with agarose-bound anti-HA followed by immunoblotting with a PKC-specific antiphosphoserine antibody. As shown in Fig. 6 D, the mutant P0 forms are hypophosphorylated when compared with the wild-type control.

Mutations in P0 that eliminate p65 binding also eliminate the ability of P0 to mediate cell–cell adhesion

The data thus far presented imply that p65 acts as a bridge bringing RACK1 and thus PKCα to the cytoplasmic domain of P0. Because the phosphorylation of serine residues 199 and 204 by PKCα is essential for P0 adhesion function (Xu et al., 2001), loss of the P0–p65 interaction should also result in the loss of P0-mediated adhesion. This is indeed the case: L cells expressing P0 deletion mutants Δ4 and Δ5 as well as the G184R mutation, all of which compromise p65 binding to P0, show much reduced P0-mediated cell–cell adhesion when compared with cells expressing equal levels of wild-type P0 (Fig. 7 A). To ensure that the loss of adhesion is not caused by altered cell surface expression, intact cells were biotinylated using a cell-impermeable biotinylation reagent followed by lysis and immunoprecipitation with anti-HA antibody. All three mutant P0s are found at the cell surface (Fig. 7 C). The importance of the G184 residue is further reflected in the fact that cells expressing this mutant do not form an adhesive interface as do cells expressing wild-type P0 (Fig. 7 B).

A mutant that mimics the serine phosphorylation of residues 199 and 204 abrogates the need for P0–p65 interaction

We have previously shown that serine residues 199 and 204 are essential for P0 adhesion function, presumably acting as substrates for PKCα (Xu et al., 2001). If the function of p65 is indeed to position PKCα so it can phosphorylate P0, a mutation at serine residues 199 and 204 that mimics phosphorylation should abolish the need for p65. Thus, we introduced the S199, 204E double mutation into the P0 cDNA constructs containing the Δ4 and Δ5 deletions as well as the G184R point mutation, which are all mutations that abolish p65 binding. As predicted, the ability of cells expressing the P0 deletion mutants and the G184R mutation to form adhesions is rescued by the presence of the double mutation S199, 204E (Fig. 7 A; compare G184R with GR/SE).

Discussion

The importance of the cytoplasmic region of P0 in the formation and/or stabilization of P0-mediated adhesion is well

