Mapping the Binding Site on Small Ankyrin 1 for Obscurin*§

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Small ankyrin 1 (sAnk1), an integral protein of the sarcoplasmic reticulum encoded by the ANK1 gene, binds with nanomolar affinity to the C terminus of obscurin, a giant protein surrounding the contractile apparatus in striated muscle. We used site-directed mutagenesis to characterize the binding site on sAnk1, specifically addressing the role of two putative amphipathic, positively charged helices. We measured binding qualitatively by blot overlay assays and quantitatively by surface plasmon resonance and showed that both positively charged sequences are required for activity. We showed further that substitution of a lysine or arginine with an alanine or glutamate located at the same position along either of the two putative helices has similar inhibitory or stimulatory effects on binding and that the effects of a particular mutation depended on the position of the mutated amino acid in each helix. We modeled the structure of the binding region of sAnk1 by homology with ankyrin repeats of human Notch1, which have a similar pattern of charged and hydrophobic residues. Our modeling suggested that each of the two positively charged sequences forms pairs of amphipathic, anti-parallel α-helices flanked by β-hairpin-like turns. Most of the residues in homologous positions along each helical unit have similar, though not identical, orientations. CD spectroscopy confirmed the α-helical content of sAnk1, ~33%, predicted by the model. Thus, structural and mutational studies of the binding region on sAnk1 for obscurin suggest that it consists of two ankyrin repeats with very similar structures.

The intracellular membrane systems of striated muscle, transverse tubules, and sarcoplasmic reticulum (SR), play essential roles in the contractile cycle. Muscle contraction requires the release of Ca2+ from the terminal cisternae of the SR, through a process that is initiated by an action potential propagating along the plasma membrane and transverse tubules into the interior of the muscle cells (1, 2). The rise in intracellular Ca2+ promotes cross-bridge formation between thin filaments of actin and myosin head groups, leading to contraction. Relaxation follows the re-uptake of Ca2+ into the network compartment of the SR membrane by the Ca2+-ATPase of the SR membrane (3). The regular organization of the network compartment of the SR around each sarcomere, at the level of the Z-disks and M-bands, is typical of skeletal muscle, and essential for activity (4).

We and others have proposed that at least two molecules are responsible for aligning the SR around each sarcomere, small ankyrin 1 (sAnk1) and obscurin (5, 6). sAnk1 (also known as Ank 1.5 and AnkR;3) is concentrated in the network compartment of the SR membrane in skeletal muscle (7, 8). As a product of the ANK1 gene (9), it is a member of the ankyrin superfamily, which is composed of proteins that are ubiquitously expressed and typically found within the membrane-associated cytoskeleton. Three genes in vertebrates, ANK1, ANK2, and ANK3, encode ankyrin proteins 1, 2, and 3 (also known as R, B, and G, respectively (10, 11)) and their splice variants. Canonical ankyrins contain ~24 ankyrin repeat domains within their N-terminal region (12), which mediate binding to other macromolecules (13), a spectrin-binding domain, and a C-terminal regulatory domain (14, 15) that has evolved to bind titin and obscurin (16, 17). Ankyrin repeats are typically found in tandem arrays and are composed of 33 amino acid residues, with high conservation of the positions of the hydrophilic and hydrophobic amino acids within each repeat. Characteristic of these sequences is the formation of short anti-parallel α-helices separated by a short loop and linked to neighboring repeats by β-hairpin-like turns (18).

At ~17 kDa in mass, sAnk1 differs from the much larger ankyrins 1, 2, and 3 (~200 kDa) by the absence of the N-terminal ankyrin repeat domain (~90 kDa), responsible for binding integral membrane proteins, and the central domain (~65 kDa), responsible for binding β-spectrin (9, 17, 19, 20). These large binding domains are replaced at the N terminus of sAnk1 by a unique, alternatively spliced sequence of 73 amino acids (Fig. 1A). The most N-terminal 22 amino acids are highly hydrophobic and are sufficient to confer on sAnk1 the properties of an integral membrane protein (7). Following the 22-amino acid transmembrane segment, the unique sequence contains a stretch of positively charged residues interspersed with hydrophobic residues that can be modeled as an amphipathic α-helix, which is identical in rat, mouse, and human (see Fig. 1A). The C-terminal 83 amino acids of sAnk1 are common to larger forms of Ank1 and share considerable homology with the
C-terminal regions of splice isoforms of Ank2 and Ank3 (Fig. 1B). This conserved region also contains a sequence, similar to that in the unique sequence, that can be modeled as a positively charged, amphipathic α-helix and binds the co-chaperone Hdj1/Hsp40 in Ank2.2 (21). Our previous results suggested that the portion of sAnk1 containing these putative helices is exposed on the cytoplasmic surface of the SR membrane (7), where it localizes around the M-bands and Z-disks of skeletal muscle and forms a high affinity binding site for obscurin, its major ligand in striated muscle (6).

