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The binding stoichiometry of CIN85 SH3 domain A and Cbl-b

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To the editor

Receptor tyrosine kinase (RTK) activation results in the initiation of several crucial cellular biological processes, including proliferation, adhesion and apoptosis¹. However, for normal cell function, RTK activity must be tightly regulated and controlled either by its inhibition or through endocytic trafficking and degradation². An important adaptor protein that has a key role in the latter process is the human Cbl-interacting protein of 85 kDa, CIN85 (also known as SETA, Ruk or SH3KBP1)³. The main known function of CIN85 is to downregulate RTKs and facilitate endocytosis by interacting with the endophilin-associated ubiquitin ligase Cbl proteins^{3,4}. Complex formation between CIN85 and Cbl proteins takes place through CIN85's three SH3 domains (referred to as CIN85A, B and C). The interaction is mediated via atypical proline-arginine motifs of the form PXXXPR (where X is any residue) located in the C-terminal region of Cbl proteins⁵⁻⁷. *In vitro* studies of the binding of either isolated or linked CIN85 SH3 domains provide an important insight into the mechanism of recognition and recruitment of Cbl proteins to these domains. Furthermore, because CIN85 exists as a dimer and/or tetramer *in vivo* (based on the N-terminal coiled-coil interactions between monomers)⁸, *in vitro* studies provide an understanding of the implications of such oligomerization states of the protein in achieving its function and, in particular, its interaction with Cbl proteins. The existence of such oligomeric states of the protein are likely to be important in the function of CIN85 for several reasons, for example, in ensuring high local concentration of the protein, in maximizing the clustering of Cbl proteins via formation of multimeric complexes, or simply for the folding and stability of the protein under conditions of cell stress.

A recent study by Jozic *et al.*⁹ reported an X-ray crystal structure that showed that the binding of CIN85A to a PXXXPR-type peptide from Cbl-b protein (with the sequence SQAPARPPKPRPRRTAY corresponding to residues 899–915 of Cbl, henceforth referred to as Cbl-b) results in a heterotrimeric complex formed between two of these SH3 domains and one peptide. Using isothermal titration calorimetry (ITC), the authors confirmed the stoichiometry seen in the crystal structure. Immunoprecipitation experiments using affinity-tagged CIN85 revealed the presence of possible trimeric complexes that incorporated an engineered monomeric Cbl protein and CIN85.

The Cbl-b peptide contains a pseudo-symmetrical binding motif (RPPKPRPR) that seems to be important in sustaining the heterotrimeric complex. However, close inspection of the X-ray structure (Fig. 1a) reveals that the two SH3 monomers, A and B, bind with clear

differences. For example, Asn51 in monomer A forms a hydrogen bond with the Lys907 backbone oxygen in Cbl-b, whereas Asn51 in monomer B does not form a similar hydrogen bond. Also, using proton-proton distances ($\sim 4.5 \text{ \AA}$) between the monomers and Cbl-b as a measure of the interface proximity, we find that nearly 20% of such distances are absent between monomer B and Cbl-b (mainly at the N-terminal residues of Cbl-b). These differences suggest that the binding of Cbl-b to CIN85A may have a preference for one orientation over the other. This observation and the apparent failure to observe the expected one-to-one complex on saturation of CIN85A titrated with peptide9 prompted us to investigate the complex formation in solution using both ITC and high-resolution NMR spectroscopy.

ITC experiments of CIN85A titrated with Cbl-b under the same conditions as previously used (except the experimental temperature was 25°C rather than 18°C)⁹ result in a stoichiometry of $n = 0.9 (\pm 0.2)$ and $K_d = 9.9 (\pm 3.8) \mu\text{M}$ (Fig. 1b). These data are consistent with previously reported 1:1 complex formation⁵.

Figure 2 shows the overlaying of ^1H - ^{15}N HSQC spectra of CIN85A in its apo form and in complex with Cbl-b. Inspection of the spectrum shows no sign of the two binding interfaces of a heterotrimer complex, such as new cross-peaks, signal doubling or dramatic intensity changes. Instead, the signals simply shift to their final position in the complex form (see selected residues from the binding site of CIN85A; Fig. 2). To verify further the stoichiometry of the complexes formed by CIN85A and Cbl-b in solution, the rotational correlation times (τ_C) of the apo and complex forms of CIN85A have been determined using NMR relaxation as previously reported¹⁰. The τ_C values of free and bound SH3 domain were $6.3 (\pm 0.3) \text{ ns}$ and $8.6 (\pm 0.2) \text{ ns}$, respectively. Taking into account the anisotropic nature of the complex due to the disordered termini of both the domain and/or the peptide, the τ_C values provide strong evidence that the CIN85A–Cbl-b complex is formed from monomeric species binding with a 1:1 stoichiometry.

Under the crystallization conditions reported⁹, a heterotrimer is clearly formed. In contrast, here we provide evidence that in solution CIN85A forms a heterodimeric complex with Cbl-b. From a thermodynamic perspective, the heterotrimeric complex could form under low relative concentrations of Cbl-b. However, as the peptide concentration is increased, the homodimer would prevail. We see no evidence for this two-state binding. If the heterotrimeric complex does exist in solution, it is likely to be only as an intermediate at low peptide concentrations, and as there is no apparent change of signals for the formation of the two SH3-peptide interfaces in the NMR spectrum, the population of this complex is always likely to be low ($\sim 5\%$). Thus, we conclude that the heterotrimeric complex of CIN85A and Cbl-b observed in the X-ray crystal structure is likely to be the consequence of the crystallization process trapping the two SH3 domains bound to the pseudo-symmetrical Cbl-b. Finally, our results suggest that there is no physiological relevance to the heterotrimer formation; oligomerization of CIN85 via interaction with Cbl-b *in vivo* cannot be responsible for higher-order complex clustering, as recently reported⁹. Instead, the formation of dimers with CIN85 SH3 domains improves the efficiency of recruitment of the ubiquitin ligase.

Supplementary Material

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Figure 1.

(a) X-ray structure of CIN85A–Cbl-b heterotrimeric complex. The pseudo-symmetry of Cbl-b peptide is shown by the opposite pairs Arg904-Pro905 and Pro910-Arg911 binding to the acidic pocket at the RT loop of either monomer A or B of CIN85A. Note the absence of a hydrogen bond between Asn51 in monomer B and the Lys907 backbone oxygen of the Cbl-b peptide (PDB 2BZ8). (b) ITC titration data for CIN85A with Cbl-b (see Supplementary Methods for more details).

Figure 2.

Overlaid ^1H - ^{15}N HSQC spectra of apo-CIN85A (blue) and CIN85A in complex with Cbl-b peptide (red). Residues shown are located in the binding site of CIN85A. NMR data were collected with ~ 0.6 mM protein samples containing 20 mM phosphate buffer at pH 6.3, 100 mM NaCl, 2 mM DTT, 1 mM EDTA and 0.01% NaN_3 (see Supplementary Methods for more details).