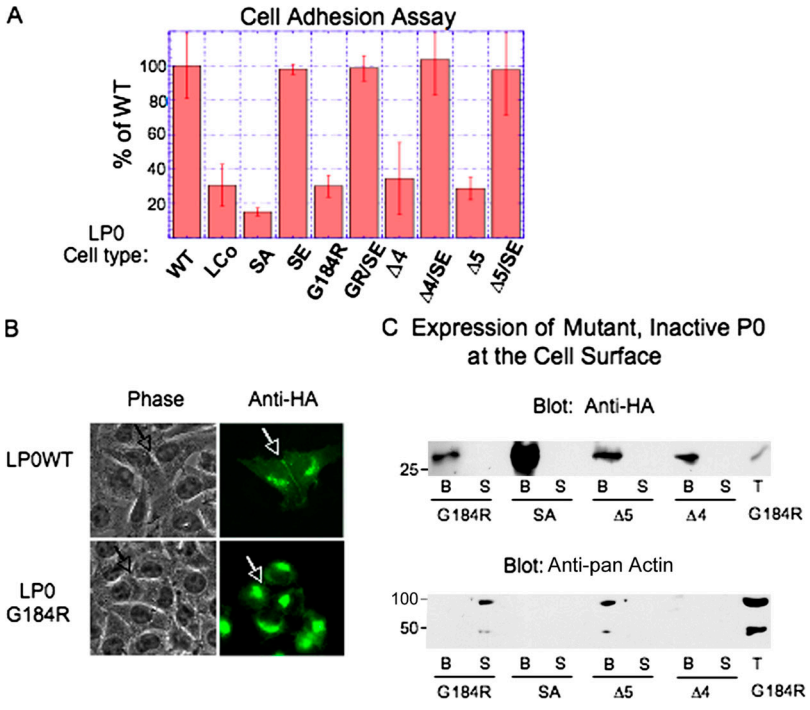

Figure 7. **Effect of p65 interaction on P0 adhesion function and cell surface expression.** (A) P0-mediated cell–cell aggregation. Cultures of L cells expressing similar levels of the indicated P0 constructs were dissociated into single cells and allowed to aggregate for 4 h at 70 rpm and 37°C. The number of cells present in clusters was then compared with the total number of cells using Image Pro Plus (MediaCybernetics). Notice that the double mutation S199, 204E abolishes the effect of deletions Δ4 and Δ5 and point mutation G184R. The figure represents results from five different experiments normalized to 100% aggregation for wild-type P0 cells (the observed number of wild-type cells in clusters was around 50–70%). (B) L cells expressing wild-type or G184R P0 were grown on coverslips and reacted with anti-HA followed by fluorescein-conjugated secondary antibody. Cells were visualized using an inverted laser-scanning confocal microscope. Notice that wild-type P0 localizes at areas of cell–cell contact (arrow), whereas the mutant G184R shows a more diffuse distribution and is absent from regions where cells are in close proximity (arrow). (C) Confluent cell layers were incubated with cell surface–impermeable biotinylation reagent (B) or buffer alone (S). Cell lysates were reacted with avidin-conjugated magnetic beads, and the eluted material was fractionated by SDS-PAGE and immunoblotted with anti-HA antibody. The blots were reacted with antiactin antibody to control for labeling of intracellular peptides; actin is readily detected in the total lysate lane but is weak or not detectable in the avidin-bound material. The amount of cell lysate loaded in the lane marked T (total lysate) was ∼1/50th of that loaded in the avidin bead (B) and control bead (S) lanes. Error bars represent SD.

established (Wong and Filbin, 1994; Xu et al., 2001). The studies presented here were targeted at understanding the mechanistic basis for the role of the cytoplasmic domain. We previously demonstrated that PKCα-mediated phosphorylation of serines 199 and 204 is essential for function and that RACK1 played a role, possibly in mediating the binding of PKCα with P0 (Xu et al., 2001). We now demonstrate the requirement for an adaptor protein, p65, that links RACK1/PKCα to P0. Our functional analysis shows that p65 interacts directly with a well-defined region of P0; that, in the absence of binding, RACK1 and PKCα are absent from the cytoplasmic domain of P0; and that P0 is hypophosphorylated and is unable to mediate the formation of cell–cell adhesions in spite of the fact that it still expressed at the cell surface.

p65 has been previously reported, but in an entirely different context. It was originally cloned from an expression library using an antibody to the rat synaptonemal complex antibody (Chen et al., 1992) and was subsequently cloned from a human expression library and localized to the interphase nucleolus (Ochs et al., 1996). These previous results were quite surprising; however, it is not unusual to find proteins or alternate transcripts fulfilling very different functions in the cell. This dual functionality is consistent with the redistribution we find when parental L cells are compared with L cells expressing P0. The two putative functions (one nuclear and related to cell division and one cell surface and related to cell–cell interactions) are completely separate temporally and spatially, as Schwann cells in the process of ensheathment are in the final stages of a terminal differentiation program.

Deletion analyses of the P0 cytoplasmic domain in conjunction with competition assays using peptides mimicking specific regions of the cytoplasmic domain were used to map the p65-binding region. The P0 site to which p65 binds spans ∼18 amino acids (residues 179–197). The use of peptides mimicking regions of the cytoplasmic domain of P0 as competitors suggests that the p65-binding site has two component parts. This is based on the fact that peptides mimicking the N-terminal half of the binding region (amino acids 179–189) when used in pull-down or in vitro binding assays enhance binding, appearing to prime p65 for stable binding or to stabilize binding to P0. In contrast, peptides mimicking the C-terminal half of the binding region (amino acids 190–199) inhibit binding. Furthermore, in the presence of both peptides, the binding of p65 to P0 is inhibited, suggesting that the C-terminal half site is dominant. However, both regions are essential for binding, as the deletion of either region abrogates binding. Our interpretation of these data is that binding of p65 is stabilized by a change in configuration that requires interaction with the N-terminal half site of P0.

Human mutations in the cytoplasmic domain of P0 give rise to CMT with variable severity (Shy et al., 2004). Truncations of the cytoplasmic domain and point mutations are among those associated with the disease. Therefore, it is not surprising to find that the p65-binding region is the site of a point mutation, G184R, giving rise to the disease. What is quite interesting is that this mutation is within the N-terminal half of the p65-binding site, the site we suggest is essential for priming or stabilizing p65 binding. A peptide spanning the N-terminal half of the P0 p65-binding site but containing the G184R mutation no longer is able to inhibit or stabilize binding. The neuropathy resulting from the G184R mutation is late onset and extremely variable in penetrance (unpublished data). Because mutations in P0 are dominant, variable penetrance may well be the result of the relative ratios of the normal versus mutant P0 synthesized. Additionally, because P0 function is suggested to be based on cis-tetramers (Shapiro et al., 1996), this particular mutation may be compensated by wild-type cis-partners carrying the p65–RACK1–PKCα complex that phosphorylate mutant partners. This is consistent with the fact that mutation of serines 198 and 204 to glutamic acid restores wild-type function to P0s carrying deletions or point mutations that compromise the binding of p65.