Obscurin is a multifunctional, ~800-kDa protein composed of multiple Ig and FnIII domains, as well as several signaling motifs (22–25). The C-terminal region of obscurin A, the predominant giant isoform of obscurin in striated muscle (26, 27), hereafter referred to simply as obscurin, is composed of a non-modular sequence of ~400 amino acids and harbors the binding site for sAnk1 (5, 6). Obscurin is firmly anchored to the contractile apparatus, where it is concentrated around the periphery of the Z-disks and M-bands of each sarcomere and so co-distributes with sAnk1 (5, 6). Obscurin is firmly anchored to the network SR around Z-disks and M-bands. In agreement with these results, homology modeling suggests that the two regions are indeed helical and are organized as non-modular, C-terminal region of obscurin (5, 6), and by sAnk1 to obscurin is mediated primarily by sequences in the amino acids 61–130 of sAnk1, which include the two positively charged regions that are likely to form amphipathic α-helices. Here we focus on the role of these charged residues in binding obscurin. We use site-directed mutagenesis to show that both sequences are essential for high affinity binding to obscurin, and that the two residues at homologous positions along the putative helices play qualitatively similar roles in binding, consistent with a binding site composed of two functionally similar units. In agreement with these results, homology modeling suggests that the two regions are indeed helical and are organized as two ankyrin repeats, with very similar structures. These form a large charged region on the surface of sAnk1, similar to the C-terminal repeats of the ankyrin repeat domain of human Notch1 (31), that mediates binding to obscurin.

**EXPERIMENTAL PROCEDURES**

*Generation of sAnk1 Mutation Constructs*—Mutated constructs were produced with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sense and antisense oligonucleotides (see supplemental Table S1) were used with cDNA encoding the cytoplasmic domain (amino acids 29–155) of wild-type (or mutant, for sequential mutations) sAnk1 (sAnk1154_130). The cDNA was subcloned in the pMal-c2X vector (New England Biolabs, Beverly, MA) using the EcoR1 and SalI restriction sites. The authenticity of all constructs was verified by sequence analysis.

*Generation and Purification of Fusion Proteins*—All mutant and wild-type forms of the cytoplasmic portion of sAnk1 (sAnk1140_130), prepared as a 10X histidine-tagged protein in pET-19b (Novagen, San Diego, CA), was also expressed in BL21 Star (DE3) pLysS One Shot (Invitrogen) bacterial cells. A truncated version of the cytoplasmic portion of sAnk1 (sAnk160_130), produced as MBP fusion proteins, and its primary binding site on obscurin (Obsc6316–6436 (6)), prepared as a GST fusion protein in pGEX-4T1 (Amersham Biosciences), were expressed in BL21 Star (DE3) pLysS One Shot (Invitrogen) bacterial cells. A truncated version of the cytoplasmic portion of sAnk1 (sAnk160_130), prepared as a 10X histidine-tagged protein in pET-19b (Novagen, San Diego, CA), was also expressed in BL21 Star (DE3) pLysS One Shot. Expression of all fusion proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3.5 h for the GST and MBP fusion proteins and 5 h for the His-tagged protein. Proteins were purified by affinity chromotography on amylose resin (for MBP fusion proteins), glutathione-agarose (for GST fusion proteins), or nickel-nitriilotriacetic acid-agarose (for His-tagged protein), following the manufacturers’ instructions. After purification, MBP and GST fusion proteins were dialyzed in phosphate-buffered saline, containing 10 mM NaCl and phenylmethylsulfonyl fluoride (1.5 mM). His-sAnk1140_130 was dialyzed into 20 mM sodium tetraborate, 10 mM sodium phosphate, pH 7.4. The purity of all proteins was >90%, as determined by SDS-PAGE and staining with Coomassie Blue.

*Blot Overlay Assays*—Blot overlays were performed as described (6), with minor modifications. Aliquots containing 1.0 µg of bacterially expressed, affinity-purified GST-Obsc6316–6436 protein were separated by SDS-PAGE on 4–12% gradient gels and transferred electrophoretically to nitrocellulose. Equivalent loading and transfer were confirmed by staining with Ponceau Red. Blots were incubated in buffer A (50 mM Tris, pH 8, 120 mM NaCl, 3% bovine serum albumin, 2% dithiothreitol, 0.5% Nonidet P-40, 0.1% Tween 20, 0.2% maltose) for 16 h at room temperature, followed by buffer A containing 0.75 µg/ml of either MBP, wild-type MBP-sAnk129–155, or mutants of MBP-sAnk129–155 for 4 h at room temperature. Blots were washed extensively with buffer A and twice with buffer B (phosphate-buffered saline, pH 7.2, 10 mM NaCl, 0.1% Tween 20, 3% dry milk). Membranes were probed with antibodies to MBP (1:5000, New England Biolabs), followed by goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000). Labeled bands were visualized by chemiluminescence (Western Light Detection, Tropix Laboratories, Bedford, MA).

*Surface Plasmon Resonance*—The binding of GST-Obsc6316–6436 to wild-type and mutant forms of MBP-sAnk129–155 was also studied by surface plasmon resonance (SPR) with a Biacore 3000 biosensor (GE Healthcare, Biacore, Uppsala, Sweden), as described previously (6), with minor modifications. After activation of the sensor chips, ~14,000 resonance units of affinity-purified goat anti-GST antibody were immobilized on flow cells 1 and 2 (FC1 and FC2). GST-obscurin6316–6436 was used as ligand and MBP-sAnk1 constructs as analytes. Control lanes were bound to GST alone.

Two sets of experiments were performed: (i) binding of all sAnk1 mutants to obscurin was evaluated and compared with the binding of the wild-type proteins; (ii) kinetic analysis of binding was performed using selected mutant forms of MBP-sAnk129–155. For the former, we used wild-type and mutant forms of MBP-sAnk129–155 at a standard concentration of 1 µM. The kinetics of binding between GST-Obsc6316–6436 and wild-type or mutant MBP-sAnk129–155 proteins was studied.
over a range of concentrations from well below to well above the measured dissociation constant determined for each construct. All kinetic data were evaluated with the 1:1 Langmuir model (BIAevaluation Software 3.1, Biacore). Statistical significance was calculated with the Student’s t test.

