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Electrostatic Interactions Mediate Binding of Obscurin to Small Ankyrin 1: Biochemical and Molecular Modeling Studies

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Abstract

Small ankyrin 1 (sAnk1; also Ank1.5) is an integral protein of the sarcoplasmic reticulum in skeletal and cardiac muscle cells, where it is thought to bind to the C-terminal region of obscurin, a large modular protein that surrounds the contractile apparatus. Using fusion proteins *in vitro*, in combination with site directed mutagenesis and surface plasmon resonance measurements, we previously showed that the binding site on sAnk1 for obscurin consists in part of six lysine and arginine residues. Here we show that four charged residues in the high affinity binding site on obscurin for sAnk1, between residues 6316–6345, consisting of three glutamates and a lysine, are necessary, but not sufficient, for this site on obscurin to bind with high affinity to sAnk1. We also identify specific complementary mutations in sAnk1 that can partially or completely compensate for the changes in binding caused by charge-switching mutations in obscurin. We used molecular modeling to develop structural models of residues 6322–6339 of obscurin bound to sAnk1. The models, based on a combination of Brownian and molecular dynamics simulations, predict that the binding site on sAnk1 for obscurin is organized as two ankyrin-like repeats, with the last α -helical segment oriented at an angle to the nearby helices, allowing lysine-6338 of obscurin to form an ionic interaction with aspartate-111 of sAnk1. This prediction was validated by double mutant cycle experiments. Our results are consistent with a model in which electrostatic interactions between specific pairs of side chains on obscurin and sAnk1 promote binding and complex formation.

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Supplementary Data Domain structure and sequence of obscurin and interacting regions with sAnk1, SPR traces, RMSD of sAnk1₅₇₋₁₂₂ in an aqueous solution with respect to the initial homology model, cluster transition diagram of 4-cluster model of sAnk1₅₇₋₁₂₂, RMSD of backbone atoms in Obsc₆₃₂₄₋₆₃₃₈, cluster transition diagram of 2-cluster model and rapid local bending of Obsc₆₃₂₄₋₆₃₃₈, and time-series and distribution of distance between Obscurin Lys 6338 and sAnk1 Asp 111 are provided in supplementary data. Characteristic distributions of distance between ionic side chain interactions (Asp/Glu-Arg and Asp/Glu-Lys) are shown. Schematic diagram of BD simulation is also provided.

Keywords

Small Ankyrin 1; Obscurin; protein-protein interaction; molecular dynamics simulation; Brownian dynamics simulation

Introduction

Small ankyrin 1 (sAnk1) is a small, integral membrane protein encoded by the *ANK1* gene.^{1, 2} *ANK1* typically encodes a large (1880 amino acids), canonical ankyrin, known as ankyrin R or ankyrin 1, which links the spectrin cytoskeleton to integral membrane proteins in erythrocytes, striated muscle, epithelia, and neurons.^{3, 4, 5} The sAnk1 isoform is one of five small muscle-specific isoforms^{2, 6, 7} with an alternative start codon, produced by alternative splicing, that concentrates in the sarcoplasmic reticulum (SR) and that is likely required for its formation and stability.⁸

sAnk1 (or Ank1.5, as it is also known) is comprised of three distinct regions. The N-terminal region is composed of 29 amino acids, the majority of which are hydrophobic, that anchor the protein to the membrane of the sarcoplasmic reticulum.⁹ The following 44 amino acids, once considered unique to the small ankyrin isoforms, in fact share identifiable sequence homology with a variety of cell cycle regulatory factors, such as TNIP2, cdc42bpa, and the penultimate ankyrin repeat of tankyrase. This region also shares some exonal structure with RFX-B, a protein that contains ankyrin repeats. The next 82 amino acids share 100 percent identity with the far C-terminus of isoform 3 of ankyrin R. Although neither is algorithmically predicted, Borzok, et al.,¹ used homology modeling based on human Notch 1 to model both the unique and the conserved regions of sAnk1 as ankyrin-like repeats. Experimentally verified examples of non-algorithmically predicted ankyrin-like repeat domains can be found in the Protein Data Bank,¹⁰ with the terminal flanking repeats of p53BP2 (1YCS) providing a notable example.¹¹ Despite numerous attempts, we have not been able to obtain crystals of the ankyrin-like repeats of sAnk1, or sufficient concentrations in solution, to confirm this prediction in structural studies. We have therefore used molecular modeling to test this prediction more rigorously and to show that this region is indeed organized as two modified ankyrin-like repeats.

The major ligand identified thus far for sAnk1 in striated muscle is obscurin A, a large, ~720 kDa modular protein encoded by the *OBSCN* gene, which has two different but nearby binding sites for sAnk1 in its C-terminal, non-modular region.^{12, 13} Obscurin A is one of two giant proteins encoded by the *OBSCN* gene; the other, obscurin B, is larger due to the presence of C-terminal protein kinase domains that are alternatively spliced, replacing the binding sites for sAnk1^{14, 15}. The A isoform is the more prevalent in both cardiomyocytes and skeletal muscle¹⁶, where it can interact with proteins of the contractile apparatus,^{17, 18, 19, 20, 21, 22, 23} signaling proteins^{24, 25, 26, 27} and members of the ankyrin superfamily.^{1, 12, 13, 28, 29, 30} Its interactions with these proteins allow obscurin A to associate with and apparently encircle the contractile apparatus at the level of the M-band, with its C-terminal region extended towards the SR, which it approaches within molecular distances.¹⁶

