Mapping the allosteric network within a SH3 domain

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SH3 domains are very abundant protein-protein interactions modules, involved in the regulation of several cellular processes. Whilst they have been associated to allosteric communication pathways between contiguous domains in multi-domain proteins, there is lack of information regarding the intra-domain allosteric cross-talk within the SH3 moiety. Here we scrutinize the presence of an allosteric network in the C-terminal SH3 domain of Grb2 protein, upon binding the Grb2-associated binding 2 protein. To explore allostery, we performed double mutant cycle analysis, a powerful quantitative approach based on mutagenesis in conjunction with kinetic experiments. Data reveal the presence of an unexpected allosteric sparse network that modulates the affinity between the SH3 domain and its physiological partner.

SRC homology 3 (SH3) domains are among the most abundant protein-protein interaction (PPI) modules, found in a large amount of human proteins and implicated in the control of critical cellular processes, such as cell scaffolding, signal transduction, cytoskeletal modification and proliferation. From a structural point of view, the SH3 domains comprise of approximately 60 amino acid residues and display a characteristic β-barrel fold formed by five or six β-strands arranged as two antiparallel β-sheets. Generally, these adapter domains recognize substrates with proline-rich motifs typically organized in a polyproline type II (PPII) helix; the interactions are highly specific and promote the assemblage of the molecular complexes involved in signaling transduction. Furthermore, SH3 domains are also able to regulate the catalytic activity or the binding site accessibility of the proteins containing them.

Despite SH3 domains share a highly conserved structure and a relatively simple binding pocket, they are nevertheless very specific. Among the numerous potential proline-rich binding targets, the distinct role of each SH3 domain in the crowded cellular environment demands precise interactions, while avoiding potentially harmful non-specific reactions. One possible strategy to achieve such specificity is to modulate binding affinity through allosteric effects, whereby residues far from the binding pocket may tune the energetics of binding to a specific ligand.

Whilst allosteric effects in SH3 domains have been previously explored to investigate the structural communication between contiguous domains in complex multidomain proteins, there is lack of information regarding the intra-domain allosteric cross-talk within the SH3 moiety. One of the most powerful methods to describe intra-domain allostery is the measurement of inter-residue energetic communication upon binding through the so-called double mutants cycle analysis. This experimental approach provides a quantitative measurement of the energetic coupling between distal residues which are involved in the binding reaction.

By employing double mutant cycles, we provide here the first demonstration of an allosteric network within an SH3 domain. In particular, we investigated the energetic coupling upon binding between the C-terminal SH3 domain of the adapter protein Grb2 and the intrinsically disordered protein Gab2. The experimental results allowed us to map the presence of an allosteric network that might be implicated in balancing the selectivity of this domain for its physiological partners.

Results and Discussion
Double mutant cycles as a tool to investigate protein allostery. The classical view of protein allostery relies on a detectable conformational change, regulating the functions of the active site(s). However, allosteric modulation of macromolecule might also be more elusive and allostery might be at play even in the absence of a prominent conformational modification, involving either dynamic or long range interactions modulating amyloidogenic effects. In these cases, the characterization of the allosteric...
A powerful method to investigate protein allostery is represented by the measurement of the energetic coupling between the ligand and residues of the protein that are spatially distant from the binding site. In fact, rather than observing detectable conformational changes, it is possible to define and quantify interaction networks that modulate the allosteric communication within the protein moiety. The quantification of these allosteric connections allows to pinpoint the structural elements critical for the binding processes and, therefore, to obtain a description in energetic terms of such structural communication.

Double mutant cycle analysis is a mighty method based on mutagenesis and measurements of binding free energies, providing a quantitative description of the allosteric energetic connections upon binding. The experimental approach can be described by considering a system $P-AB$ containing two residues under investigation $A$ and $B$. The measurement of the energetic contributions of these residues to a given reaction might be explored by producing the deletion mutants $P-A$, where $B$ is deleted, $P-B$, where $A$ is deleted, and $P$, where both residues are deleted. Quantitatively, the changes in free energy for single mutants at positions $A$ and $B$ can be then calculated assuming the following equations:

$$
\Delta \Delta G_{P-AB} \rightarrow P \rightarrow B = \Delta G_{P-AB} - \Delta G_{P-B}
$$

$$
\Delta \Delta G_{P-AB} \rightarrow P \rightarrow A = \Delta G_{P-AB} - \Delta G_{P-A}
$$

$$
\Delta \Delta G_{P-AB} \rightarrow P = \Delta G_{P-AB} - \Delta G_{P}
$$

By applying a squared thermodynamic cycle, it may be noted that the change in energy upon deleting both $A$ and $B$ ($\Delta \Delta G_{P-AB} \rightarrow P \rightarrow B$) should equal the sum of the change in energies for the single mutants ($\Delta \Delta G_{P-AB} \rightarrow P \rightarrow B + \Delta \Delta G_{P-AB} \rightarrow P \rightarrow A$), if $A$ and $B$ do not interact energetically. The coupling energy $\Delta \Delta \Delta G$ can be derived subtracting the effects of the single mutants to that of the corresponding double mutant:

$$
\Delta \Delta \Delta G = \Delta \Delta G_{P-AB} \rightarrow P \rightarrow B - \Delta \Delta G_{P-AB} \rightarrow P \rightarrow A
$$

The $\Delta \Delta \Delta G$ measures the energetic strength of the interaction between positions $A$ and $B$ and when its value is different from zero represents the signature for the two residues to interact energetically.