**Modeling**—We used the structure of the ankyrin repeat domain of human Notch1 (31) and the Homology and Biopolymer modules of Insight software (Accelrys, Inc., San Diego, CA) to model the portion of the cytoplasmic sequence of sAnk1 (amino acids 29–122) that contains the binding site for obscurin. The pattern of hydrophobic and hydrophilic residues in the cytoplasmic region of sAnk1 is very similar to the sequence of ankyrin repeats 4–6 of Notch1 (IYYH, Protein Data Bank (31)). Our initial model was created by aligning the hydrophobic residues of the helical regions of sAnk1 (amino acids 29–35, 61–76, and 94–112) with ankyrin repeats 5 and 6 from Notch1. Some manual folding of the long loop regions between these helices (amino acids 36–60 and 85–92) was performed to alleviate steric hindrances and reduce hydrophobic surface area. The most N-terminal portion of sAnk1, included in the working model but not shown (amino acids 29–56), was fit to the C-terminal portion of the fourth ankyrin repeat of Notch1. This included an α-helix (amino acids 29–35) and a large loop region (amino acids 36–56) bridging the gap from an initial α-helix to the first full ankyrin repeat. The axis of the N-terminal α-helix was rotated ~45° and shifted ~4 Å (measured at the level of Cys-34) from that of Notch1 to permit disulfide-linked dimerization through cysteine 34 (7). Modeling of the last 33 amino acids was not performed, due to reduced homology to Notch1.

The modeled structure underwent three rounds of unrestrained minimization plus dynamics in a periodic water box (300 steps minimization, 3000 steps dynamics, 1-fs timestep, and constant valence force field) followed by one round of similar minimization plus dynamics (consistent force field 91), with restraints applied to hydrogen bonds in α-helical structures to preserve the helices. Insight and Discover software (Accelrys Inc., San Diego, CA) were used for all simulations.

**CD Spectroscopy**—CD spectroscopy was performed on a Jasco-810 spectropolarimeter (Jasco, Easton, MD) at 25 °C. Spectra were collected from 192 to 260 nm with a 0.2-mm resolution and a 1.0-cm bandwidth. His-sAnk160–130 was dialyzed against 20 mM sodium tetraborate, 10 mM sodium phosphate, pH 7.4, and diluted in 50% trifluoroethanol to a final concentration of 10 μM. Background signal (buffer alone) was subtracted, and the mean residue ellipticity (deg cm² dmol⁻¹) was calculated using CDPRO software supplied by the manufacturer. The percent α-helical content was determined from the spectra with the CONTINLL and SELCON3 methods within CDPro analytical software (32).

**Materials**—Unless otherwise noted, all reagents were from Sigma and were of the highest grade available.

**RESULTS**

**Binding Requires Unique and Conserved Sequences**—Yeast two-hybrid assays have shown that the minimal binding site for Obsc6316–6436 on sAnk129–155 is confined to a 70-amino acid sequence within the cytoplasmic region of sAnk1. This sequence (61–130) is composed of residues that are conserved among the larger ankyrin isoforms (amino acids 75–130), C-terminal to residues that are unique to sAnk1 (amino acids 61–74) (6). We performed overlay assays with Obsc6316–6436 and wild-type as well as truncated forms of sAnk129–155 to learn if portions of both the unique and conserved regions mediate binding in vitro.

A set of sAnk1 deletion constructs was generated that contained truncations of the cytoplasmic portion of sAnk1. One construct included amino acids 29–89 and contained most of the unique cytoplasmic sequence of sAnk1 along with the first 16 amino acids of the conserved sequence, whereas a second included amino acids 90–155, in the conserved sequence only (Fig. 1). As a positive control, we also expressed a construct containing the minimal binding site (amino acids 61–130 (6)), consisting of the last 12 amino acids of the unique sequence and most of the conserved sequence. Fig. 2A shows the results of SDS-PAGE analysis of the purified fusion proteins that were used. Equivalent amounts of Obsc6316–6436 were probed in far Western blots with MBP fusion proteins of these sAnk1 variants, or with MBP alone. sAnk161–130 bound almost as well to Obsc6316–6436 as to the full cytoplasmic construct, sAnk129–155 (Fig. 2B, lanes 1 and 4), confirmed by kinetic analysis that revealed a binding affinity very close to wild-type (data not shown). However, the two other constructs did not bind nearly as well (Fig. 2B, lanes 2 and 3). We found no significant binding of MBP alone (Fig. 2B, lane 5). These results confirm in vitro the results of yeast two-hybrid assays (6), i.e. that the binding of sAnk1 to Obsc6316–6436 requires amino acids in both its unique and conserved sequences.

**Mutating the Lysines and Arginines of the Proposed Binding Site to Alanine**—The region of sAnk1 that binds obscurin includes two stretches of positively charged amino acids that can be modeled as amphipathic α-helices (see below), one in the sequence unique to sAnk1, the other in the sequence conserved among many ankyrin isoforms. We hypothesized that these residues in each of the putative helical regions were essential for binding to obscurin. We systematically mutated these residues individually and in groups to test this. A complete list of the mutations and the oligonucleotides used to prepare them are given in supplemental Table S1.