Here we focus on the binding of obscurin A to sAnk1. We have previously compared the two known binding sites for sAnk1 in the C-terminal region of obscurin A.³¹ Both contain short stretches of ~30 amino acids each or less, are separated by only 51 amino acid residues, and bind to sAnk1 with affinities in the submicromolar range. We found that the more C-terminal site, Obsc₆₃₁₆₋₆₃₄₅, binds with several fold higher affinity to sAnk1 than the second site, located at residues 6231-6260.³¹

The central region of the high affinity site in obscurin is rich in glutamate residues. This observation and our previous finding, that positively charged residues on sAnk1 are necessary for binding to this region of obscurin,¹ suggest that at least some of the glutamate residues are necessary for binding, and that particular glutamate side chains form ionic bonds with specific lysine or arginine residues exposed on the surface of sAnk1. We test this idea through site-directed mutagenesis studies and double mutant cycle experiments that identify specific K- or R-to-E mutations in sAnk1 that restore binding that is lost as a result of specific E-to-K mutations of obscurin. To facilitate interpretation of our experimental results, we used Brownian and conventional molecular dynamics simulations to develop structural models of the binding region of obscurin docked to the ankyrin-like repeats of sAnk1. The resulting models are consistent with our experimental data. In addition, our models predict the presence of an additional specific ionic interaction between K6338 of obscurin and D111 of sAnk1, a prediction that we verified by double mutant cycle experiments.

Results

We previously demonstrated³¹ that residues 6316-6345 of obscurin isoform A are necessary and sufficient to mediate the binding of the C-terminal region of obscurin to sAnk1. Borzok et al.¹ demonstrated that this binding is mediated at least in part by a set of 3 positively charged residues exposed on the surface of the ankyrin-like repeat domains of sAnk1 (Supplemental Figure S1C, green). We hypothesized that many of these K and R residues of sAnk1 interact in a specific manner with the three glutamate residues (E6327, E6329, E6330) in the high affinity binding site on obscurin (Supplemental Figure S1B, orange).

To test the hypothesis that the glutamate residues of Obsc₆₃₁₆₋₆₃₄₅ are involved in binding to sAnk1, we mutated each of these residues to alanine, both individually and simultaneously. We then assayed peak binding of each construct to an MBP fusion protein of sAnk1₂₉₋₁₅₅ by both SPR and far Western blot (blot overlay). Our results demonstrated that individual mutations of each of these glutamates to alanines reduced binding of GST-Obsc₆₃₁₆₋₆₄₃₆ to ~40% of control levels (Figure 1A, bars 1-4). We were not able to assay binding of the E6330A to sAnk1, however, due to the insolubility of the MBP fusion protein carrying this sequence. We then made multiple mutations of these glutamates, and found that double mutations of the two N-terminal glutamates to alanines reduced binding further, to ~10% of control, and that mutation of an additional glutamate residue reduced binding to ~3% of control. Mutation of all glutamate residues eliminated binding almost completely (Figure 1A, bars 5-7). Blot overlays that confirm these results are shown in Figure 1B. These results indicate that the glutamate residues in the Obsc₆₃₁₆₋₆₃₄₅ sequence are necessary for binding to sAnk1.

We hypothesized that these residues were directly involved in specific interactions with the positively charged residues on the sAnk1 molecule, previously shown to be involved in binding.¹ To test this further, we also mutated glutamates E6327, E6329 and E6330 and E6333 individually to lysines. Figure 2 shows that, with the exception of E6333K, there is a trend of these E-to-K mutations to inhibit binding to a greater extent than the corresponding E-to-A mutations, and E6330K shows a marked decrease in binding in both grouped SPR data (Figure 2A) and blot overlay experiments (Figure 2B). Representative SPR traces of individual data points used to calculate the results in Figure 2A are shown in supplementary Figure S2. Collectively, these results indicate that electrostatic interactions involving glutamate residues in Obsc₆₃₁₆₋₆₃₄₅ mediate binding of this region of obscurin to sAnk1.

The results presented above indicate that ionic residues of opposite charges on obscurin and sAnk1 mediate binding, but they do not show if these ionic interactions are non-specific or if

binding involves specific interactions of pairs of residues. We addressed this in double mutant cycle experiments, which have been used previously to determine the residues of a protein or peptide involved in binding to a specific target.³² In these experiments, the residue in question on one peptide or protein is converted to a residue with the same charge as the hypothesized partner residue on the peptide or protein to which it binds. Similarly, residues of the second protein are converted individually to residues of the opposite charge. Binding of each set of mutant pairs is then assayed and compared to the binding of wild type fusion proteins and to the binding of each individual mutant to the wild type of the paired protein. To fulfill the criteria for complementarity, binding of a particular mutant of one protein to its complementary mutant must show higher avidity than to the wild type protein. Furthermore, this effect must be specific, i.e., other mutants of the partner must not show increased binding. Applying this approach provided strong evidence that binding of Obsc₆₃₁₆₋₆₃₄₅ to sAnk1 involves the specific interactions of several pairs of amino acid residues of opposite charge.

When we screened all of the E-to-K mutants of obscurin against the K-to-E or R-to-E mutants of sAnk1 that affect binding¹, we found 3 specific pairs of mutants that showed increased binding compared to each mutant alone (Figure 3): ObscE6329K and sAnk1-K105E (panel 3A, final hatched bar), ObscE6330K and sAnk1-R104E (panel 3B, final hatched bar), and ObscE6327K and sAnk1-R69E (panel 3C, final hatched bar). These effects were specific, and not simply due to a loss of electrostatic repulsion or steric hindrance, as alanine mutants of each (with the exception of E6330A, which is insoluble: see above) showed poorer binding than the corresponding charge-switched residue (Obscurin mutants, grey bars; sAnk1 mutants, not shown). All data were normalized to binding of WT sAnk1 to WT Obsc₆₃₁₆₋₆₃₄₅, acquired the same day. These results indicate that the effects of particular K- or R-to-E mutations of sAnk1, and of particular E-to-K mutations of obscurin, are specific. They are consistent with the hypothesis that specific ionic interactions between the glutamate residues of obscurin and the lysine and arginine residues of sAnk1 mediate binding.

