The apo-structure of the leucine sensor Sestrin2 is still elusive

Robert A. Saxton,1,2,3,4,5 Kevin E. Knockenhauer,1,* Thomas U. Schwartz,1† David M. Sabatini1,2,3,4,5†

Sestrin2 is a GATOR2-interacting protein that directly binds leucine and is required for the inhibition of mTORC1 under leucine deprivation, indicating that it is a leucine sensor for the mTORC1 pathway. We recently reported the structure of Sestrin2 in complex with leucine [Protein Data Bank (PDB) ID, 5DJ4] and demonstrated that mutations in the leucine-binding pocket that alter the affinity of Sestrin2 for leucine result in a corresponding change in the leucine sensitivity of mTORC1 in cells. A lower resolution structure of human Sestrin2 (PDB ID, 5CUF), which was crystallized in the absence of exogenous leucine, showed Sestrin2 to be in a nearly identical conformation as the leucine-bound structure. On the basis of this observation, it has been argued that leucine binding does not affect the conformation of Sestrin2 and that Sestrin2 may not be a sensor for leucine. We show that simple analysis of the reported “apo”-Sestrin2 structure reveals the clear presence of prominent, unmodeled electron density in the leucine-binding pocket that exactly accommodates the leucine observed in the higher resolution structure. Refining the reported apo-structure with leucine eliminated the large \( F_{\text{obs}} - F_{\text{calc}} \) difference density at this position and improved the working and free \( R \) factors of the model. Consistent with this result, our own structure of Sestrin2 crystallized in the absence of exogenous leucine also contained electron density that is best explained by leucine. Thus, the structure of apo-Sestrin2 remains elusive.

INTRODUCTION

The mechanistic target of rapamycin complex 1 (mTORC1) couples cell growth with the availability of biosynthetic inputs such as amino acids (1, 2). Leucine, in particular, robustly activates mTORC1, and leucine deprivation inhibits mTORC1 signaling in a wide variety of experimental systems (3–5). Recently, we identified Sestrin2 as a key leucine sensor in mammalian cells (6, 7). Leucine binds directly to Sestrin2 in vitro, and genetic loss of Sestrin2 and its closely related homologs renders mTORC1 signaling resistant to inhibition by leucine starvation. Mechanistically, Sestrin2 inhibits mTORC1 in the absence of leucine by binding to GATOR2, an upstream activator of mTORC1 (7–9). The addition of leucine triggers the dissociation of Sestrin2 from GATOR2 both in vitro and in cells, suggesting that leucine binding induces a conformational change in Sestrin2 that disrupts this interaction (6, 7). Consistent with this, leucine substantially increases the thermal stability of purified Sestrin2, as is often observed for ligand-receptor complexes (6, 7, 10).

We solved the crystal structure of human Sestrin2 in complex with leucine at a 2.7 Å resolution, revealing insights into the mechanism of leucine sensing (6). However, Sestrin2 failed to crystallize in the absence of leucine, and the structure of apo-Sestrin22 remained elusive. Subsequently, a 3.5 Å structure of Sestrin2 that crystallized without the addition of exogenous leucine was reported in the same crystal form, showing Sestrin2 in a nearly identical conformation as the leucine-bound structure (11). On the basis of this observation, Lee et al. argued that the similarities between the reported “apo”-structure (11) of Sestrin2 and the leucine-bound structure (6) are inconsistent with Sestrin2 being a sensor for leucine (12, 13).

To reconcile our structural, biochemical, and cell biological data with the claims made by Lee et al. (12), we reanalyzed their reported apo-Sestrin2 structure [Protein Data Bank (PDB) ID, 5CUF], revealing the clear presence of a ligand bound in the leucine-binding pocket. In addition, we report our own 3.0 Å structure of Sestrin2 that we obtained without adding exogenous leucine during the purification process, in which leucine is also present.