In the case of protein-protein interaction, double mutant cycles can be promptly applied to measure the functional role of residues that are not physically located in the binding site of a protein. In fact, by considering the example described above, if the residue $A$ is located in one of the interacting protein and $B$ in the other, the energetic coupling between $A$ and $B$ can be associated to the strength of their interaction, independently on whether they physically interact in the complex. For these reasons, this methodology represents an ideal technique to monitor protein allostery quantitatively.

### Experimental set up to investigate allostery on SH3 by double mutant cycles.

In order to investigate the allosteric network in a SH3 domain we resorted to perform a double mutant cycle analysis of the C-terminal SH3 domain of Grb2 and a peptide mimicking its physiological ligand Gab2, denoted as Gab2503–524. In analogy to a typical ligand for an SH3 domain, Gab2503–524 corresponds to a proline rich sequences that undergoes a disorder to order transition upon binding, forming a polypeptide type II helix. As briefly recalled above, the double mutant cycle methodology relies on the measurement of changes in binding free energy upon mutation ($\Delta \Delta G$). When perturbing positions that are directly involved in binding, it is therefore of critical importance to design the mutants carefully and focus on those positions that display a $\Delta \Delta G$ that is high enough to be experimentally determined but low enough not to perturb binding. In fact, if a reference mutation destabilizes the complex too much, the propagation of the associated errors may jeopardize the quantitative analysis of the corresponding $\Delta \Delta \Delta G$, see for example ref 28. In the case of Gab2503-524, it was recently shown that mutations P510A and P512A display a detectable effect in the stabilization of the complex, whilst the other amino acids involved in binding appear to display a dramatic effect (with mutations of P511A, R515A, K518A and P519A yielding to a very pronounced change in $K_d$)26,27. In an effort to detect the presence of allosteric networks in a SH3 domain, we therefore subjected the C-terminal SH3 domain of Grb2 to extensive site directed mutagenesis and challenged 18 variants of with (i) WT Gab2503–524, (ii) P510A Gab2503–524 and (iii) P512A Gab2503–524. In analogy to what previously discussed in protein folding studies, the mutants were designed following standard procedures, which has been extensively discussed previously by Fersht and co-workers26. In turn, each mutant was constructed to introduce a small deletion of the side-chain without changing the steric properties of the residues. Importantly, previous detailed investigation on the folding and stability of these variants of SH3 revealed that the all well-folded stable proteins displaying a similar (un)folding co-operativity compared to wild type SH329, confirming that none of the mutations had a relevant effect on the structure of the domain.

We carried out pseudo-first-order binding experiments at 10 °C using a stopped-flow apparatus and mixing a constant concentration of C-SH3 Grb2 (0.5 μM) with increasing concentrations of Gab2503–524 (2–12 μM). As described previously, the binding reaction was monitored following changes in fluorescence upon binding of Trp35 and Trp36 residues of C-SH3 Grb2. The resulting kinetic trace was fitted to a single exponential function to obtain the observed rate constant $k_{obs}$. Under all the investigated conditions, observed kinetics were consistent with a single-exponential behavior, suggesting the lack of accumulation of intermediates. Figure 1 compares the kinetic data obtained for the binding of wild type C-SH3 Grb2 and its site-directed mutants to WT Gab2503–524, P510A Gab2503–524 and P512A Gab2503–524 respectively. It is evident that, in all cases, a plot of the observed rate constant ($k_{obs}$) as function of Gab2503–524 concentration is consistent with a linear behaviour, further supporting a...
two-state mechanism at the explored experimental condition. By applying the pseudo-first order approximation, the association and dissociation rate constants were obtained from the dependence of the observed rate constant on [Gab2$_{503-524}$].
Interestingly, several mutations displayed a detectable change in $K_D$, despite the position being distant from the binding pocket (Table 1). For example, mutation T53S showed an increase in about 10 fold in $K_D$ when challenged with P512A Gab2$_{303-524}$, despite not being in direct contact with the peptide. This finding suggests the presence of an allosteric behaviour in C-SH3 Grb2, which cannot be rationalized in a simple manner from a structural point of view and demands careful investigation by taking advantage of coupling energies, as detailed below.