We examined all our sAnk1 mutant forms with dynamic light scattering (data not shown) to assess the effects of mutagenesis on the sizes of the mutant proteins in solution. Most mutations showed no significant differences in the relative size of the molecules compared with wild type, suggesting that the mutations did not disrupt the overall structure of the cytoplasmic domain of sAnk1. We then evaluated the ability of these mutant forms of sAnk1 to bind to Obsc6316–6436 by blot overlay and SPR. Below, we first consider the conserved sequence and then examine the unique sequence.

Initially, we used blot overlays to assay the relative abilities of wild-type MBP-sAnk129–155 and alanine mutants of the lysines and arginines in the conserved region (Fig. 3) to bind to GST-Obsc6316–6436. MBP alone served as a negative control. The effects of individual K-to-A and R-to-A substitutions varied with their position in the sequence. Mutating the first amino acid in the conserved sequence (K100A) had a greater inhibi-
tory effect on binding than mutating any of the middle residues (K101A, R104A, and K105A), whereas mutating the last residue (R108A) increased binding significantly (Fig. 3B, lanes 3–7). Mutating these amino acids in combination also gave differing results, again depending on their locations. Mutation of three or more residues simultaneously appeared to abolish binding completely (all five are mutated in K100A/K101A/R104A/K105A/R108A, Fig. 3B, lane 2; others not shown). Mutating the first and last amino acids (K100A/R108A) resulted in a protein that bound to Obsc6316–6436 better than the K100A construct alone but not as well as wild-type sAnk1 (see also Fig. 3B, lane 8). As in Fig. 2, no specific binding was detected when the identical blot was overlaid with MBP alone (not shown). These results show that the first four positively charged residues in the conserved region of sAnk129–155 (Lys-100, Lys-101, Arg-104, and Lys-105) promote binding to Obsc6316–6436, but that the last positively charged amino acid (Arg-108) inhibits binding to a modest extent.

We used SPR to confirm and quantify the binding of wild-type and mutant forms of sAnk1 to Obsc6316–6436. In these experiments, we immobilized GST-Obsc6316–6436 on one flow cell of the sensor chip, and immobilized GST, on a separate flow cell, as a control. We then introduced wild-type or mutant forms of MBP-sAnk129–155 at concentrations of 1 μM onto the GST-obscurin and GST surfaces and measured binding and dissociation (see "Experimental Procedures"). Data from representative sensograms are shown in Fig. 3C, and, after normalization to binding of the wild-type proteins (100%), as bar graphs in Fig. 3D. These results confirmed quantitatively all the results of our blot overlay assays of the effects of mutating the lysine and arginine residues in the conserved region of sAnk1 on binding to GST-Obsc6316–6436. Differences from wild type were statistically significant (p < 0.01).

We used identical approaches to study the role of the homologous, positively charged amino acids in the unique region of sAnk129–155 on binding (Fig. 4). All constructs were purified as
stable fusion proteins and were assayed as above. As we observed with the conserved sequence, mutation of the first amino acid (R64A) had a greater inhibitory effect than similar mutations of the three middle residues (R67A, R68A, and R69A), whereas mutation of the last amino acid (K73A) enhanced binding to GST-Obsc6316–6436 compared with wild-type sAnk1. Double mutation of the first and last residue (R64A/K73A), however, produced a fusion protein that bound GST-Obsc6316–6436 better than wild-type sAnk1, in contrast to the K100A/R108A mutant form. The blot overlay assays described above, and SPR studies (Fig. 4, B–D) gave similar results. Collectively, these results show that each of the positively charged sequences in the unique and conserved regions of sAnk1 plays a qualitatively similar role in the binding of sAnk1 to obscurin and sug-

FIGURE 2. Regions of small ankyrin 1 involved in binding to the C-terminal region of obscurin. A, GST-Obsc6316–6436 and a series of deletion mutants of sAnk1, produced as MBP fusion proteins, were affinity-purified and subjected to SDS-PAGE, followed by staining with Coomassie Blue. The molecular masses of MBP fusion proteins of wild-type sAnk129–155 and smaller deletion mutations are ~56–62 kDa; the molecular mass of GST-Obsc6316–6436 is ~38 kDa. B, in blot overlays, visualized after incubation with antibodies to MBP, MBP-sAnk1-wt but not the deletion mutants bound to immobilized GST-Obsc6316–6436; MBP alone did not bind to GST-Obsc6316–6436.

FIGURE 3. Alanine mutations of positively charged amino acids of the conserved region of sAnk1 inhibit binding to obscurin. A, site-directed mutagenesis was used to create a series of MBP fusion proteins containing alanine substitution mutations of the positively charged residues of the conserved region of sAnk1. These were affinity-purified, subjected to SDS-PAGE, and stained with Coomassie Blue. The chart summarizes the residues that were mutated and the nomenclature used for each construct; sAnk1-cALL denotes a variant mutated at all five residues in the conserved region (Lys-100, Lys-101, Arg-104, Lys-105, and Arg-108). B, recombinant, purified MBP-sAnk1 (wild-type and mutants) bound to immobilized GST-Obsc6316–6436 to varying degrees in blot overlay assays. C, surface plasmon resonance (SPR) was used to evaluate binding of MBP-sAnk1 mutants to the COOH terminus of obscurin quantitatively. GST-Obsc6316–6436 was used as ligand and MBP-sAnk1 (wild-type and mutants) served as analytes. The representative plots are recordings of the response differences, in resonance units versus time in seconds. D, SPR data are represented in a bar graph normalized to wild-type (100%). Most of the mutants showed decreases in binding of at least 75%. However, a single mutation, sAnk1-R108A (shown in dark turquoise) caused a >35% increase in binding. Compared with wild-type, all mutations showed a significant change in binding (p < 0.01).
gest that they form a binding site on sAnk1 for obscurin that is composed of two similar structures, both of which are required for binding.