Modeling of sAnk1, its binding region on obscurin, and their complex

We used MD and BD simulations to obtain a molecular model of the interaction of sAnk1 with its high affinity binding site on obscurin. To facilitate our calculations, we limited our analysis to the two putative ankyrin-like repeat domains of sAnk1 (sAnk1₅₇₋₁₂₂) and to the central region of Obsc₆₃₂₂₋₆₃₃₉, where its essential glutamates are located. We first focused on each of these polypeptides alone, to obtain stable structures via MD simulations. The resulting structures were then used in BD simulations, followed by MD simulations, to generate molecular models of the obscurin-sAnk1 complex. The final models were then compared with the double mutant cycle experiments, to select optimal models.

The homology model of sAnk1₅₇₋₁₂₂ developed by Borzok, et al.,¹ was subjected to a 30 ns MD simulation to assess the stability of the model and then to identify the most likely conformations assumed by this region of sAnk1 in aqueous solution. Analysis of the MD simulation showed an initial relaxation of the structure away from the initial homology model during the initial 10 ns, following which the structure fluctuated in the vicinity of 8 Å from the initial model (see Figure S3A of supplementary material). Clustering based on root-mean-square (RMS) differences³³ showed the presence of 4 clusters of conformations. Analysis of the cluster transitions (see Figure S4 of supplementary material) showed one cluster to be associated with the original homology model; the remaining three clusters are sampled during the final 20 ns of simulation time. Notably, cluster two, which is most similar to cluster one, is sampled again towards the end of the 30 ns MD simulation, indicating that sAnk1₅₇₋₁₂₂ is sampling a range of conformations. Representative conformations from the three sampled clusters are shown in Figure 4. Visual analysis of the

structures in Figure 4 shows all three to maintain the 4 helical core associated with the two ankyrin-like repeats in the previously published homology model. This is supported by RMS differences for the backbone atoms in the helical regions of the four cluster structures, which are 2.9, 3.2, 3.4 and 3.2 Å, compared to the published homology model. Of those helices, the biggest structural differences occurred in the second helix of the second ankyrin-like repeat, including residues 104-114, which projects from the planes of the preceding helices (Figure 4D, arrow). Although this change may be an artifact of only having modeled residues 57-122, and therefore omitting a terminal structure to “cap” the second ankyrin repeat, two ankyrin repeats alone can be stable,³⁴ and crystal structures of several ankyrin repeat proteins^{11,35} show similar projections of the terminal helix relative to the preceding helices. Thus, although the structure calculated by MD simulation of sAnk1₅₇₋₁₂₂ differs slightly from the model based on homology to Notch 1, the two models are quite similar, overall. The present calculations therefore confirm the earlier prediction that the positively charged residues shown to mediate binding to obscurin, R69, R104 and K105, are on the same surface of sAnk1 (Figure 4D), where they are all exposed to the solvent. MD simulations suggest that this same surface contains a solvent-exposed aspartate residue, D111, which, we show below, is also involved in binding sAnk1 to obscurin.

We also performed MD simulations on the central region of Obsc₆₃₁₆₋₆₃₄₅, specifically Obsc₆₃₂₂₋₆₃₃₉, the segment containing the glutamate and lysine residues involved in binding. The initial model for the simulations was an ideal α -helix with all the side chains in fully extended conformations. During the 30 ns simulation the structure stayed primarily helical with the largest structural change being local unwinding of the N- and C-terminal turns of the helix (see Figure S5 C, D, of supplementary material). In addition, local, rapid bending of the helix was observed (see Figure S6 of supplementary material).

The complex between Obsc₆₃₂₂₋₆₃₃₉ and sAnk1₅₇₋₁₂₂ was modeled using 10,000 BD simulations, followed by the minimization of the bound complexes, with solvation based on the Generalized-Born model. Docking was performed using the representative structures of Obsc₆₃₂₂₋₆₃₃₉ (Cluster 2, see Figure S5D of supplementary material) and sAnk1₅₇₋₁₂₂ (Cluster 4, Figure 4C) from the MD simulations. Of the 10⁴ BD simulations, 3122 diffused to within 30 Å of the center of mass of sAnk1 (Figure 5A). Each of those structures was energy minimized, yielding 326 pairs with an interaction energy less than -50 kcal/mol (Figure 5B). These were then clustered based on the spatial position using the neural network-based clustering algorithm ART-2^{36, 37} in CHARMM,³⁸ from which 16 clusters were obtained. Ten of these clusters were eliminated because they contained 10 or fewer members, yielding 6 clusters. Representative structures from each cluster and their orientations relative to sAnk1 are shown in Figure 5C. The structures are all located around the same region of sAnk1 containing R69, R104, and K105, in agreement with experimental results indicating that these residues interact with Obsc₆₃₁₅₋₆₃₄₅.

