Structure of the Ankyrin-binding Domain of α-Na,K-ATPase

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The ankyrin 33-residue repeating motif, an L-shaped structure with protruding β-hairpin tips, mediates specific macromolecular interactions with cytoskeletal, membrane, and regulatory proteins. The association between ankyrin and α-Na,K-ATPase, a ubiquitous membrane protein critical to vectorial transport of ions and nutrients, is required to assemble and stabilize Na,K-ATPase at the plasma membrane. α-Na,K-ATPase binds both red cell ankyrin (AnkR, a product of the ANKI gene) and Madin-Darby canine kidney cell ankyrin (AnkKα, a product of the ANK3 gene) utilizing residues 142–166 (SYYYQAEKSSKIMFNVMPQQALV) in its second cytoplasmic domain. Fusion peptides of glutathione S-transferase incorporating these 25 amino acids bind specifically to purified ankyrin (Kd = 118 ± 50 nM). The three-dimensional structure (2.6 Å) of this minimal ankyrin-binding motif, crystallized as the fusion protein, reveals a 7-residue loop with one charged hydrophilic face capping a double β-strand. Comparison with ankyrin-binding sequences in p53, CD44, neurofascin/L1, and the inositol 1,4,5-trisphosphate receptor suggests that the valency and specificity of ankyrin binding is achieved by the interaction of 5–7-residue surface loops with the β-hairpin tips of multiple ankyrin repeat units.

Tethering interactions between the cytoplasmic domains of integral membrane and other proteins, mediated by ankyrin or proteins containing ankyrin-like repeat structures, play fundamental roles in diverse biological activities including growth and development (1–5), intracellular protein trafficking (6–8), and mRNA transcription (20, 21). Ankyrin, including its many isoforms in diverse biological activities including growth and development (1–5), intracellular protein trafficking (6–8), and mRNA transcription (20, 21). Ankyrin, including its many isoforms

MATERIALS AND METHODS
Preparation and Purification of Recombinant Peptides—Oligonucleotides were designed to amplify constructs IIa, IIb, and IIc from a construct encoding CDII of α-Na,K-ATPase (28). Amplification products were subcloned into TA vectors (Invitrogen) and sequenced by the dideoxynucleotide chain termination method (U. S. Biochemical Corp.). Constructs were expressed as fusions with Schistosoma japonicum glutathione S-transferase (SGST) using the pGEX-2T prokaryotic expression vector (Amersham Pharmacia Biotech) and purified using glutathione-Sepharose (28). SGST was expressed as a control peptide. All peptides were eluted with 50 m M Tris-HCl, 5 m M reduced glutathione, pH 8.0, and dialyzed into ankyrin-binding buffer (ABB: 50 m M NaCl, 1 m M dithiothreitol, 1 m M EDTA, 1 m M EGTA, 1 μ M phenylmethylsulfonyl fluoride, 1 μ M Pefabloc SC). Proteins were analyzed by SDS-PAGE.

Ankyrin Binding Assay—Each fusion protein (50 μ g at 1 μ g/ml) was conjugated to 50 μ l of a 50% slurry of glutathione-agarose for 1 h at 4 °C with gentle rotation. Ankyrin (AnkR) was purified by extraction of spectrin-depleted fresh erythrocyte inside-out vesicles with 1 M KCl, followed by ion exchange chromatography on DEAE cellulose (31). Ankyrin-binding was assayed by adding 25 μ g to the peptide-conjugated beads in a total volume of 500 μ l in ABB, overnight incubation at 4 °C, and analysis of the bead fraction by SDS-PAGE. Ankyrin was detected by Western blotting with specific antibodies (28). Confluent MDCK cells extracted in situ were used to prepare a high salt extractable cytoskeletal fraction (Fx2) enriched in MDCK cell ankyrin (AnkKα) (28). Conjugated beads were incubated with Fx2 (300 μ g of total protein), and bound ankyrin was detected as above. Other procedures and antibodies were as before (7, 28).