The machinery we have identified to be associated with the cytoplasmic domain of P0 suggests that like members of the cadherin (Lilien et al., 2002; Lilien and Balsamo, 2005; Potter et al., 2005) and integrin (Webb et al., 2004) families of adhesion molecules, P0 function is modulated through a set of associated cytoplasmic components designed to regulate phosphorylation (M. Xu et al., 2000). This further suggests that dephosphorylation also plays a critical role in regulating P0 function (Bolino et al., 2000; Houlden et al., 2001; Berger et al., 2002).

Regulated phosphorylation may play a role in the early stages of myelination, as phospho-P0 is most prevalent during the period of maximal myelination (Eichberg and Iyer, 1996). Possibly, P0 adhesion is turned off and on during this time to regulate the rate of compaction. Additionally, as suggested by Eichberg (2002), phosphorylation may be a component of the machinery that is critical for the regulation of trans-intracellular membrane interactions essential for normal myelin compaction. In this scenario, phosphorylation would serve to retard maturation by inhibiting interactions of the cytoplasmic domain with phospholipids on the opposing membrane; thus, during maximal periods of myelin formation, intercellular adhesion predominates, whereas maturation is prevented. After completion and/or stabilization of wrapping, phosphorylation is decreased, and compaction ensues. Although the phosphorylation of P0 is clearly essential for adhesion function, it may also play a role in other aspects of myelination. We (Menichella et al., 1999; W. Xu et al., 2000) and others (Giese et al., 1992) have reported that transcription of myelin-associated genes is deregulated in the absence of P0, and, thus, phosphorylation may play an important role in downstream signaling processes. These are among the questions that we will be pursuing in the near future.

Materials and methods

Antibodies

Antibodies used in this study are as follows: anti-GST (Ab-3; Calbiochem), anti-FLAG M2 (Stratagene), anti-HA (Roche Applied Science), anti–maltose-binding protein (MBP; New England Biolabs, Inc.), anti-RACK1, anti-PKCα, and antiflotillin (BD Biosciences), antiphosphoserine antibody specific for PKC (Cell Signaling Technology), HRP-conjugated secondary antibodies (Invitrogen or Jackson ImmunoResearch Laboratories), and agarose-conjugated anti-HA (Bethyl Laboratories). The anti-p65 antibody was prepared in rabbits using amino acid sequence 104–118 as antigen. Antibody AH6

(nucleolar antigen; Developmental Studies Hybridoma Bank at the University of Iowa) was used as a nuclear marker.

Yeast two-hybrid screening
The assay used was based on the interaction mating method (Kolonin et al., 2000). cDNA encoding the cytoplasmic domain of P0 was introduced into yeast (strain RF231/pSH18-34) as a fusion with LexA. mRNA from the sciatic nerve of 20–30-d-old rats was use to create a cDNA library fused to a transcription activation domain. The library was then introduced in yeast to create the prey strains. Bait and prey strains grown on selective medium were mated and grown on indicator plates. The cytoplasmic domain of myelin-associated glycoprotein, β1-integrin, and TGFβ type I receptor were used as bait protein controls.

Northern blots
p65 and P0 probes were obtained by PCR using the p65 fragment identified in the yeast two-hybrid screen and cDNA for the P0 cytoplasmic domain as templates. The probes were labeled with [^{32}P]dATP and [^{32}P]dGTP (to normalize for GC content) using random priming and Klenow DNA polymerase. The blots were hybridized and washed using the methods described in Church and Gilbert (1984). Sciatic nerve transection and regeneration were performed as described previously (Menichella et al., 2001).

P0 constructs and expression in L cells
Full-length P0 (P0WT) and P0 deletion mutant cDNA was cloned into the phCMV3 expression vector (Gene Therapy Systems) using a PCR-based technique. All constructs were created using the same forward primer containing a Kpn1 restriction site, a Kozak consensus sequence, and the same reverse primer containing a BamH1 restriction site; for the deletion mutants, two unique additional primers in a triple PCR strategy were used. PCR site-directed mutagenesis was used to introduce point mutations in the P0 cytoplasmic domain. All constructs were confirmed by sequencing.

cDNA constructs were transfected with LipofectAMINE (Invitrogen) into mouse L cells, and stable clones were selected using G418. Clones expressing high levels of P0, as determined by Western blotting with anti-HA antibody, were chosen for assays.