**Complementarity between Conserved and Unique Regions**—We tested the possibility that the last positively charged residues in the unique (Lys-73) and conserved (Arg-108) sequences would not only compensate for the loss of activity caused by alanine mutations in the first amino acid in the same helical region but also in the other. We created double mutants, K100A/K73A and R64A/R108A, and assayed their binding to Obsc6316–6436 in blot overlays and SPR (Fig. 5). These experiments showed that alanine mutation of the last amino acid of either the conserved or the unique series of positive charges could rescue some of the binding activity lost by mutations of the first amino acids of these series, regardless of whether they belonged to the conserved or the unique portions of sAnk1.

**FIGURE 4.** Alanine mutations of positively charged residues within the unique region of sAnk1 inhibit binding to obscurin. A, a series of alanine substitution mutations of the cluster of positive charges within the unique region of sAnk1 were produced as MBP fusion proteins, as described in Fig. 3. The chart summarizes the residues mutated and the nomenclature used for each construct; sAnk1-uALL denotes a mutation at all five residues in the unique region (Arg-64, Arg-67, Arg-68, Arg-69, and K73). B, wild-type and mutant forms of MBP-sAnk1 bound to immobilized GST-obsc6316–6436 to varying degrees in blot overlay assays. C and D, binding was analyzed quantitatively by SPR, as in Fig. 3. Most of the mutations reduced binding by at least 60%. One mutation, sAnk1-K73A, alone or together with R64A, increased binding by ~40%. The differences in binding between each of the mutations and wild type were significant ($p < 0.01$).

**TABLE 1**

| Mutation          | Binding affinity, $K_d$  | Association rate constant, $K_a$  | Dissociation rate constant, $k_d$ |
|-------------------|--------------------------|-----------------------------------|-----------------------------------|
|                   | $nM$                     | $nM^{-1} s^{-1}$                   | $s^{-1}$                          |
| sAnk1-wt          | 35.2 ± 3.5               | 2.8 ± 0.5                         | 1.0 ± 0.6                         |
| sAnk1-R108A       | 17 ± 1.1                 | 8.4 ± 0.5                         | 1.4 ± 0.01                        |
| sAnk1-K100A/R108A | 240 ± 13                 | 0.9 ± 0.07                        | 2.2 ± 0.03                        |
| sAnk1-K73A        | 6.5 ± 0.1                | 6.0 ± 0.6                         | 0.4 ± 0.01                        |
| sAnk1-R64A/K73A   | 22 ± 1.0                 | 6.5 ± 0.1                         | 1.4 ± 0.04                        |
| sAnk1-K100A/K73A  | 56 ± 3.1                 | 1.9 ± 0.1                         | 1.0 ± 0.03                        |
| sAnk1-R108E       | 55 ± 2.0                 | 4.1 ± 0.2                         | 2.3 ± 0.06                        |
| sAnk1-K73E        | 9.5 ± 1.9                | 8.6 ± 1.5                         | 0.8 ± 0.004                       |

*p < 0.05.  
**p < 0.01.
Rescue between regions was somewhat less effective than rescue within a region, however. Although the results were qualitatively similar, mutating the last amino acid in the unique sequence to alanine had a higher impact on binding than mutating the last amino acid in the conserved sequence.

**Mutating the Lysines and Arginines within the Proposed Binding Site on Small Ankyrin for Obscurin**

Mutating the last amino acids in the proposed binding sites (Arg-67, Arg-68, Arg-69, Lys-101, Arg-104, and Lys-105) in both the unique and conserved regions significantly inhibited binding, whereas binding was only modestly inhibited by the R64E and K100E mutations. Binding was either moderately enhanced or not affected by replacing the last positively charged amino acids in each sequence with glutamates (K73E and R108E). Notably, the behavior of each of the homologous mutations on the conserved and unique sequences was similar. These results indicate the importance of the positive charges in the middle of each sequence and suggest that these residues are involved directly in binding to obscurin through electrostatic interactions. Likewise, they suggest that the first residues of each series (Arg-64 and Lys-100) are not directly involved in binding.

**Kinetic Studies**—We used SPR to analyze the kinetic parameters of the binding of wild-type and mutant forms of sAnk1 29–155 to Obsc6316–6436. We limited these studies to two constructs of the protein that bound to a significant extent, even if reduced when compared with wild type, in blot overlays and SPR experiments (see above). We introduced different concentrations of wild-type and mutant forms of MBP-sAnk1 (adjusted for each fusion protein to ensure that a wide range of concentrations around the concentration that gave half-maximal binding was investigated; see “Experimental Procedures”) over immobilized GST-Obsc6316–6436 or GST alone. After subtraction of nonspecific binding to GST alone, we used Biacore software to fit our binding data to Langmuir binding isotherms with a stoichiometry of 1:1, and to derive values of the association and dissociation constants for binding ($K_D$, Fig. 7, Table 1).