Further refinement of the six models of the Obsc₆₃₂₂₋₆₃₃₉ - sAnk1₅₇₋₁₂₂ complex was performed using 30 ns MD simulations in aqueous solution. During these simulations, the obscurin peptide dissociated from the ankyrin-like repeats of sAnk1 in models 3 and 7. The remaining 4 models were then analyzed to identify those that were consistent with the results of our double mutant cycle studies, as judged by the presence of the following pairwise, residue-specific interactions; sAnk1-K105 to Obsc-E6329, sAnk1-R104 to Obsc-E6330 and sAnk1-R69 to Obsc-E6327 (see above). Two of the models, 2 and 10, had such an interaction pattern. MD simulations of both models were further extended to 70 ns. The two models are shown in Figure 6A and 6B, with residues identified as involved in binding by double mutant cycle experiments indicated by yellow arrows. Time series and distribution of distances between specific ionic residues are shown in Figure 7 for the simulation in model 10. Two pairs, Obsc-E6227E to sAnk1-R69 and Obsc-E6330 to sAnk1-

R104, sample near ideal interaction distances (see Figure 7, legend), whereas the Obsc-E6329 and sAnk1-K105 pair samples distances of ~ 10 Å. As ionic interactions can occur over longer distances, such interactions may be favorable, even in an aqueous environment,³⁹ consistent with the data from our double mutant cycle experiments (Figure 3). However, the ionic interactions in all cases are not well-defined, direct contacts, but are sampling a range of distances. This suggests dynamic binding between sAnk1 and this region of obscurin in muscle, consistent with the affinity we measure (~ 130 nM),¹³ and with possible adaptations of the complex to changes in sarcomere length as muscle contracts and relaxes.

Additional analysis of the selected models focused on the identification of other charged residues that may mediate binding between sAnk1 and its high affinity site on obscurin. We again used site-directed mutagenesis, this time to learn if any of the positively charged amino acids (K6324, R6335, K6337, K6338) in the central region of Obsc₆₃₁₆₋₆₃₄₅ were involved in binding. In blot overlays, mutation of K6324 to a glutamate, but not to an alanine, significantly reduced binding, but as it did not dramatically decrease peak binding of Obsc₆₃₁₆₋₆₃₄₅ to sAnk1 in SPR experiments (Figure 8A, red), we did not study it further. Mutating K6338 to an alanine decreased binding of Obsc₆₃₁₆₋₆₃₄₅ to sAnk1, and mutation to a glutamate residue decreased binding further (Figure 8A, last bars). Representative SPR traces for these panels are shown in supplementary Figure S8. Blot overlays indicated that this response was specific to K6338, as the K6337A or K6337E mutations had no inhibitory effect on binding. (Figure 8B, C). These results suggest that K6338 plays a role in binding Obsc₆₃₁₆₋₆₃₄₅ to sAnk1.

Our molecular models suggested that D111 of sAnk1 is close enough to K6338 of obscurin to contribute to binding (Figure 6, white arrows; see also Figure S9 of supplementary material). We used double mutant cycle experiments to test their interaction, by creating MBP-sAnk1-D111A and -D111R mutants and then comparing their binding to GST-fusion proteins of wild type Obsc₆₃₁₆₋₆₃₄₅ or of this same region of obscurin carrying the K6338A, K6338E, and K6337E mutations. The ratio of Obsc-K6338E binding to sAnk1-D111R, compared to wild type sAnk1, approximately 3.7, was significantly greater than that for binding of wild type Obsc₆₃₁₆₋₆₃₄₅ to sAnk1 D111R (~ 1.7 ; Figure 9; $p \sim .002$). This suggests that Obsc-K6338 and sAnk1-D111 interact directly, consistent with the molecular models. Controls were done as described above. Peak binding of Obsc-K6337E or Obsc-K6338A to wild type sAnk1 versus sAnk1-D111R, although increased, did not give the same elevated ratio seen with Obsc-K6338E; both gave values of approximately 1.7, as seen for binding to wild type obscurin to sAnk1-D111R. We also assayed the binding of sAnk1 D111A to the same obscurin mutants and found no significant difference from the relative binding of sAnk1 wild-type (not shown). Finally, we created several additional mutants of sAnk1 in which other aspartate or glutamate residues were switched to arginines or lysines (D92R, D93K, E87K, E88K, D118R), but found no significant increase in the ratio of their binding to Obsc-K6338E compared to wild type Obsc₆₃₁₆₋₆₃₄₅ and wild type sAnk1 (not shown). Thus, the interaction between sAnk1-D111 and Obsc-K6338 is specific.

Discussion

The regions of sAnk1 and its high affinity binding site on obscurin, Obsc₆₃₁₆₋₆₃₄₅, are rich in positively and negatively charged amino acid residues, suggesting that their binding might be mediated by electrostatic interactions. We previously demonstrated that six positively charged residues in sAnk1, which homology modeling suggested were exposed on the surface of two ankyrin-like repeats in the molecule, mediated binding to obscurin. Here we use site-directed mutagenesis to investigate the contributions of the four glutamate residues in the high affinity binding site of obscurin to binding to sAnk1. Double mutant cycle

methodology identified the likely lysine and arginine residues on sAnk1 to which 3 of the 4 glutamates of Obsc₆₃₁₆₋₆₃₄₅ bind. Computational modeling of the ankyrin-like repeat domains of sAnk1 and the glutamate-rich sequence in its high affinity binding site on obscurin refined earlier structural models of the individual binding domains and predicted possible structures of the docked complex. Correlation of these structures with the results of double mutant cycle experiments led to the selection of two models of the docked complex consistent with experimental results. We used those models to predict an additional pair of interacting charged residues, an aspartate on sAnk1 and a lysine on obscurin, which we then confirmed by double mutant cycle analysis. Our results suggest that the binding of Obsc₆₃₁₆₋₆₄₃₆ to sAnk1 is at least partially mediated by ionic interactions between at least 4 specific pairs of charged residues (Figure 10). These interactions contribute to the strength of binding and orient the obscurin peptide with respect to the ankyrin-like repeats of sAnk1 in the docked complex.