P0 fusion peptides
Full-length wild-type P0 cDNA was used as a template for PCR to create P0 cytoplasmic construct (C-terminal 69 residues). The PCR fragment was cloned into the BamHI–SalI cloning site of the pCAL-N-FLAG expression vector (Stratagene) to generate a fusion with CBP. The constructs were confirmed by sequencing. CBP–wild-type P0 was prepared and purified according to the manufacturer's protocol. Purity of the peptide was assessed by Coomassie staining and Western blotting.

Generation of p65 fusion peptides
The 1.1-kb cDNA identified in the yeast two-hybrid assay was used as a template to generate four truncated p65 constructs: p65-1 (residues 178–431), p65-2 (residues 178–210), p65-3 (residues 266–298), and p65-4 (residues 299–431). p65-1, -2, and -3 constructs were ligated into the XbaI–HindIII cloning site of the pGEX 2T expression vector (GE Healthcare), and p65-4 was ligated into the BamHI–SalI cloning site of pGEX 4T2. All constructs were verified by restriction enzyme cleavage and sequencing. Peptides were purified by glutathione affinity chromatography and assayed by Coomassie staining and Western blotting.

Expression and purification of MBP-RACK1
MBP–full-length human RACK1 was provided by D. Mochly-Rosen (Stanford University, Palo Alto, CA). The plasmid was transformed into *Escherichia coli* strain BL21, and MBP-RACK1 protein was purified from the bacterial cleared lysate by maltose affinity chromatography (New England Biolabs, Inc.). The purity of fusion peptide was confirmed by Coomassie staining after SDS-PAGE.

GST pull-down assays
GST-p65 peptides bound to glutathione-Sepharose were equilibrated in binding buffer (20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.02% NP-40, 1.5% goat serum, 2 mM sodium-ortho-vanadate, and protease inhibitor cocktail) for 1 h at 4°C. L cells stably expressing P0 were lysed in mild lysis buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 3 mM KCl, 2.5% NP-40, 1 mM sodium-ortho-vanadate, 10 μg/ml DNase, and protease inhibitor cocktail [Sigma-Aldrich]) and cleared by centrifugation at 16,000 *g* for 20 min. The supernatants were incubated with the glutathione-Sepharose–bound GST-p65 peptides overnight at 4°C. Beads with bound protein complexes were collected by centrifugation at 500 *g* and washed three times with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40, 2 mM Na-o-vanadate, and protease inhibitor cocktail (Sigma-Aldrich). Beads were resuspended in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

Synthetic peptides mimicking P0 sequences were prepared by Alpha Diagnostic International. For peptide competition assays, GST-p65 immobilized on glutathione-coated beads was incubated with increasing amounts of peptide for 30 min before the addition of cell lysate and overnight incubation. The amounts of P0 pulled down at plateau peptide concentrations (20 μM) were compared with the amount of P0 pulled down by p65 in the absence of any peptide competitor.

In vitro binding assays
High protein binding wells (Pierce Chemical Co.) were incubated with 50 μl of 20 μg/ml CaM in 50 mM bicarbonate buffer overnight at 4°C. The wells were washed in PBA (PBS with 0.25% BSA and 0.02% NaN_3), blocked for 1 h at 37°C in 5% nonfat milk in PBS, washed in PBA three times, and incubated with a previously determined saturating concentration of CBP-P0 cytoplasmic domain in PBA for 1 h at 37°C. After washing three times in PBA, the wells were incubated with increasing concentrations of GST alone or GST–p65-1 for 2 h at room temperature followed by several washes with PBA. The wells were then incubated for 1 h with 1:1,000 anti-GST antibody in PBS followed by washing and incubation with HRP-conjugated goat anti–mouse IgG (1:1,000) in PBS with 0.25% BSA. The wells were thoroughly rinsed with PBS and incubated with *o*-phenylene diamine substrate for 30 min. The reaction was stopped by adding 2 M H_2SO_4, and absorbance was determined at 450 nm in a spectrophotometer (Spectramax Plus; Molecular Devices). All samples were assayed in triplicate, and the experiment was repeated three times. Statistics and the dose-response graph were computed with Kaleidagraph software (Synergy).