The average $K_D$ for the binding of the MBP fusion protein of wild-type sAnk1 29–155 to GST-Obsc6316–6436 was 35 nM, and all mutations that resulted in inhibition of binding caused a 2 to 20-fold increase in $K_D$. Consistent with this, the mutations that increased binding invariably showed increased binding affinities (i.e., lower $K_D$ values). The latter mutations involve eliminating the positive charge in the last position of each sequence we studied (Lys-73 and Arg-108), alone or in combination with mutating other residues, consistent with the idea that a positive charge at this position is inhibitory. Notably, the values for the average $K_D$ values of mutations contained within the conserved region were more severely affected (i.e., the affinities were lower), compared with the ones corresponding to mutations in the unique region. This suggests that the conserved region plays a more important role than the unique region in binding obscurin.

The kinetic constants we measured are consistent with our measurements of affinities. In particular, the five mutations that increased affinity, all of which involve mutations of the last positively charged amino acids in the unique or conserved sequences, increased the rate constant for association compared with wild type, with variable effects on the rate constant for dissociation. Mutations that reduced binding affinity slightly had effects on association and dissociation rates that were smaller and less consistent. These results are in agreement with the idea that the presence of positively charged amino acids in the last positions in each sequence mildly inhibits bind-

### Table: Binding Affinities

| Mutation         | % Binding | Standard Deviation |
|------------------|-----------|--------------------|
| sAnk1-wt         | 100       | 0                  |
| sAnk1-K100A/K73A | 69.5      | 1.4                |
| sAnk1-R64A/R108A | 16.4      | 0.7                |

**FIGURE 5. Interaction between the two positively charged sequences of sAnk1.** A, two constructs with paired mutations were produced as MBP fusion proteins, and purified and assayed as in Fig. 3 (see chart). B, recombinant, purified MBP-sAnk1 (wild type and mutants) showed differential binding to immobilized GST-obs6316–6436 in blot overlay assays. C and D, SPR was used to evaluate binding quantitatively. Both K100A/K73A and R64A/R108A mutations showed an increase in binding, compared with the R64A or K100A mutations alone. The K73A mutation was more effective than R108A in restoring binding. Differences from wild type were significant ($p < 0.01$).
The sole exception was the mutation with the largest effect that we were able to study, K100A/R108A, which decreased the binding affinity 7-fold compared with wild type by decreasing the association constant as well as increasing the dissociation constant.

Homology Modeling of the Binding Site—We used homology modeling to the ankyrin repeat domain of human Notch1 to model the structure of the cytoplasmic region of sAnk1 that contains the binding site for obscurin. The structure of the ankyrin repeat domain of Notch1 (1YYH) has been determined by x-ray crystallography (31) and is composed of seven ankyrin repeats that are involved in regulating the protein’s signaling activity through intermolecular interactions (33–36). We used repeats five and six of Notch1 as a template for the cytoplasmic portion of sAnk157–122. Although the sequence homology between the two is low, the order of hydrophobic and hydrophilic residues is conserved (Fig. 8A), suggesting high structural homology.

Our model predicts that sAnk1 contains two complete ankyrin repeats, one composed both of amino acids in the unique and conserved sequences (amino acids 57–89) and the other of residues in only the conserved sequence (amino acids 90–122). Each repeat contains two short anti-parallel α-helices (amino acids 61–67 and 71–77 in the first repeat; 95–101 and 104–112 in the second) separated from each other and from flanking sequences of the molecule by loops created by a short turn and a short loop followed by a random coil, respectively. The short loop between the two helices of one repeat is composed of only three residues and allows the second α-helix to bend back under the first in an anti-parallel fashion. In some minimization and dynamics runs, the last turn of the first α-helix in the ankyrin repeat formed by the unique sequence tended to open slightly, suggesting that that portion of the molecule may be more flexible.

Agreement between the modeled structure of the two ankyrin repeats of sAnk1 with the fifth and sixth repeats of Notch1 was good, with the highest degree of structural homology in the helical and immediately adjacent regions (Fig. 8, A and B). The seventh repeat of Notch1 may serve as a template for a third ankyrin repeat on sAnk1, which would lie outside the binding site for obscurin, but the homology between these sequences is much weaker and so we have not modeled it here.

The proposed structure of sAnk1 orients the five positive charges of each region in a parallel fashion such that each corresponding positive residue pair (i.e. Arg-64 and Lys-100, Arg-67 and Lys-101, and others) is oriented similarly (Fig. 8, B and D), consistent with the results of our studies using site-directed mutagenesis. The six amino acids (Arg-67, Arg-68, Arg-69, Lys-101, Arg-104, and Lys-105), determined experimentally to promote binding to obscurin, are arranged on the convex surface near the short turns of the two ankyrin repeats.
The “solvent-exposed surface” model, colored by residue charge (Fig. 8C), shows these six residues clustering on the surface of the molecule in approximately the same plane, and surrounding three exposed hydrophobic residues (Val-70, Ile-102, and Ile-103), that are oriented appropriately to be buried upon interaction with obscurin. The first and last amino acids of each series of positively charged residues (Arg-64, Lys-100, Lys-73, and Arg-108) are located closer to the ends of each of the four helices behind the plane containing the positively charged residues involved in binding (Fig. 8, C and D), consistent with our mutagenesis studies that suggest that these four residues are not directly involved in binding but may instead play a structural role.

