Characterization of a Novel PLZF Mutant with Suppressed Biological Activity

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Introduction

Background

The 673aa transcription repressor promyelocytic leukemia zinc finger (PLZF), a member of the BTB/POZ family of evolutionarily conserved proteins[1], is widely known for its role in the molecular pathogenesis of a rare, retinoic-acid resistant subset of acute promyelocytic leukemias[2] (APLs) and has recently been implicated as an important regulator of the development of invariant natural killer T (iNKT) cells[3]. Wild-type PLZF is also known to have roles in limb and myeloid development, spermatogenesis, and the repression of oncogenes like c-myc. Like other POZ family transcription factors, PLZF possesses a 120aa N-terminal BTB/POZ domain as well as 9 C-terminal C_2H_2 Kruppel-type DNA-binding zinc fingers. The POZ domain has been shown to regulate biological activity by modulating protein-protein interactions, dimerization, and nuclear localization[4].

C-terminal PLZF residues are also highly conserved

mouse	peeippdwriektylylcyv
human	----------
cow	----------
zebrafish	--d-------
rat	----------
opossum	----------
horse	----------
chicken	-----t----

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1   mdltkmgmiq lqnpshptgl lckanqmrla gtlcdvvimv dsqefhahrt vlactskmfe ilfhrnsqhy tldflspktf qqileyayta tlqakaedld
101 dllyaaeile ieyleeqclk iletiqasdd ndteatmadg ggeeeedrka rylknifisk hsseesgyas vagqslpgpm vdqspsvsts fglsamsptk
201 aavdslmsig qsllggtlqp pagpeeptla gggrhpgvae vkmemmqvde apgqdspgaa essisggmgd kleerskegp gtptrgsvit sarelhygre
301 esgeqlsppv eaggqppgrq eplappvekh lgiysvlpnh kadavlsmps svtsglhvqp alavsmdfst ygqllpqgfi qrelfsklge lavgmkaesr
401 plgeqcsvcg velpdneave qhrklhsgmk tygcelcgkr fldslrlrmh llahsagaka fvcdqcgaqf skedalethr qthtgtdmav fgllcgkrfq
501 aqealqqhme vhagvrsyic secnrtfpsh talkrhlrsh tgdhpyecef cgsefrdest lkshkrihtg ekpyecngcg kkfslkhqle thyrvhtgek
601 pfeclkchqr srdysamikh lrthngaspy qctictevcp slssmqkhmk qhkpeeippd wriektylyl cyvdspghga aenslspssn
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Figure 1. PLZF contains an N-terminal BTB/POZ domain (crystal structure, *upper right*) that occupies the first 120aa of the PLZF coding region (*bottom*). Functional domains in the mouse coding region are color-coded: BTB/POZ (red) and DNA-binding zinc fingers (green). The hCD2-PLZF mutant tail is shown in blue (see below). Alignment of PLZF sequences from various vertebrates (*middle right*) shows that the last 30 C-terminal residues are also highly conserved.

Aims and Scope

Recent hCD2-PLZF transgene (Tg) overexpression experiments studying the effect of forced PLZF expression on the development of iNKT cells identified a novel PLZF mutant with an aberrant C-terminal 17aa tail that confers significantly diminished biological activity *in vivo*. We describe here the cloning and characterization of this hCD2-PLZF mutant, toward the specific goal of understanding how the aberrant domain suppresses biological activity and the more general goal of shedding light on potentially novel PLZF interactions that might be leveraged to develop PLZF inhibitors. Such inhibitors may have widespread biological and therapeutic applications, not only for RA-intractable APLs, but also for other pathologies modulated by iNKT cells, like asthma.

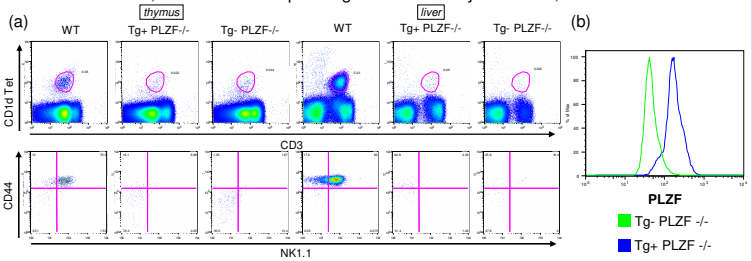


Figure 2. (a) hCD2-PLZF Tg (with an aberrant C-terminal 17aa tail) does not rescue iNKT cell development *in vivo*. Flow cytometry shows that Tg+ and Tg- PLZF deficient mice (*second and third columns in each panel*) have similar mature iNKT cell populations in the thymus (*left panel*) and liver (*right panel*), both significantly decreased as compared to wild-type B6 mice. **(b)** An anti-PLZF antibody directed against the hinge region of the WT protein binds hCD2-PLZF in Tg+ PLZF deficient thymocytes, suggesting that the mutant protein's structure is not globally disrupted and that it is still expressed and accumulated in cells.

Results

Preparation of PLZF mutants.

