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Heterogeneity in the Properties of Mutant SLURP1 Proteins in *Mal de Meleda*

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To the Editor

Genetic defects in SLURP1, a “lymphocyte antigen 6” (Ly6)–like protein, cause *mal de Meleda*, a palmoplantar keratoderma (PPK). The hallmark of Ly6 proteins is an 80– amino acid domain containing 10 cysteines, all arranged in a characteristic spacing pattern and all disulfide-bonded, creating a three-fingered structural motif (Fig. 1). This sequence motif was initially found in cobra and viper toxins, but was later identified in ~25 mammalian proteins. Most mammalian Ly6 proteins are tethered to the plasma membrane by a GPI anchor (*e.g.*, uPAR, CD59, GPIHBP1), but several, including SLURP1, are secreted proteins. SLURP1 is produced in keratinocytes but it diffuses away and can be detected in sweat and urine.¹

Many of the *SLURP1* mutations in *mal de Meleda* patients are missense mutations. Thus far, no one has yet compared the properties of mutant SLURP1 proteins. We chose to investigate properties of mutant SLURP1 proteins as a first step in a longer-term effort to define SLURP1 protein interactions. We embarked on this project in the wake of discoveries on disease-causing mutations in GPIHBP1, an Ly6 protein that binds lipoprotein lipase (LPL) avidly and transports it to the capillary lumen. Certain *GPIHBP1* missense mutations abolish LPL binding, leading to severe hypertriglyceridemia.^{2–5} Mutations involving cysteines in GPIHBP1's Ly6 domain promote GPIHBP1 dimerization and multimerization (*i.e.*, the presence of an unpaired cysteine promotes intermolecular disulfide bonds).⁶ Protein multimerization is highly relevant to disease pathogenesis because multimers are incapable of binding LPL. Mutations of many other GPIHBP1 residues (aside from the cysteines) also

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impaired disulfide bonding and led to protein dimerization/multimerization.^{5,6} Mutations in a few residues (*e.g.*, W109) abolished LPL binding without causing protein multimerization; those residues likely play a direct role in LPL binding.⁶

Most GPIHBP1 missense mutations, including those involving cysteines, have little effect on GPIHBP1 trafficking to the cell surface;⁷ however, a cysteine mutation in CD59 (identified in a patient with paroxysmal nocturnal hemoglobinuria) prevented CD59 trafficking to the cell surface.⁸ Thus, similar types of mutations can have different effects in different Ly6 proteins. Importantly, defining properties of different GPIHBP1 mutants yielded insights into residues likely to play a direct role in protein interactions.⁶

Here, we compared properties of eight SLURP1 missense mutations identified in *mal de Meleda* patients, including three involving cysteines in the Ly6 domain (p.Cys77Arg, p.Cys94Ser, p.Cys99Tyr).^{9–11} Expression vectors encoding untagged SLURP1 were transfected into CHO cells; all yielded roughly similar amounts of expression, as judged by western blots of cell extracts (Fig. 2a). To assess SLURP1 secretion, the cell culture medium was electrophoresed under reducing conditions. Large amounts of wild-type SLURP1 (WT-SLURP1) were found in the medium (Fig. 2a), but most of the mutant proteins (*e.g.*, C77R, C94S, C99Y, L98P, R71H, R71P) were secreted poorly. Two mutants (G86R, P82S) were secreted in a robust fashion—similar to WT-SLURP1. To determine if mutant SLURP1 proteins were susceptible to protein multimerization (akin to GPIHBP1 mutants), we examined the medium under nonreducing conditions. High-molecular weight multimers were observed with WT-SLURP1, but most of the WT-SLURP1 was monomeric. [WT-GPIHBP1, when expressed in CHO cells, also yields some multimers.⁶] Most SLURP1 mutants (C94S, C99Y, L98P, R71H, R71P) yielded increased amounts of dimers and multimers, and monomers were either undetectable or present in small amounts. Like WT-SLURP1, SLURP1-G86R was predominantly monomeric. SLURP1-P82S was intermediate; there were moderate amounts of monomers and increased amounts of multimers.

Similar results were observed in a human keratinocyte cell line (HaCaT). Like WT-SLURP1, SLURP1-G86R and SLURP1-P82S in the medium was mostly monomeric. The other SLURP1 mutants were barely detectable in the medium, and little was monomeric (Fig. 2b). We also expressed SLURP1 containing a uPAR tag in *Drosophila* S2 cells. Once again, WT-SLURP1 and the P82S and G86R mutants were largely monomeric (nonreduced samples, Fig. 2c). With the other mutants, only small amounts of monomers were observed, and the ratio of dimer to monomers was increased. Thus, consistent results were obtained in three different expression systems. Most SLURP1 mutants, including all three cysteine mutants, were produced in cells but secreted poorly. In these cases, the amount of monomers in the medium was low, and a higher percentage of the protein was in the form of dimers/multimers.