**CD**—We used CD spectroscopy to estimate the α-helical content of the structure required for binding obscurin (e.g. Fig. 2). We produced sAnk160–130 as a 10× histidine-tagged peptide and dialyzed it into sodium tetraborate buffer, as described under “Experimental Procedures.” Spectra were collected at a final protein concentration of 10 μM in the presence of 50% trifluoroethanol, which we used to optimize folding of His-sAnk160–130 into a conformation closely resembling its native state (37, 38). The CD spectrum (Fig. 9) showed minima at 220 and 208 nm, consistent with the presence of significant α-helical content, as seen in CD spectra of other ankyrin repeat domains (39, 40). Our homology model suggests a helical content of 38%, including as helical the last turn of the first of the two parallel helices in the unique portion of the binding site (predicted to be more flexible; see above) or 34%, excluding this region. In good agreement with the predictions of homology modeling, evaluation of the spectra and established databases (see “Experimental Procedures”) indicates that the α-helical content of His-sAnk160–130 is 33%.

**DISCUSSION**

The network compartment of the sarcoplasmic reticulum of skeletal muscle fibers is aligned precisely around the M-bands and Z-disks of the sarcomere and is closely associated with the contractile apparatus. sAnk1, an integral membrane protein of the network SR (7, 8), binds with high affinity to obscurin, a giant protein that surrounds the contractile...
apparatus, primarily around M-bands but also around Z-disks (6). We have suggested that their binding is essential for the proper alignment of the network SR with nearby elements of the contractile apparatus. Testing this hypothesis requires detailed knowledge of the binding domains of both proteins and how they interact. Here we describe the results of experiments in which we used site-directed mutagenesis and molecular modeling to examine the binding site of sAnk1 for obscurin. We postulated that two positively charged sequences in the cytoplasmic domain of sAnk1 mediate binding to the C-terminal region of obscurin. Our experimental data and homology modeling support and extend this hypothesis, by showing that the charges are not only involved in binding but also arrayed into two parallel ankyrin-like repeats with very similar structures.

Our results confirm that the binding site on sAnk1 for obscurin requires positively charged sequences in both the conserved and unique regions. The conserved sequence, which is found in many forms of ankyrin (Fig. 1), has been modeled as an amphipathic helix (21) and is clearly essential for binding, but it is not sufficient: the unique sequence is also necessary. Isoforms 1–4 of ankyrin 1 lack the unique sequence, whereas ankyrins 1.6 and 1.7 lack the positive stretch of amino acids in the conserved region (5, 9). sAnk1 (equivalent to Ank1.5) and Ank1.9 are the only known ankyrin 1 isoforms that contain both positively charged binding sequences (30), consistent with the requirement of both sequences for binding to obscurin, confirmed in this study. However, the abundance of Ank1.5 in striated muscle (30) suggests that it is the major ankyrin 1 isoform capable of binding to obscurin in vivo. It is noteworthy that some muscle-specific isoforms of Ank G also show high homology to both positive sequences of sAnk1 (Fig. 1) (16), suggesting that they too may bind obscurin. Published evidence (41, 42) indicates that they associate primarily with the sarcolemma, however, making it unlikely that they contribute significantly to the organization of the SR.

![FIGURE 8. Structural model of the binding region of sAnk1 for obscurin.](image)
We also studied the kinetics of binding for selected mutants. The wild-type protein bound with an average binding affinity of 35.2 nm, significantly stronger than our previously published value of 130 nm (6). Our most recent purification methods lead to less degraded protein (Fig. 3A, lane 1) and appear to preserve activity more effectively than our earlier methods. Most mutant proteins that we subjected to kinetic analysis show essentially no degradation in SDS-PAGE. The stronger binding seen here may therefore be a result of the use of higher concentrations of active protein.

All of our kinetic data confirm the idea that the binding site on sAnk1 for obscurin is composed of two sets of positively charged residues, and that one of each (the last, or Lys-73 and Arg-108, residues) has the unusual property of inhibiting binding by the wild-type protein. The bilateral nature of the binding site is confirmed by the observation that mutations of each of these residues can restore some or all of the binding lost as a result of mutating the first (Arg-64 or Lys-100) residue, regardless of whether this mutation is introduced into the unique or conserved sequence. The bilateral organization of this region of sAnk1 is consistent with the results of molecular modeling, which showed that, although sAnk1 does not contain ankyrin-like repeats that could be predicted from the presence of consensus amino acids, the binding region of sAnk1 shares significant structural homology with other proteins of the ankyrin-repeat superfamily. In particular, the pattern of hydrophilic and hydrophobic amino acids in its binding region resembles that of many ankyrin repeats, especially the fifth and sixth repeats of human Notch1, the molecular structure of which has recently been reported (31).

The ankyrin repeat region of Notch1, a transmembrane protein essential for the Notch signaling pathway (43), is located within the intracellular domain and, after cleavage by γ-secretase, is transported into the nucleus via a nuclear localization sequence. Ligands for the ankyrin repeats of Notch1 include EM5, Deltex, and RBP-J, all of which act as downstream effectors in the Notch signaling pathway and positive regulators of transcription (35, 36, 43). It is unlikely, however, that the regions of Notch1 and sAnk1 that we have compared structurally share the same ligands, because the binding site for obscurin on sAnk1 is highly positively charged, whereas Notch1 has negatively charged residues in the repeats we have used for modeling.