For quantitative binding measurements, purified ankyrin was labeled with sulfo-N-hydroxysuccinimide biotin (Pierce), further purified by gel filtration, and concentrated using a Centricon ultradialysis membrane (7). Aliquots (10 μ g) of purified fusion peptide IIa (GST-MAB) or GST were incubated as above in ABB with varying concentrations of biotinylated ankyrin. Bound ankyrin was visualized by ECL after direct overlay with horseradish peroxidase-avidin (Vector). Relative binding was measured by densitometry of autoradiograms, with precautions taken to assure linearity of detection. Because the absolute free concentration of ankyrin could not reliably be measured using this biotinylated assay, free ankyrin was assumed to equal total ankyrin. This

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Printed in U.S.A.
The Minimal Ankyrin-binding Domain of α-Na,K-ATPase—Recombinant peptides derived from CDII of rat α-Na,K-ATPase (28) were prepared as fusion proteins with GST and assayed for their ability to bind AnK₉ (from red cells) or AnK₉ (from Madin-Darby canine kidney cells) (Fig. 1). All peptides were of the predicted molecular mass, soluble, and readily purified (Fig. 1B). Peptide IIA (residues 142–166 of α-Na,K-ATPase numbered according to Ref. 38) retained full binding activity to both AnKᵢ and AnKᵢ and constituted the MAB sequence. CDII peptides not encompassing MAB, such as peptide IIC (residues 168–286), were devoid of activity.

GST-MAB Binds Ankyrin with High Affinity—Prior studies have detected Na,K-ATPase affinities for ankyrin in the range of 50–2600 nM (11, 39, 40). Of interest was whether GST-MAB bound ankyrin with comparable affinity. Purified ankyrin (AnkR) was biotinylated, and its binding to GST-MAB or GST was measured (Fig. 2). Nonlinear regression analysis based on a bi-molecular binding model indicated that ankyrin specifically bound GST-MAB with a Kᵦ = 118 ± 50 nM and a Bₘ₉90% = 6.1 ± 0.7 × 10⁵ (arbitrary units).

RESULTS AND DISCUSSION

The Minimal Ankyrin-binding Domain of α-Na,K-ATPase—A schematic representation of the five cytoplasmic domains of α-Na,K-ATPase and their relationship to the ankyrin-binding peptide sequences identified here. Codon positions defining each peptide are shown. B, each depicted peptide (II–IIC) was prepared as a fusion construct with GST and examined for its ability to bind at various concentrations either purified AnKᵢ (ANKI, from human red cells) or kidney ankyrin AnKᵢ (ANK3) derived from whole MDCK cell lysates. The top panel shows Coomassie Blue-stained SDS-PAGE analysis of each peptide, as well as the entire MDCK cells extract applied to the ankyrin column to detect AnKᵢ (ANKI) binding. To detect AnKᵢ (ANKI) binding, purified erythrocyte ankyrin was used. Peptide IIA, sequence SYYQEAKSSKIMESFKNMVPQQALV, represents the minimal active sequence detected.

approximation will systematically overestimate slightly the Kᵦ (weaker apparent affinity than the true value). Nonspecific binding to GST was subtracted prior to nonlinear regression fitting. Errors are expressed as ± 2 S.D.

Structure Determination—Fusion peptide IIA was purified by high pressure liquid chromatography gel filtration into 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM β-mercaptoethanol. Crystals were prepared at room temperature by hanging drop vapor diffusion against a reservoir of 30% polyethylene glycol 4000, 100 mM bis-tris-propane, 150 mM NaCl, 40 mM β-mercaptoethanol, pH 8.8 (32). Diffraction data were collected with a R-AxisII imaging plate detector mounted on a Rigaku 200HM generator at −170 °C using a crystal flash frozen in crystallization buffer. Data were processed using the program DENZO and SCALEPACK (33) and were 86% complete (>2σ). The structure was determined by molecular replacement using the published crystal structure of GST from S. japonica (34) and the program AMORE (35) of the CCP4 program suite (33). Successive rounds of model building and simulated annealing refinement were carried out with the program X-PLOR version 3.851 (36) and the CCP4 program suite. The graphic display program O, version 5.10 (37) was used to build and correct the structure manually. Each residue of the final model was checked by the omit map.