In our studies, SLURP1-P82S yielded monomers but in reduced amounts. Of note, the *mal de Meleda* patient carrying the p.Pro82Ser mutation appeared to have a mild form of the disease (the soles of the feet were spared).¹² We speculate that the milder disease in this case could relate to partial function of SLURP1-P82S monomers.

Large amounts of SLURP1-G86R monomers were secreted, similar to WT-SLURP1. Previously, Favre *et al.*¹ transfected *myc*-tagged SLURP1-G86R into HEK293 cells and concluded that it was secreted poorly. In those studies, a *myc*-tagged SLURP1 protein in the medium migrated as a doublet band, and the susceptibility of the mutant to multimerization was not assessed.

The first paper linking SLURP1 to *mal de Meleda* proposed that SLURP1 binds to a cell surface protein.¹³ Later, several studies proposed that WT-SLURP1 modulates acetylcholine signaling through the α -7-nicotinic acetylcholine receptor (α 7nAChR),¹⁴ but no one has directly documented binding of SLURP1 protein to α 7nAChR. [We did not find SLURP1 binding to α 7nAChR in our cell-based binding assays.¹⁵] To understand *mal de Meleda*, it will be important to identify proteins that interact with SLURP1. In any such studies, it will be important to assess binding of mutant SLURP1 proteins that cause *mal de Meleda*. Our studies suggest that SLURP1-G86R could be useful for those sorts of studies. SLURP1-G86R can be expressed at high levels in insect cells and, like WT-SLURP1, is secreted as monomers.

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Abbreviations

PPK	palmoplantar keratoderma
Ly6	lymphocyte antigen 6
GPIHBP1	glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1

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Fig 1.

Schematic of SLURP1 based on the crystal structure of other Ly6 proteins. The homology model of human SLURP1 was generated with the protein fold recognition server Phyre 2 using the 3D structure of LYNX1 as the template. The protein (excluding the signal peptide) is visualized with the PyMOL Molecular Graphics System. SLURP1 contains 10 cysteines, and all are disulfide-bonded (C25 with C50, C28 with C37, C43 with C73, C77 with C93, and C94 with C99). The five disulfide bonds are depicted in yellow. The locations of all missense mutations causing *mal de Meleda* are noted; three involve conserved cysteines of the Ly6 domain. The protein, like all Ly6 proteins, forms a three-fingered structural motif. The mutants that were secreted in low amounts and which led to protein multimers were located in the core of the protein; the two mutants that yielded a significant amount of monomers (G86R and P82S) are located at the tip of the third finger of the three-fingered motif. C-term, carboxyl terminus; N-term, amino terminus.

Fig 2.

Heterogeneity in the properties of SLURP1 mutants associated with *mal de Meleda*. All experiments shown were repeated twice with virtually identical results. All samples were denatured in 1% lithium dodecyl sulfate for 10 min at 70° C. Where indicated, samples were reduced in 50 mM dithiothreitol. For each expression system, actin was used as a loading control. (a) Western blots depicting the expression of untagged WT-SLURP1 and SLURP1 mutants in transfected CHO cells. Shown (from the top) is a western blot of medium under nonreducing conditions (media, NR), a western blot of medium under reducing conditions (media, R) and a western blot of the cell extracts under reducing conditions (cells, R). Western blots were performed with a polyclonal antibody against human SLURP1 (Novus). All mutants were expressed, but they exhibited different levels of secretion. The secretion of SLURP1-R71H, SLURP1-R71P, SLURP1-C77R, SLURP1-C94S, SLURP1-L98P, and SLURP1-C99Y was reduced by 83–95% when compared to WT-SLURP1. For WT-SLURP1, monomers (~11 kDa, arrowhead) accounted for 37% of secreted SLURP1; for SLURP1-G86R, 24%; for SLURP1-P82S, 13%; for other SLURP1 mutants, 0.3–2%. (b) Western blots depicting the expression of untagged WT-SLURP1 and SLURP1 mutants in transfected HaCaT cells (a human keratinocyte cell line). In HaCaT cells, the secretion of the R71H, R71P, C77R, C94S, L98P, and C99Y mutants were reduced by >97%; the effects of the G86R and P82S mutations were more modest, and monomers were easily detected. SLURP1-W15R (with a mutation in the signal peptide) was not expressed, consistent with findings with a myc-tagged SLURP1-W15R construct.¹ (c) Western blots depicting the expression of WT and mutant SLURP1 proteins in *Drosophila* S2 cells. These proteins were tagged at the carboxyl-terminus with sequences encoding uPAR Ly6 domain III,⁶ and therefore had a higher molecular weight. Nonreduced fusion proteins were detected with a

uPAR-specific monoclonal antibody (R24);⁶ reduced proteins were detected with the SLURP1 polyclonal antibody. In *Drosophila* S2 cells, the production and synthesis of the different SLURP1 constructs was variable. Monomers (~20 kDa, arrowhead) accounted for 81–92% of WT-SLURP1, SLURP1-G86R, and SLURP1-P82S in the medium, whereas they accounted only for 7–43% of the SLURP1 with the other mutants.