Although MD and BD models have considerable value, their application to the study of obscurin-sAnk1 binding was significantly strengthened by double mutant cycle experiments, which tested the interactions between particular pairs of charged residues on these two proteins. These studies involved the use of site-directed mutagenesis to change positively charged residues on sAnk1, previously shown to mediate binding to obscurin,¹ to glutamates, and to change glutamates on obscurin, which we show here are important for binding to sAnk1, to lysines. The rationale behind these experiments is simple: if ionic interactions mediate sAnk1-obscurin binding, then preserving the presence of opposite charges of paired residues should preserve high affinity binding when the charges are switched between the ligand pair. Our results confirm this prediction. Controls show that mutations to alanines are insufficient to preserve binding, and that charge-switching mutations of nearby residues are also insufficient, suggesting that the results are specific for particular pairs of residues. In short, they indicate that particular lysine or arginine residues on sAnk1 interact specifically with particular glutamates in the region of obscurin we have characterized here.

Studies in our laboratory to determine the structure of obscurin or sAnk1 alone by NMR or crystallographic methods have been unsuccessful, in part due to insolubility of the protein. This motivated us to use computational methods to obtain structural models of the regions of sAnk1 and obscurin involved in high affinity binding. In earlier work,³¹ the high affinity binding site on obscurin for sAnk1 was predicted to contain a significant amount of α -helical character based on CD experiments, predictions of secondary structure and considerations of homology. The helical region was predicted to be centered in the middle of Obsc₆₃₁₆₋₆₃₄₅ and flanked by less ordered sequences.³¹ Because such simulations are computationally expensive, we focused on the core 18 amino acids, predicted to be in the α -helical region in the high affinity sAnk1-binding region of obscurin, to develop model structures of sAnk1-obscurin complex. This region contains all the residues that were experimentally shown to be involved in binding to sAnk1.¹ Consistent with these earlier ideas, we show here that MD simulations model the central residues of this polypeptide as primarily α -helical on the 30 ns timescale. However, during the simulation bending of the helix up to $\sim 40^\circ$ is observed (see Figure S6). Such bending is also consistent with our original model of Obsc₆₃₁₆₋₆₃₄₅.³¹

We also subjected our previously published model of sAnk1, based on the ankyrin repeats of Notch1, to MD simulation. The simulation confirmed the general features of the structure, especially a pair of ankyrin-like repeats, with the positively charged residues that mediated binding to obscurin exposed to the solvent in the loops between the helices of each repeat. Thus, although the ankyrin-like repeats are not predicted by algorithms based on sequence homology to other ankyrin-repeat proteins, they are likely to be the predominant structural

motif in the region of sAnk1 exposed to the cytoplasm in striated muscle.¹ There are several other examples of non-algorithmically predicted ankyrin repeats in the PDB.^{11, 40} Our MD simulations do not agree with our original model in all respects, however. In particular, the second α -helical segment of the second ankyrin-like repeat was oriented at a $\sim 30^\circ$ angle to the more N-terminal helices. The angled position of the final helix of the second repeat is consistent with the structure of other ankyrin-repeat proteins.^{11, 40} Furthermore, MD simulations emphasize the dynamic nature of the structure of sAnk1, which samples a range of conformations on the time scale of the simulation, but which in most respects are consistent with our original homology model.

To model the interaction between obscurin and sAnk1, structures from the monomer MD simulations were docked using BD simulations. From 10,000 BD simulations, followed by additional energy minimization and clustering analysis, 6 docked complexes were identified and subjected to MD simulations in aqueous solution. From analysis of the complex structures from the MD simulations, two contained the pattern of ionic interactions observed in the double mutant cycle experiments. Although model one is the best model, it has a very small population showing interactions between sAnk1-D111 and Obsc-K6338. In contrast, the other model has a very stable interaction between sAnk1-D111 and Obsc-K6338 throughout the trajectory, even though it is the second best model based on the rest of the interactions. These models can be reconciled by more significant helix bending, as suggested in Figure S6. Analysis of these structures led to the identification of one additional residue-specific ionic interaction, namely K6338 on obscurin with D111 on sAnk1. The computational prediction was confirmed in a final round of double cycle mutagenesis. The reliability of the Obsc₆₃₂₂₋₆₃₃₉-sAnk1 model is supported by its consistency with the experimental data and with its ability to predict an additional ionic interaction.

Our results further suggest that specific binding of obscurin to sAnk1 is the result of four specific ionic interactions between amino acids along the helical segment of obscurin's high affinity binding site and amino acids exposed on the surface of the ankyrin-like repeats of sAnk1. This binding behavior may be of particular significance for short sequences of amino acids, such as those found in the ankyrin-binding motifs of obscurin, as tertiary structural considerations may become less important. The significance of amino acid position may also be important for the evolution of protein domains. When residues important for binding of one domain to another are altered, a compensatory change in the domain that binds to it may occur, if preserving this interaction is advantageous. The physiological implications of position-specific binding are significant. With such tightly regulated electrostatic interactions, a change in a single base pair could switch the chemical character of a given residue, potentially changing the ligand specificity of the altered domain. This study provides evidence that specific residue interactions between the ankyrin-like repeats of sAnk1 and its high affinity binding domain on obscurin mediate specific binding. They suggest that only a few changes in sequence can significantly alter binding, leading to changes in affinity or specificity. They are also potentially useful in devising models for how short sequences of amino acids can form binding motifs capable of interacting with a variety of protein folds, and in particular, how particular ankyrin-binding motifs interact with particular ankyrin repeats. Experiments to test these ideas are currently in progress in our laboratories.

