Several Human PATCHED1 Mutations Block Protein Maturation

Evans C. Bailey, Lei Zhou, and Ronald L. Johnson

Departments of Cell Biology and Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

ABSTRACT

The tumor suppressor gene PATCHED1 (PTCH1) is mutated in sporadic and inherited forms of basal cell carcinoma. PTCH1 binds Hedgehog proteins and inhibits signaling in the absence of ligand. Although PTCH1 mutations are proposed to reduce or abolish protein function, few mutations have been tested for activity. We introduced six PTCH1 missense mutations into mouse patched1 and tested them in murine cells deficient for patched1 function. Three mutants retained significant activity. Three other mutants had little or no function, and of these, two were retained in the secretory pathway. These studies indicate that missense mutations can abolish PTCH1 function by blocking protein maturation.

INTRODUCTION

Human PTCH13 mutations occur in several sporadic tumors, including BCC and medulloblastoma (reviewed in Ref. 1). Individuals with BCNS are heterogeneous for PTCH1 mutations (2, 3). Affected individuals display a range of tumor types and developmental abnormalities, including spina bifida and polydactyly (4). In human tumors that carry PTCH1 mutations, both alleles are altered, indicating that PTCH1 function is compromised or absent (5).

Little is known about how PTCH1 mutations alter function. Disease-associated PTCH1 mutations are dispersed over much of the coding sequence and ~75% are predicted to truncate the protein (reviewed in Ref. 6). The remaining 25% are amino acid changes and in-frame insertions and deletions. To ascertain the consequences of human PTCH1 mutations, we introduced six missense alterations at the corresponding position in mouse ptc1 and tested their activity in murine cultured cells deficient for endogenous ptc1.

MATERIALS AND METHODS

Cell Culture. ptc1−/− cells were maintained, infected, and purified by fluorescence-activated cell sorting as described previously (7).

Vectors. Site-directed mutations were made in a full-length mouse ptc1 cDNA (8) using Quick Change (Stratagene). ptc1 mutants were cloned proximal to internal ribosomal entry site-GFP in a MSCV-based vector (7).

β-Gal Activity Determination. Cells were plated at 8.4 × 104 cells/cm2 (95–100% confluent) in 24-well plates and after 1 day were trypsinized, resuspended in 0.25 ml of (95–100% confluent) in 24-well plates and after 1 day were trypsinized, resuspended in 0.25 ml of β-gal lysis buffer, and 50 μl of cell extracts were assayed after 24 h at 37°C. β-Gal activity was calculated by (A472/A492) × (μg protein) × (extinction coefficient of 2 × 105) and was normalized to total cellular protein using Bio-Rad protein assay reagent. For each time point, duplicate wells were plated, and each well was assayed in triplicate. Values shown are averages of these six values with the associated experimental error.

Immunoblot and Glycosidase Treatment. Cells were lysed in radioimmunoprecipitation assay buffer with Complete protease inhibitors (Roche) for 45 min on ice. For glycosidase treatment, NP40 was added to the lysates at 1.5% final concentration and incubated for 45 min at 37°C with either 1000 units of Endo H or 500 units PNGase F (New England Biolabs) in 50-μl volumes. Lysates were solubilized in an equal volume of 2× Laemmli buffer at room temperature, size-fractionated on 7.5% polyacrylamide gels, and transferred to Protran (Schleicher & Schull). The membranes were incubated with HA.11 (Convance) or GM130 (Transduction Laboratories) antibodies and visualized using enhanced chemiluminescence reagent (Amersham).

RESULTS AND DISCUSSION

We used several criteria to select six missense mutations in human PTCH1 that were likely to alter functionally important residues (Table 1). First, the chosen mutations affected residues that were identical among at least 10 of 12 Ptcl and Ptc2 homologues. Second, several mutations were identified in sporadic tumors in which both PTCH1 alleles were altered, indicating a loss of heterozygosity at the PTCH1 locus. Finally, for the R294C and Δ816 mutations, the same residue has been found mutated in independent patients. These criteria indicated that the mutations were involved in tumor formation and were not benign polymorphisms.

To test for function, the PTCH1 mutations were introduced at the corresponding positions of a mouse ptc1 cDNA (9) by site-directed mutagenesis. This full-length cDNA contains a COOH-terminal HA epitope that does not interfere with function (7). ptc1 constructs were cloned into an MSCV retrovirus proximal to an internal ribosomal entry site followed by GFP. ptc1−/− cells were infected and stable integrants were selected. Using fluorescence-activated cell sorting, cell populations expressing high and low levels of Ptc1 transgenes were isolated on the basis of high or low GFP fluorescence. For each transgene, there was a 5–7-fold difference in Ptc1 expression between the high and low GFP-expressing populations (data not shown).

Total cell lysates were immunoblotted with an anti-HA antibody to detect Ptc1 protein (Fig. 1). Both wild-type and mutant Ptc1 proteins migrated with a similar apparent molecular weight, between M, 165,000 and 175,000. For each Ptc1 transgene, cell were sorted for similar levels of GFP fluorescence and thus should produce similar levels of Ptc1 protein. Lower steady-state levels indicated that some mutants were unstable. Only D499Y and P1111L were produced at amounts comparable with the wild-type transgene; all other mutants were expressed at several fold lower levels. The pattern of relative Ptc1 stabilities was the same whether cells expressed low (Fig. 1) or high (data not shown) levels of transgene.