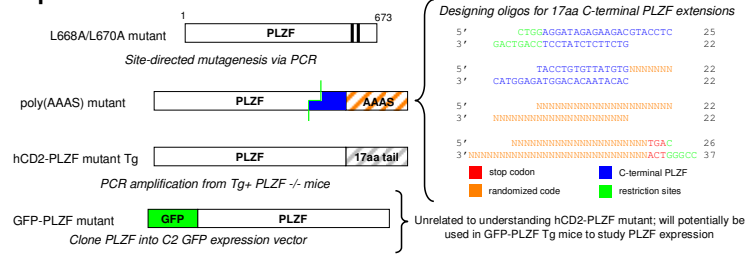


Figure 3. PLZF mutants successfully cloned. Whereas the hCD2-PLZF Tg and L668A/L670A mutants were PCR amplified before inserting into expression vectors, the poly(AAAS) mutant was prepared by annealing a series of 30bp oligonucleotides with complementary overhangs and directly ligating this extension construct into an expression vector.

Probing biological activity of mutants.

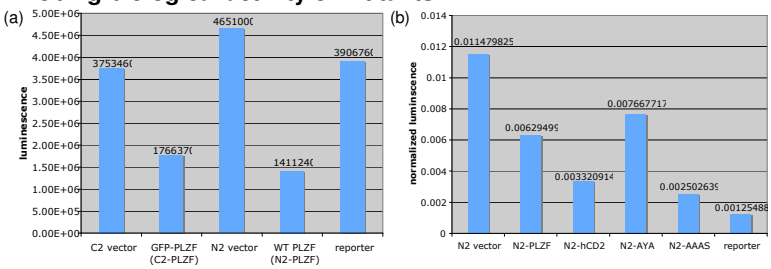


Figure 4. (a) GFP-PLZF and wild-type PLZF exhibit similar repressor activities *in vitro*. **(b)** PLZF mutants with 17aa extensions show unexpectedly high repressor activity as compared to wild-type PLZF *in vitro*, while the L668A/L670A mutant shows slightly impaired repression. Experiments were normalized based on transfection efficiency using an independent renilla luciferase expression vector, which encodes a luciferase the emits light at a different wavelength.

Conclusions & Future Directions

While previous work has typically associated the regulation of PLZF transcription repression with its N-terminal BTB/POZ domain, the results reported here show that an aberrant C-terminal 17aa tail affects biological activity both *in vitro* and *in vivo* without disrupting global structure or preventing the protein from accumulating in cells. In the hopes of gaining a more thorough understanding of PLZF's function, as well as interactions that could potentially be targeted in order to inhibit PLZF, we set out to characterize how this aberrant tail confers suppressed biological activity and have derived the following conclusions thus far:

- GFP-PLZF and wild-type PLZF show similar levels of repressor activity *in vitro*, validating potential experiments that will knock in a GFP-PLZF fusion protein in mice in order to study PLZF expression.
- Intriguingly, preliminary data shows that the hCD2-PLZF Tg and poly(AAAS) mutants with aberrant 17aa C-terminal extensions both exhibit higher repressor activity than wild-type PLZF. These results may suggest a more critical and tightly regulated role for PLZF in the development of iNKT cells.

Future experiments will be aimed at confirming these results and studying the cellular localization and DNA-binding abilities of these mutants.

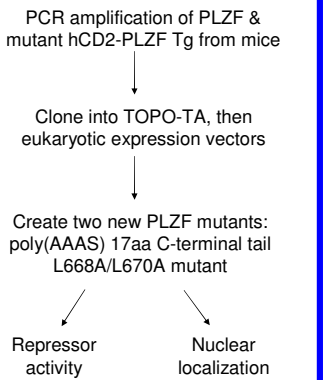
Experimental Design

Hypotheses and preparation of mutants. We hypothesized that the aberrant 17aa tail interacts non-specifically to sterically shield some critical interaction or process required for biological activity, possibly local protein folding. Under this assumption, it is likely that a randomized 17aa extension would demonstrate a similar level of biological activity as that of the hCD2-PLZF mutant. Thus, to test this hypothesis we developed a method for cloning randomized C-terminal extensions into wild-type PLZF. We then used this scheme to prepare a PLZF mutant with an aberrant 17aa C-terminal poly(AAAS) tail.

We further sought to propose a specific mechanism by which non-specific steric shielding might disrupt transcription repression, besides folding interference. Computational screens for predicted phosphorylation sites revealed no potential sites within 40aa of the aberrant 17aa tail. An LYL motif, however, appears near the end of the PLZF coding sequence (aa 668-670), and the LXL motif has been shown to mediate kinase binding in certain proteins[5]. To determine whether the LYL motif was required for PLZF biological activity, we used site-directed mutagenesis to prepare an L668A/L670A mutant.

Probing biological activity of mutants. A luciferase reporter system was then used to probe the repressor activity of PLZF mutants by comparing their abilities to knock down the transcription of luciferase and thereby decrease output luminescence.

We also asked whether PLZF mutants retained the ability to localize in the nucleus. In order to answer this question, we performed colocalization analysis on HeLa cells transfected with PLZF mutants by staining cells with anti-PLZF and DAPI.



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