To analyze the binding of RACK1 to p65, 96-well glutathione-coated microplates (Pierce Chemical Co.) were rinsed with PBS and incubated with GST–p65-1 peptide. The wells were blocked with 4% BSA in PBS for 1 h, rinsed with PBS, and increasing concentrations of MBP-RACK1 or MBP peptide in PBS were added. After 1 h of incubation, the wells were washed three times with PBS and incubated with anti-MBP mouse monoclonal antibody in PBS with 0.5% BSA followed by HRP goat anti–mouse IgG in TBS with Tween 20 (0.1%) with 0.5% BSA. The wells were thoroughly washed with TBS with Tween 20 (0.1%), and color reagent was added and analyzed as described in the previous paragraph.

Coimmunoprecipitation assays
Confluent cell layers were washed in PBS and lysed in buffer containing 20 mM Hepes, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM NaF, and protease inhibitor cocktail (1 ml/10-cm plate; Sigma-Aldrich) for 10 min at 4°C. The lysates were cleared by centrifugation at 14,000 *g*, and the supernatant was incubated with anti-HA or control antibody for 2 h at 4°C, with rotation followed by 1 h with anti–rat IgG covalently attached to magnetic beads. The beads were then collected using a magnetic stand, washed extensively with PBS containing 0.5% Triton X-100, and eluted with SDS sample buffer. Eluted material was fractionated on SDS-PAGE, transferred to polyvinylidene difluoride, and assayed by immunoblotting with the appropriate antibody.

Aggregation assays
Single cells were prepared from semiconfluent cell layers: layers were washed with PBS, incubated for ~2 min with 0.002% trypsin in PBS, and collected in complete medium (DME with 5% FBS) with 5 μg/ml of added antipain and 10 μg/ml DNAase. Cells were collected by centrifugation at 1,000 *g*, resuspended in DME (wih 20 mM Hepes, pH 7.4, antipain, and DNAase), and counted in a hemocytometer (AO-Spencer Brightline; Reichert Scientific Instruments). The cell suspensions were diluted to a concentration of ~10^4/ml, and 1 ml was added to 30-mm Petri plates containing 2 ml DME/Hepes. Dishes were rotated at 70 rpm in a humidified chamber at 37°C. After ~4 h, cells were observed and photographed under a microscope (Axiovert 25CFL; Carl Zeiss MicroImaging, Inc.). Several fields of cells were used to quantify cell numbers using ImagePro Plus (Media Cybernetics). Results were tabulated as ratios of cells in aggregates versus total cell number for a minimum of five fields.

Biotinylation of cell surface P0 and cell fractionation
For biotin labeling of cell surface P0, cell layers were washed in PBS and incubated with the membrane-impermeable biotinylation reagent sulfo-*N*-hydroxysuccinimide-SS-biotin (Pierce Chemical Co.) at a concentration of 1 mg/ml in PBS for 30 min at room temperature. Cell layers were washed three times with ice-cold PBS, pH 8.0, and lysed as described in the

Coimmunoprecipitation assays section. Cleared cell lysates were incubated with streptavidin-conjugated magnetic beads (Roche Applied Sciences) for 1 h, the beads were extensively washed, and bound material was eluted with SDS sample buffer and analyzed by immunoblotting with anti-HA antibody. For cellular fractionation, confluent layers of control L cells or cells expressing wild-type P0 (LP0) were washed in ice-cold PBS and scraped in 0.25 M sucrose in Hepes buffer, pH 7.9, containing protease inhibitor cocktail (Sigma-Aldrich). Cells were homogenized, and the nuclear and membrane fractions were separated using Optiprep (Sigma-Aldrich) according to the manufacturer's directions. Alternatively, L control and LP0 cells were fractionated into nuclear and cytoplasmic fractions using the Ne-PER kit (Pierce Chemical Co.).

P0 immunostaining

L cells expressing P0 or the P0 mutant G184R were grown on poly-L-lysine–coated coverslips, washed free of serum, fixed in 4% PFA for 20 min at room temperature, and permeabilized in 0.1% Triton X-100 for 5 min. After washing in PBS, the coverslips were incubated with rat anti-HA for 1 h at room temperature. The coverslips were washed extensively and incubated for another hour in AlexaFluor488 anti–rat antibody diluted in PBS with 5% goat serum. After several washes, the coverslips were mounted on glass slides, and images were captured using an inverted laser-scanning confocal microscope (TCS SP2 AOBS; Leica).

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