Materials and Methods

Generation of obscurin mutants

The Quik-Change II mutagenesis kit (Stratagene, <http://www.stratagene.com/>) was used to generate single or serial mutants in residues 6316-6345 of obscurin. Briefly, primers were

made to cover the site of interest and mutations were introduced via PCR. Template DNA was removed with Dpn-1 and the remaining mutated plasmid was transformed into XL-1 competent cells. DNA was extracted and sequence was verified (Biopolymer Core Facility, UMB). Sequences of PCR primers used for generating constructs are shown in Supplemental Table 1.

Purification of Glutathione-S-Transferase and Maltose-Binding Protein fusion proteins

Following sequence verification, DNA was transformed into BL21* pLysS competent cells (Stratagene). These cells minimize degradation of the expressed proteins and have a strong dependence of expression on chloramphenicol. Cells were grown in sequentially diluted cultures, induced with 1 mM IPTG, and allowed to produce protein for 4 hr. Soluble fusion constructs were purified from sonicated culture supernatants via affinity chromatography, as described.¹

Assays of Binding

Binding of MBP fusion proteins of sAnk1 to GST fusion proteins containing short sequences of obscurin were assayed by blot overlay and surface plasmon resonance, as previously described.^{1, 13, 31}

Computational methods

Molecular dynamics (MD) simulations—Empirical force field calculations were performed with the programs CHARMM³⁸ and NAMD.⁴¹ Calculations used the CHARMM 22 all-atom protein force field⁴² including the CMAP correction.⁴³ Preparation for the MD simulations involved overlaying the structures of sAnk1₅₇₋₁₂₂ developed by Borzok *et al.*,¹ part of a partially α -helical model of obscurin₆₃₁₆₋₆₃₄₅ developed by Busby *et al.* 2010³¹ or the sAnk1₅₇₋₁₂₂-Obsc₆₃₂₂₋₆₃₃₉ complex with, respectively, 55 Å, 50 Å, and 76 Å pre-equilibrated cubic boxes of TIP3P water that contained 150 mM NaCl. Solvent molecules with non-hydrogen atoms within 2.8 Å of protein non-hydrogen atoms were deleted. These were then subject to MD simulations. Each system was minimized and heated to 298K at a rate of 10K/ps and equilibrated in the NPT ensemble (1atm, 298K)⁴⁴ for 100ps. Production runs were performed in the NPT (1atm, 298K) ensemble. Simulations were performed with a 2fs integration timestep using the SHAKE algorithm⁴⁵ to constrain covalent bonds to hydrogens. Electrostatic forces were calculated with the particle mesh Ewald method⁴⁶ using a real space cutoff of 12 Å with a kappa value of 0.4 Å⁻¹ and a 4th order spline interpolation. Van der Waals (VDW) forces were truncated with a cutoff distance of 12 Å with smoothing performed using a force switching function starting at 10Å. Time frames from the trajectories were saved every 5 ps for subsequent analysis.

Brownian dynamics simulation—Brownian dynamics (BD) simulations were performed with the program MacroDox version 3.2.2.⁴⁷ BD simulations were performed with sAnk1₅₇₋₁₂₂ fixed at the center of the simulation system, while Obsc₆₃₂₂₋₆₃₃₉ was placed in random positions and orientations on the surface of a sphere 69 Å away from the center (B-surface, see Figure S10 of the supplementary material). 10,000 Brownian dynamics simulations were performed in which Obsc₆₃₂₂₋₆₃₃₉ was docked on sAnk1₅₇₋₁₂₂. The simulations were terminated once the obscurin fragment either escaped from a spherical surface 200 Å away from the center (C-surface, see Figure S10 of the supplementary material) or interacted with sAnk1₅₇₋₁₂₂, as defined as coming within 30 Å of the center of the simulation system.

Representative structures of free sAnk1₅₇₋₁₂₂ from cluster 4 (see below) and Obsc₆₃₂₂₋₆₃₃₉ from cluster 2 (see below) obtained from the MD simulations were used for the BD simulations. Atomic charges and masses were assigned based on the CHARMM22 force

field.⁴² Tanford-Kirkwood theory was used to estimate the pK_a values and determine the protonation states of titratable residues at pH 7.4, consistent with experimental conditions. Solvent accessibility was calculated using the method of Lee and Richards.⁴⁸ The electrostatic field grid around sAnk1₅₇₋₁₂₂ was generated by solving the linear Poisson-Boltzmann equation. A 3.6Å low resolution grid was first generated followed by the generation of higher resolution grid of 1.2Å to obtain a more accurate boundary potential. Dielectric constants for bulk solvent and protein were set to 78 and 4, respectively, the ionic strength was set to 150mM, and the temperature was set to 298°K.

Minimization using the Generalized-Born molecular volume (GBMV) method—

The docked structures of the sAnk1₅₇₋₁₂₂-Obsc₆₃₂₂₋₆₃₃₉ complex from the BD simulations were subjected to energy minimization to further refine the structures, prior to evaluating the energy of the complexes. The aqueous environment was treated implicitly using the GBMV method^{49, 50} with dielectric constant of 80. Energy minimization was performed based on 500 steps of steepest descent (SD) and an additional 500 steps of adopted basis Newton-Raphson (ABNR).