The Ptc1 mutants were tested for their ability to complement constitutive ptc1−/− lacZ activity in ptc1−/− cells. In these cells, a lacZ gene insertion in the ptc1 locus disrupts ptc1 function and places lacZ under the control of the ptc1 promoter. ptc1 is induced by Hedgehog signaling (9), and in ptc1−/− cells, the absence of Ptc1 function causes persistent expression of ptc1−/− lacZ and production of high levels of β-gal protein. Expression of wild-type Ptc1 reduces β-gal activity in these cells by ~8-fold (7, Fig. 2). P1111L functioned as well as the wild-type transgene, whereas the activities of R280C and D499Y were 2-fold less. Δ802 function was four times as weak as wild-type and L346R, and Δ472-3 had little or no function. Similar profiles of activity were observed for cells expressing low (Fig. 2) or high (data not available).
not shown) levels of the Ptc1 transgenes. The cells expressing functional Ptc1 mutants also responded to ligand; high levels of ptc1-lacZ were induced upon incubation with media containing Sonic hedgehog (data not shown).

The Ptc1 mutants that had greatly reduced or absent activity may not properly mature in the secretory pathway, preventing the protein from reaching its site of action. To test this idea, we used the sensitivity of Ptc1 to the Endo H glycosidase as a way to monitor the protein’s cellular location. Proteins containing N-linked carbohydrates are sensitive to Endo H before reaching the medial Golgi but become resistant upon entering this compartment (10).

We examined ptc1−/− cells expressing high levels of wild-type, L346R, Δ472-3, and Δ802 Ptc1 for sensitivity to PNGase F and Endo H. Treatment of Ptc1 with PNGase F, a glycosidase that removes all N-linked carbohydrates (11), decreases the protein’s apparent molecular weight (Fig. 3). However, Ptc1 is partially resistant to Endo H, indicating that some of the protein has transited into or beyond the medial Golgi (7, Fig. 3). In contrast, L346R and Δ472-3 were completely sensitive to Endo H, whereas Δ802 was partially resistant. This suggests that L346R and Δ472-3 have little or no function because they are arrested in a compartment proximal to the medial Golgi and cannot reach the site where Ptc1 normally functions. Potentially, the mutations cause Endo H sensitivity by preventing proper carbohydrate modification rather than blocking movement through the secretory pathway. We believe this to be unlikely because both large extracellular loops of Ptc1 are glycosylated (7). If a mutation prevented access to carbohydrates in different domains of Ptc1, the protein conformation would likely be highly altered and not transit the secretory pathway.

In this and a separate study (7), we have tested a total of nine PTCH1 missense mutations in mouse Ptc1 using the ptc1−/− cells (Fig. 2). We expected that these mutants would have little or no function. However, whereas the L346R and Δ472-3 mutations were severely compromised, the seven other mutants retained significant activity. Q802L and P1111L complemented as well as the wild-type Ptc1 transgene, whereas R280C, G495V, D499Y, Δ802, and R1100W were reduced in function by 2–4-fold.

There are several explanations for the mutants retaining function. The missense mutations may have little activity in vivo but function when overexpressed in the ptc1−/− cells. To address this concern, we isolated cells expressing low levels of transgene on the basis of low GFP production and observed similar results (Fig. 2). However, even with this selection, the mutant proteins may be overproduced at levels higher than endogenous Ptc1. Indeed, Taipale et al. (12) expressed G495V or D499Y at physiological levels in ptc1−/− cells and found these mutants are 7–8-fold less active. It is also possible that Ptc1 has multiple functions and that some missense mutations disrupt Ptc1 control of cell proliferation, although not affecting its ability to regulate ptc1-lacZ. Alternatively, some missense mutations may cause only modest reductions in activity. This would suggest that small reductions in Ptc1 could give rise to tumors. Consistent with this idea is the finding that in ptc1+/− mice, medulloblastoma can arise from haploinsufficiency (13, 14).

Of the missense mutations that we have tested, there appears to be no correlation between their position in the protein sequence and the degree to which they disrupt function. Of the four mutations that reside in the putative large extracellular loops, L346R and Δ802 were severe, whereas R280C and Q802L were mild (7, Fig. 2). Because L346R blocked maturation of Ptc1 (Fig. 3), this large loop may be involved in an early folding or glycosylation step. In the predicted transmembrane regions, only Δ472-3 blocked function, whereas G495V, D499Y, and P1111L had minor effects. Because the Δ472-3 mutation removes two amino acids from the predicted fourth transmembrane domain, this might prevent proper membrane insertion, leading to reduced protein levels and the inability to mature in the secretory pathway. R1100W, the only mutation predicted to be in a cytosolic loop, caused minor reductions in function.

Table 1 Human-derived mutations tested in mouse ptc1

<table>
<thead>
<tr>
<th>No.</th>
<th>Human PTCH1</th>
<th>Mouse Ptc1</th>
<th>Source</th>
<th>Conservation</th>
<th>LOH</th>
<th>Reference</th>
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<tr>
<td>1</td>
<td>R294C</td>
<td>R280C</td>
<td>BCC</td>
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<td>(15)</td>
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<td>2</td>
<td>L360R</td>
<td>L346R</td>
<td>BCC</td>
<td>12/12</td>
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<td>(16)</td>
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<tr>
<td>3</td>
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<td>Δ472-3</td>
<td>Medulloblastoma</td>
<td>11/12</td>
<td>Yes</td>
<td>(17)</td>
</tr>
<tr>
<td>4</td>
<td>D513Y</td>
<td>D499Y</td>
<td>BCNS</td>
<td>11/12</td>
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<td>(18)</td>
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<tr>
<td>5</td>
<td>Δ816Δ</td>
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<td>12/12</td>
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<tr>
<td>6</td>
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<td>P1111L</td>
<td>BCC</td>
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</table>

* Number of sequences where the residue is identical between human PTCH1 and other known Ptc1 and Ptc2 homologs from human, mouse, zebrafish, chicken, frog, worm, fruit fly, and butterfly.

* Position mutated in independent tumors or individuals.
REFERENCES


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