RESULTS

Using the corresponding structure factors and atomic model deposited in the PDB, we calculated the \( 2F_{\text{obs}} - F_{\text{calc}} \) and \( F_{\text{obs}} - F_{\text{calc}} \) electron density maps for the protein with PDB ID 5CUF. Simple inspection of the \( F_{\text{obs}} - F_{\text{calc}} \) difference map, which highlights discrepancies between experimental data and the structural model, revealed the presence of substantial unexplained electron density (positive 7.5σ peak) at the exact location of the leucine-binding pocket observed in the leucine-bound Sestrin2 (PDB ID, 5DJ4; Fig. 1A). This unmodeled density is easily observed in all five crystallographically independent copies of Sestrin2 in the asymmetric unit and is the prime location where modeled density and observed density differ. Furthermore, the unexplained electron density in 5CUF resembles the density corresponding to leucine in the higher-resolution 5DJ4 structure (Fig. 1B).

To test whether this density may represent leucine, we refined the 5CUF model either with or without leucine built into it. As with 5DJ4, refining 5CUF with leucine eliminated the \( F_{\text{obs}} - F_{\text{calc}} \) map peak at the binding site and improved the \( R \) factors of the model (Fig. 1, B, C, and E), consistent with the presence of bound leucine in this structure.

We also analyzed the possibility that one of the buffer components (MES, tris, sodium, chloride, malonate, and TCEP) of the reported crystallization condition of 5CUF may be contributing to the large difference density at the leucine-binding site (11). The only ingredient that could be reasonably considered at the modest resolution of 3.5 Å as a leucine substitute is malonate (Fig. 1D). It is the main precipitant at a 1.15 M concentration, and it could at least mimic the interaction of the carboxyl group of leucine. However, the contacts formed by the hydrophobic leucine side chain, as well as by the cationic amine group, would still be unexplained. Nonetheless, refining 5CUF
The leucine-binding site of Sestrin2 consists of a shallow hydrophobic pocket flanked on each side by opposing positive and negative charges contributed by Arg^{390} and Glu^{451}, respectively, making it well suited to specifically accommodate zwitterionic \(\alpha\)-amino acids with short aliphatic side chains (6). Among the amino acids, Sestrin2 has the highest affinity for leucine, with about 15- and 30-fold lower affinity for the closely related amino acids methionine and isoleucine, respectively, and undetectable affinity for the remaining natural amino acids (7). In addition, whereas it is impossible to definitively conclude whether the density present in the 3.5 Å 5CUF structure corresponds to leucine or another ligand such as malonate, our own 3.0 Å structure clearly favors leucine as the best candidate to explain the observed density. In any case, these data conclusively demonstrate that 5CUF represents a ligand-bound form of Sestrin2, not the apo-Sestrin2 conformation as reported by Lee et al. (12) based on the data in Kim et al. (11).

Although leucine is highly abundant in bacterial cells (14), it may seem surprising that Sestrin2 can remain stably bound to leucine throughout the purification process given the reported 20 \(\mu\)M affinity of Sestrin2 for leucine (7). One possibility is that the off-rate of the Sestrin2-leucine interaction is substantially lower in the absence of additional protein components or

**DISCUSSION**
Table 1. Data collection and refinement statistics. RMS, root mean square. Values in parentheses are those for the highest resolution shell.

<table>
<thead>
<tr>
<th>Protein</th>
<th>“Pseudo-apo”-Sestrin2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>PDB ID</td>
<td>5T0N</td>
</tr>
<tr>
<td>Data collection</td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>I23</td>
</tr>
<tr>
<td>a, b, and c (Å)</td>
<td>292.26, 292.26, and 292.26</td>
</tr>
<tr>
<td>α, β, and γ (°)</td>
<td>90.0, 90.0, and 90.0</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.9792</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>200.0–3.0 (3.05–3.00)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>40.7 (41.9)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; (%)</td>
<td>28.0 (&gt;100)</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt; (%)</td>
<td>4.4 (37.8)</td>
</tr>
</tbody>
</table>