Estimation of distance between ionic interactions of amino acid residues—

Acetate and methyl-guanidinium was used as a model for side chain interaction between glutamate and arginine, while acetate and methylammonium was used for glutamate and lysine. Each two molecule system was minimized in the gas phase with two cycles of 500 steps of SD and three cycles of 500 steps of ABNR. Each system was heated to 298°K at a rate of 15K/ps and equilibrated at 298°K for 100ps. Production runs of 30ns were performed in the gas phase at 298°K with 2fs integration time step using the SHAKE algorithm⁴⁵ to constrain covalent bonds to hydrogens. The trajectories were saved every 5 ps for subsequent analysis. The distances between the side chains were defined based on the carboxyl carbon in acetate, the guanidinium carbon in methyl-guanidinium and the ammonium nitrogen in methylammonium.

Materials—Unless otherwise noted, all reagents were from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BD	Brownian dynamics
CD	circular dichroism
GST	glutathione S-transferase
MBP	maltose-binding protein

MD	molecular dynamics
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
sAnk1	small ankyrin
SPR	surface plasmon resonance
SR	sarcoplasmic reticulum

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Figure 1.

Individual and serial mutations of glutamates in Obsec₆₃₁₆₋₆₄₃₆ to alanines reduce binding to sAnk1. (A) Bar graph summarizing surface plasmon resonance (SPR) data for the binding of wild type and mutant forms of GST-Obsec₆₃₁₆₋₆₄₃₆ to MBP- sAnk1₂₉₋₁₅₅; n = 3 for all except E6327A, where n = 6. The various glutamate residues mutated to alanine are indicated. All data are normalized to binding of the wild type fusion protein to sAnk1 (WT). *Some multiple mutations cannot be reliably statistically analyzed because of lack of appreciable binding. (B) Corresponding blot overlay studies of binding using the same site directed mutants as in A. Some lanes in the blot may appear darker than expected from SPR measurements, as mutant fusion proteins do not always behave identically in blots and SPR experiments.

Figure 2.

Glutamate-to-lysine mutations reduce binding of Obsc₆₃₁₆₋₆₄₃₆ to sAnk1. (A) Bar graph showing grouped SPR data of binding of the obscurin mutants to MBP-sAnk1 normalized with respect to WT levels. E6327K, n = 11; E6329K, n = 14; E6330K, n = 7; E6333K, n = 5 (p < .05 for all mutants). (B) Corresponding blot overlay of binding of fusion proteins, with same residues mutated as A; n = 5. Two gels are separated with a space.

Figure 3.

Double mutant cycle experiments demonstrate residue-specific electrostatic interactions between sAnk1₂₉₋₁₅₅ and Obsc₆₃₁₆₋₆₃₄₅. SPR results for the double mutant cycle studies of Obsc₆₃₁₆₋₆₃₄₅ constructs in which glutamates were mutated to lysines, showing specific enhancement of binding to sAnk1₂₉₋₁₅₅ in which positively charged residues were mutated to glutamates. All data on mutants of Obsc₆₃₁₆₋₆₃₄₅ and sAnk1 are compared to binding of the obscurin mutants to WT sAnk1₂₉₋₁₅₅. The first solid bar indicates maximal binding, measured for the wild type fusion proteins (see *Methods*). The first hashed bar indicates wild-type Obsc₆₃₁₆₋₆₃₄₅ – sAnk1₂₉₋₁₅₅ mutant, demonstrating a reduction in binding, consistent with Borzok, et al.,¹ except in the case of R69. The next set of bars indicate the binding of an obscurin construct with an adjacent glutamate – E6327K in A, E6329K in B, and C – changed to a lysine to either wild-type (solid) or mutant (hatched) forms of sAnk1. The third set of bars indicates binding of obscurin constructs, in which glutamate residues are mutated to alanines, to wild type (solid) or charge-switched mutants (hatched) of sAnk1₂₉₋₁₅₅. The last set of solid bars indicates binding of each of the individual Obsc₆₃₁₆₋₆₃₄₅ mutants to wild type sAnk1₂₉₋₁₅₅. The last set of hashed bars indicate binding of Obsc₆₃₁₆₋₆₃₄₅ mutants to sAnk1₂₉₋₁₅₅ mutants. Asterisk indicates that the difference between the ratios of pairs of solid and hashed bars has a p-value < .05. n=6, 4, 3, 6 for panel A, n=7, 6, n/a, 7 for panel B, and n= 6, 3, 3, 6 in panel C for each pair of graph bars.

Figure 4.

Three representative conformations of sAnk1₅₇₋₁₂₂ alone in an aqueous environment in 13-30 ns MD trajectory. (A) Cluster 2: 31.8%; (B) Cluster 3: 48.4%; (C) Cluster 4: 19.8%; (D) Side view of Cluster 4. After the MD simulation, sAnk1 still showed two ankyrin-like repeats, with the second helix of the more C-terminal repeat organized at an angle to the other helices (arrow). Positively charged residues (R69, R104, and K105), demonstrated to be involved in binding to obscurin (see Figure 3), are shown in blue. Aspartate 111, shown to be involved in binding (see Figure 9), is shown in red.