Modeling amino acids 56–122 of sAnk1 into the molecular folds determined for repeats 5 and 6 of human Notch1 was accomplished with little adjustment. The results revealed a structure with two ankyrin repeat domains, each consisting of two short α-helices linked by a short loop, which are connected to each other and to flanking regions of the protein by β-hairpin-like turns. Notably, the two pairs of helices consist of the unique and conserved stretches of positively charged amino acids, arrayed in parallel.

The CD spectra suggest that residues 60–130 of sAnk1 have \(\sim 33\%\) α-helical content, which is consistent with the homology model prediction of \(\sim 38\%\) α-helical content. The small discrepancy in the calculated values may be due to the greater flexibility predicted by some modeling programs at the end of the first helix of the unique region (see above). Assuming that the three residues,
which tend to form a more flexible structure, do not contribute to the CD minima and maximum typical of an α-helix; reduces the predicted helical content to 34%, in good agreement with our measurements. Further confirmation of these predictions will require verification of the structure by other means.

Both the experimental evidence and proposed model suggest a bipartite binding site. The two ankyrin repeat structures in the unique and conserved regions are composed of pairs of α-helices folded over each other. The disposition of some of the charged residues along these helices appears to be highly similar, as well (Fig. 8, B and D). Furthermore, both regions are linked to each other and to nearby sequences via β-like-turns, enhancing their overall similarity. These structural parallels are consistent with the qualitatively similar effects on binding of mutating each of the charged amino acid residues along each of the helices.

The similarities are limited, however, because not all four helices are parallel, nor are all amino acid side chains disposed identically with respect to the axis of each helix. A major difference in the orientation of the positively charged amino acids within the unique and conserved ankyrin repeats is the spacing of the three middle amino acids, which as modeled appear at the short turns. In the unique region they are found in series (Arg-67, Arg-68, and Arg-69), but in the conserved region the second residue (Lys-101) is separated from the third and fourth (Arg-104 and Lys-105) by two isoleucine residues (Ile-102 and Ile-103). This reduces the structural parallels and could help to explain the greater importance of the conserved region’s contribution to binding (see above).

In addition to its confirmation of a bipartite binding site, the structural model is consistent with the results of site-directed mutagenesis in other ways. First, the model suggests that the middle three amino acids of each positively charged sequence form a large, planar patch of positive charge on the surface of the molecule. Mutagenesis suggests that the residues making up the patch contribute to the binding site, but no individual residue is required for binding; several must be mutated before binding activity is completely lost, consistent with a role for a large cluster of positively charged amino acids in binding obscurin. These residues are found within and flanking the loop regions of each ankyrin repeat, which in ankyrins are highly variable and important in determining the specificity of binding (18). The model also predicts that the last positively charged amino acid of each helical region is positioned behind the plane that contains the residues involved in binding. The fact that their conversion to alanines (and, in the case of Lys-73, to glutamate) increases binding activity suggests that a positive charge at these positions restricts the ability of sAnk1 to bind obscurin, perhaps by modulating conformational flexibility or presenting an alternative binding surface. These possibilities are consistent with our observation that mutations of either of these amino acids to eliminate their positive charge increases the kinetic constant for association of sAnk1 to obscurin, without having a major effect on the dissociation rate.

Conformational changes linked to a shift from hydrophilic (Arg or Lys) to mildly hydrophobic (Ala) amino acids, or destabilization of the amphipathic α-helices in which they are found, are most likely to account for the changes in binding activity caused by the R64A and K100A mutations. This explanation would be consistent with our observation that the R64E and K100E mutations inhibit binding only slightly. Thus, the first amino acids of each of the ankyrin-like repeats may play a role in maintaining the helical structure of the repeats and the appropriate positioning of the charged residues involved in binding, but not in binding obscurin directly.

The partial compensatory effect of the double, R64A/K73A or K100A/R108A, mutations is consistent with results obtained by Cammett et al. (44), who studied the binding of the ankyrin repeat protein, p16, to Cdk4. As with sAnk1, the binding of unstable mutants of p16 was improved by the introduction of a second, thermodynamically favorable, mutation. If the restoration of binding of Arg-64 or Lys-100 sAnk1 mutants by the Lys-73 or Arg-108 mutations is due to similar factors, then the latter are likely to stabilize the secondary structure of sAnk1 to promote binding to obscurin. This would be consistent with the abilities of the Lys-73 and Arg-108 mutations alone to promote binding, as well.

In principle, electrostatic interactions, even between clusters of positively and negatively charged amino acids (and the region of obscurin we have used in these studies does indeed have a large cluster of glutamates), do not in themselves account for the high affinity of binding that we have measured for sAnk1 and obscurin because of the energy required for the desolvation of these charges as the protein goes from the unbound to the bound state. Studies of other ankyrin repeat proteins, like GABPβ, suggest that they form a “hot spot” for binding composed of a few hydrophobic residues surrounded by other amino acid side chains, containing at least one charged residue, that also contribute to binding (45). Leucine and isoleucine are thought to be most effective in promoting binding at “hot spots” (46). These results imply that hydrophobic side chains of sAnk1, exposed to the solvent in the unbound state, become buried as binding to obscurin occurs, accounting for the nanomolar affinities we measure. Examination of the model (Fig. 8) suggests that these residues may include isoleucine and valine residues (Ile-102, Ile-103, and Val-70) and perhaps others as well. Further studies of the complex formed by sAnk1 and obscurin are in progress to address these issues.

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