### Mapping allosteric communication on the structure of the SH3 domain.

The coupling free energies ($\Delta \Delta G$) of each mutated residue of the C-SH3 domain with both prolines (ProS10 and ProS12) of Gab2$_{303-524}$ were then quantified using the experimental association ($k_{on}$) and dissociation ($k_{off}$) rate constants obtained by kinetics (Table 1). Interestingly, we found that six residues (Val3, Leu6, Ser31, Ala39, Thr53 and Val55) showed a detectable $\Delta \Delta G$ upon mutation and binding with Gab2 P510A, i.e. with a value of $\Delta \Delta G > 0.4$ kcal mol$^{-1}$. More to the point, four of these residues, Leu6, Ser31, Ala39 and Thr53, were also found energetically coupled with the ProS12, with a $\Delta \Delta G$ of 0.6 ± 0.2, 0.4 ± 0.1, 0.9 ± 0.1 and 1.0 ± 0.1 kcal mol$^{-1}$ respectively. Upon mutation of residues Phe7, Phe19 and Tyr51 the binding reaction does not take place, suggesting that these residues play a key role of these amino acids in such reaction.

It is of interest to study the structural distribution of the residues displaying a detectable $\Delta \Delta G$ or a pronounced effect on binding (Fig. 2). In fact, whilst Phe9 and Tyr51 are physically located in the binding pocket, Val3, Leu6, Ser31, Ala39, Thr53 and Val55 do not engage any contact with Gab2$_{303-524}$. Because none of these residue is directly located in the binding pocket of C-SH3, it may be concluded that they play an allosteric role in finding parallels earlier work on PDZ domains \cite{27}, where the presence of a sparse network could not be rationalized from a structural point of view and demands careful investigation by taking advantage of coupling energies, as detailed below.

### Allosterity and selectivity – on the significance of positive and negative $\Delta \Delta G$ values.

One of the plausible mechanisms whereby protein domain selectivity is achieved is to account for long range allosteric networks\cite{2}. In fact, when and if an adaptor domain family displays a highly conserved structure and a relatively simple binding pocket, the fine-tuning of its selectivity may be achieved by long range interactions that are modulated by residues that are physically distant from the binding pocket. As previously shown on PDZ domains\cite{29}, a characteristic signature of such scenario is represented by the positive sign in the $\Delta \Delta G$ values. In other words, if a mutation of core residue in the protein will affect the binding of the peptide such that the effect of a perturbation in the peptide will be smaller in the mutant as compared to the wild type protein, it follows that the sequence of the wild type is optimized to bind the wild type sequence of the peptide. On the contrary of what previously observed for PDZ domains\cite{29}, the calculated values reported in Table 1 do not show a clear prevalence of positive $\Delta \Delta G$ values. We speculate that this arises from the complex role of the C-SH3 domain, which is involved in several cellular pathways and has been suggested to interact with different ligands\cite{31-33}.
Figure 2. Cartoon representation of the complex between the C-SH3 Grb2 and Gab2503–524 (drawn in Pymol; PDB code: 2VWF). C-SH3 Grb2 domain is represented in black, Gab2503–524 in gray while mutated residues are represented as spheres on the structure of the complex. The energetic coupling between the prolines of Gab2 in pos 510 (left; yellow sphere) and 512 (right; yellow sphere) with the residues of C-SH3 Grb2 is highlighted as magenta spheres in case of negative values of ΔΔG and as red spheres in case of positive values of ΔΔG. Blue spheres highlight mutations that abrogate binding.

Conclusions
The allosteric regulation of protein is a general mechanism whereby physically distant residues regulate the functionality of the active site through conformational changes, dynamic or chronosteric effects. In the case of SH3 domains, whilst they have been often associated to allosteric pathways, the intra-domain communication within residues has been poorly explored to date. The employment of extensive site-directed mutagenesis and double mutants cycles allowed us to map an allosteric sparse network that regulates the recognition between an SH3 domain and a peptide mimicking its physiological partner. Future work on other SH3 domains will further clarify the importance and generality of this network.

Materials and Methods

C-SH3 Grb2 wild-type and its site-directed mutants were produced as previously reported30. Kinetic experiments of binding were performed on a single-mixing SX-18 stopped-flow instrument (Applied Photophysics), recording the change of fluorescence emission. The excitation wavelength used was 280 nm while the fluorescence emission was collected using a 320-nm cut-off glass filter. The binding experiments were carried out at 10°C in pseudo-first order condition mixing a constant concentration of C-SH3 Grb2 in the wild-type and mutated forms (0.5 μM) versus increasing concentrations of Gab2503–524 wild-type and its mutants P510A and P512A (ranging from 1 to 12 μM). For all measurements the buffer used was 50 mM HEPES, 0.5 M NaCl, pH 7.0. The observed rate constants (k_on) were calculated from the average of 3–6 single traces and by fitting of the time-course for binding using a single exponential equation.

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**Author Contributions**

S.G. designed research; F.M., F.T., D.B. and A.T. performed experiments; all the authors analysed data; F.M. and S.G. wrote the first version of the manuscript and all the authors revised the manuscript.

**Additional Information**

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