![Fig. 1. Identification of the MAB domain of α-Na,K-ATPase. A, schematic representation of the five cytoplasmic domains of α-Na,K-ATPase and their relationship to the ankyrin-binding peptide sequences identified here. Codon positions defining each peptide are shown. B, each depicted peptide (II–IIC) was prepared as a fusion construct with GST and examined for its ability to bind at various concentrations either purified AnKᵢ (ANKI, from human red cells) or kidney ankyrin AnKᵢ (ANK3) derived from whole MDCK cell lysates. The top panel shows Coomassie Blue-stained SDS-PAGE analysis of each peptide, as well as the entire MDCK cells extract applied to the ankyrin column to detect AnKᵢ (ANKI) binding. To detect AnKᵢ (ANKI) binding, purified erythrocyte ankyrin was used. Peptide IIA, sequence SYYQEAKSSKIMESFKNMVPQQALV, represents the minimal active sequence detected.](image-url)
molecular replacement strategy based on the known crystal structure of S. japonica GST (SjGST) (which has one molecule in the asymmetric unit and 41% solvent) (34). The refined model displayed a continuous main chain electron density and consisted of two domains, one representing SjGST and the other MAB (Fig. 3). Each residue of MAB was verified with an electron density omit map. Both the GST and MAB domains were well defined when the total structure was checked with the $2F_o - F_i$ map, including the side chains of Leu118, His215, and Lys218, residues disordered in the published structure of SjGST (34). Backbone residues 1–210 of SjGST superimposed on those of SjGST-MAB with a RMS deviation of 1.02 Å for backbone residues 1–210. The crystallographic coordinates of GST-MAB are available from the Brookhaven Protein Data Bank as accession number PDB1bis.

that the structure of MAB as revealed in the fusion protein crystal is valid. Finally, it is clear from the graphical display that four MAB units are packed in each unit cell. Although theoretically the MAB conformation might be influenced by the packing force, these forces are usually quite small. Taken together, these considerations argue strongly that the conformation of MAB will not be influenced by the presence of SjGST and that carrier-mediated crystallization of active ankyrin-binding peptides fused with SjGST may represent an important and general approach to identifying the structural determinants of ankyrin binding activity in a variety of proteins.

**The Loop-on-a-Stalk Structure of MAB**—Detailed analysis of the ankyrin-binding domain within SjGST-MAB reveals an antiparallel double β-strand flanking a loop composed of the seven residues MESFKNM (residues 152–158 of α-Na,K-ATPase) (Fig. 4). The overall structure of MAB is suggestive of a loop on a stalk. This loop is amphipathic, presenting a hydrophobic face composed of two methionines and one phenylalanine and a hydrophilic face composed of Glu-Ser and Lys-Asn on the opposite side. A dipolar interaction between Ser149 (position 234 in the crystal) and Glu148 (crystal position 249) of the antiparallel β-strands stabilizes the stalk. Presumably, in intact α-Na,K-ATPase, flanking sequences would further stabilize the stalk and probably alter the positioning of the terminal residues in MAB. A search of nucleotide and protein sequence banks (GenBankTM, Swiss-Prot) revealed exceptional conservation of MAB across species and between isoforms of α-Na,K-ATPase (Fig. 5). Sequences partially homologous to MAB also exist in gastric H,K-ATPase (42), which associates with ankyrin in gastric parietal cells (43). No homologous sequences were noted in other well documented ankyrin-binding proteins (including the erythrocyte anion exchanger, the amiloride-sensitive sodium channel, the voltage-sensitive sodium channel, the Na$^+$/Ca$^{2+}$ exchanger, CD44, neurofascin, and IP3-R).

These findings represent the first available data on the mo-
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complexity for interacting specifically with diverse ligands (analogous to antigen recognition sites in antibodies). We envision the seven residue loop and possibly portions of the β-stranded stalk of MAB associating with the β-hairpin and sheet structures of the ankyrin repeat unit (Fig. 5). Given that the other reported ankyrin-binding domains in CD44, IP3-R, and neurofascin are also small peptides, it is likely that although lacking sequence homology to MAB, they may also assume a loop on a stalk conformation that best enables them to interact with a complimentary site on the complex ankyrin surface. Thus, the structure of MAB reported here may offer a glimpse into a general mechanism by which the profound multivalency of ankyrin is achieved.

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FIG. 5. MAB is an ATPase specific sequence that may interact with one or more ankyrin repeat units. A, sequence alignment of the minimal ankyrin-binding domain (peptide IIA) with all other proteins in GenBankTM and Swiss-Prot. Dashes represent residues identical to human Na,K-ATPase α subunit, residues 142–166. This sequence is well conserved among all known Na,K-ATPase α subunits and is partially conserved (68% identity) in only one other documented ankyrin-binding protein (gastric H/K-ATPase). There is no homology to the ankyrin-binding sequences in CD44, IP3-R, neurofascin, or p53bp2. Ankyrin-binding Domain of α-Na,K-ATPase

Ankyrin repeats (Gonna and Pavletich, 1996) α-Na, K-ATPase