Refinement

| Resolution range (Å) | 103.3–3.0 |
| R<sub>work</sub> (%) | 18.9 |
| R<sub>free</sub> (%) | 22.5 |
| Number of reflections | 82,205 |
| R<sub>free</sub> reflections | 1,637 |
| Number of nonhydrogen atoms | 14,648 |
| Protein atoms | 14,648 |
| RMS deviations | |
| Bond lengths (Å) | 0.010 |
| Bond angles (°) | 1.037 |
| Average B-factor (Å<sup>2</sup>) | 49.0 |
| Protein | |
| Ramachandran (%) | |
| Favored | 96.4 |
| Allowed | 3.0 |
| Outlier | 0.7 |

In summary, we argue that the 5CUF structure depicts Sestrin2 in the ligand-bound conformation, not the apo-conformation as reported by Lee et al. (12). The leucine-dependent dissociation of Sestrin2 from GATOR2 represents the critical first step in the activation of mTORC1 by leucine and understanding how leucine-binding alters the conformation of Sestrin2 to disrupt its interaction with GATOR2 remains a major open question in the field.

**MATERIALS AND METHODS**

**Protein production and purification**

Human Sestrin2 was expressed and purified as described previously (6). Briefly, full-length, codon-optimized human Sestrin2 was N-terminally fused with a human rhinovirus 3C protease–cleavable His<sup>10</sup>-Arg<sup>8</sup>-ScSUMO tag and cloned into a PETDuet-1 bacterial expression vector. This vector was transformed into E. coli LOBSTR (DE3) cells (Kerafast) (15). Cells were grown at 37°C to 0.6 optical density, then protein production was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at 18°C for 12 to 14 hours. Cells were collected by centrifugation at 6000g, resuspended in lysis buffer [50 mM potassium phosphate (pH 8.0), 500 mM NaCl, 30 mM imidazole, 3 mM β-mercaptoethanol (βME), and 1 mM phenylmethylsulfonyl fluoride], and lysed with a cell disruptor (Constant Systems). The lysate was cleared by centrifugation at 10,000g for 20 min. The soluble fraction was incubated with Ni-Sepharose 6 Fast Flow beads (GE Healthcare) for 30 min.
Crystallization conditions
Crystals were grown at 18°C by hanging drop vapor diffusion with 1 M Sestrin2 (5 mg/ml) mixed with an equal volume of reservoir solution containing 100 mM MES (pH 6.0), 1.2 M disodium malonate, and 1% (v/v) Jeffamine ED-2001. Drops were microseeded to obtain well-diffracting crystals. Microseed stock (0.2 μl) of crystals was then added to the 2-μl hanging drop described above (for a total final dilution of 1:2,500,000). Crystals were equilibrated in running buffer [10 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, and 1 mM DTT].

Data collection and structure determination
Data collection was performed at the Advanced Photon Source end station 24-IDC at the Argonne National Laboratory. All data processing steps were carried out with programs provided through SBGrid (17). Data reduction was performed with HKL-2000 (18). The phase problem was solved by molecular replacement using our published Sestrin2 structure (5DJ4) as the search model in the Phaser-MR program, run as part of the Phenix Autosol program (space group I23; five molecules per asymmetric unit) (19). To reduce potential model bias, we used a search model lacking the bound leucine or malonate. The resulting model was further refined by size-exchange chromatography on an SP Sepharose fast flow column (GE Healthcare) with a linear NaCl gradient. The protein was further purified by size-exchange chromatography on a Superdex S200 16/60 column (GE Healthcare) equilibrated in running buffer [10 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, and 1 mM DTT].

Data and materials availability: Coordinates and individual B-factors were refined over three cycles. All structure figures were made in PyMOL (21).

REFERENCES AND NOTES

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