Figure 5. Results from the docking of obscurin to sAnk1 using Brownian dynamics simulations. (A) Distribution of the center of mass of 3122 Obsc₆₃₂₂₋₆₃₃₉ molecules that are within 30 Å of the center of mass of sAnk1₅₇₋₁₂₂ from 10⁴ Brownian dynamics simulations. Blue dots indicate unfavorable states (mean = +100 kcal/mol); red dots indicate favorable states (mean = -50 kcal/mol); white dots indicate intermediate states (mean = 0 kcal/mol). Color intensity is scaled to reflect the relative deviation from the center of the color scale. (B) Distribution of interaction energy between Obsc₆₃₂₂₋₆₃₃₉ and sAnk1₅₇₋₁₂₂. (C) Orientation of representative structures from the 6 major clusters of all molecules with energies of interaction of Obsc₆₃₂₂₋₆₃₃₉ and sAnk1₅₇₋₁₂₂ more negative than -50 kcal/mol. sAnk1: cyan, obscurin: model 1 (blue), model 2 (red), model 3 (orange), model 7 (yellow), model 10 (green), model 13 (purple). Models 2 and 10 are in closest agreement with experimental data.

Figure 6.

Refined docked models of Obsc₆₃₂₂₋₆₃₃₉ and sAnk₁₅₇₋₁₂₂. These models were selected from six potential models obtained from Brownian dynamics simulations (see Figure 5C models 2 and 10), followed by further refinement using 70ns MD simulations. This figure is a representative structure from the 70ns MD simulation of (A) model 2 and (B) model 10. Glutamate residues in Obsc₆₃₂₂₋₆₃₃₉ involved in binding (E6327, E6329, and E6330) are shown in red; lysine and arginine residues on sAnk1 involved in binding (R69, R104, and K105) are shown in blue. These residues are also indicated by yellow arrows. K6338 of obscurin and D111 of sAnk1 (white arrows) are predicted to interact by both models. Stick and surface representation are used in Obsc₆₃₂₂₋₆₃₃₉ and sAnk₁₅₇₋₁₂₂, respectively.

Figure 7.

Time series and distribution of distances between specific ionic residues on Obsc₆₃₂₂₋₆₃₃₉ and sAnk1₅₇₋₁₂₂ based on the 5-70 ns portion of the MD trajectory of model 10 of the sAnk1-Obse complex. Plots of the time series (upper) and distributions (lower) are shown for (A, D) R69 - E6327E, (B, E) R104 - E6330RE6 and (C, F) K105K - E6329. Bin size for the probability distributions is 0.5 Å. Distances were calculated between arginine C ζ and glutamate C δ atoms, and between lysine N ζ and glutamate C δ atoms. All three ionic interactions agree with the experimental results in Figure 3. The ideal distances between arginine or lysine and glutamate residues are calculated to be 3.8 ± 0.0 and 3.0 ± 0.0 Å (average $\pm$ standard error), respectively, based on the gas phase simulations (see Figure S7 of the supporting information).

Figure 8.

Mutations of positively charged amino acids in Obsc₆₃₁₆₋₆₃₄₅ to alanine and glutamate reduce binding to sAnk1. (A) Summary graph of pooled SPR data. * indicates significance ($p < .05$; K6324E, $n = 3$; K6338E, $n = 8$). (B) Results of blot overlays demonstrating that mutation of K6338 to alanine reduces binding of GST-Obsec₆₃₁₆₋₆₄₃₆ to MBP-sAnk₁₂₉₋₁₅₅. $n = 3$. (C) Blot overlays suggest that mutation of K6324 to alanine slightly reduces binding of GST-Obsec₆₃₁₆₋₆₄₃₆ to MBP-sAnk₁₂₉₋₁₅₅, and mutation of K6338 to glutamate reduces binding of Obsc₆₃₁₆₋₆₄₃₆ to MBP-sAnk₁₂₉₋₁₅₅ more than the K6338A mutation ($n = 6$ for K6324 and K6338; $n = 4$ for R6335 and K6337). Panels B and C also show that K6337A and K6337E mutations have little effect on binding, suggesting that the effects of mutating K6338 are specific.

Figure 9.

Double mutant cycle experiment confirms that D111 of sAnk1 interacts with K6338 of obscurin. The first solid bar indicates maximal binding, measured for the wild type fusion proteins (see *Methods*). The first hashed bar indicates binding by Obsc₆₃₁₆₋₆₃₄₅ to sAnk1₂₉₋₁₅₅ D111R. Second set of bars indicates the binding of GST-Obsc K6337E to either sAnk1 wild-type (solid) or sAnk1 D111R (hatched). The third set of bars indicates GST-Obsc K6338A binding to sAnk1₂₉₋₁₅₅ wild-type (solid) and sAnk1 D111R (hatched). The last solid bar indicates GST-Obsc K6338E mutant binding to sAnk1₂₉₋₁₅₅ wild-type. The last hashed bar indicates GST-Obsc K6338E binding to sAnk1₂₉₋₁₅₅ D111R. Asterisks indicate that the difference in the ratios has a p-value < .05. In this case, the ratio of the differences in the last set of bars are significantly different than every other group, which are not significant with respect to each other (see Results).

Figure 10.

Experimentally determined electrostatic interactions of residues within sAnk1₂₉₋₁₅₅ and Obsc₆₃₁₆₋₆₄₃₆, shown in context. (A) Schematic diagram of sAnk1 protein, with the region that associates with obscurin boxed. (B) Amino acids contained in box region that are presented on the helical face of the ankyrin-like repeats. Residues experimentally identified to bind obscurin are shown in blue and orange. The center of the highest affinity known binding site on obscurin for sAnk1 is shown below. Residues experimentally identified to bind sAnk1 are shown in red and green. Black lines indicate direct residue association, as shown by double mutant cycle experiments. (C) Schematic diagram the obscurin protein, with the region that associates with sAnk1 with highest affinity boxed.