Interactions with ankyrin<sub>G</sub> are crucial to the localization of voltage-gated sodium channels (VGSCs) at the axon initial segment and for neurons to initiate action potentials. However, the molecular nature of these interactions remains unclear. Here we report that VGSC-<alpha> but not -beta subunits bind to ankyrin<sub>G</sub> using pull-down assays. Further dissection of this activity identifies a conserved 9-amino acid motif (V/A)P(I/L)AXXE(S/D)D) required for ankyrin<sub>G</sub> binding. This motif is also required for the localization of chimeric neurofascin/sodium channel molecules to the initial segment of cultured hippocampal neurons. The conserved nature of this motif suggests that it functions to localize sodium channels to a variety of “excitable” membrane domains both inside and outside of the nervous system.

The concentration of voltage-gated sodium channels (VGSCs) into excitable membrane domains is crucial to information processing and transmission in the nervous system and to excitation/contraction coupling in muscle. Localized concentrations of VGSCs at the initial segment (IS) and the nodes of Ranvier are necessary for the initiation and propagation of action potentials through myelinated axons. However, mechanisms underlying the localization of VGSCs to these excitable membrane domains remain relatively unknown. In neurons, VGSCs exist as heterotrimers composed of a large pore-forming alpha subunit associated with two smaller accessory beta subunits (reviewed in Ref. 1). At least 10 genes encoding putative alpha subunits have been identified in mammals (reviewed in Ref. 2) along with three beta subunit genes. Beta subunits not only modulate the activity of alpha subunits (3, 4) but also exhibit characteristics of cell adhesion molecules (CAMs) binding to extracellular matrix molecules such as tenascin-C and -R (5) and other CAMs such as neurofascin (6).

Polarized localization of VGSCs in the axonal membrane requires interactions with members of the ankyrin family of peripheral membrane proteins (reviewed in Ref. 7). Ankyrins function as membrane-cytoskeleton adaptors that immobilize integral membrane proteins to the spectrin-based membrane skeleton. Ankyrins co-purify with and directly bind to purified VGSCs (5), and specific isoforms of the ankyrin<sub>G</sub> gene are concentrated with VGSCs at the IS (9), the nodes of Ranvier (9), and the neuromuscular junction (10). The functional importance of the interaction between ankyrin<sub>G</sub> and VGSCs is demonstrated in knockouts of ankyrin<sub>G</sub> in the mouse cerebellum. Purkinje cells from knockout animals are deficient in localized VGSC concentrations at the IS (11) and are unable to initiate action potentials (12).

In addition to VGSCs, ankyrins also interact with a variety of other integral membrane proteins present at the node including the CAMs neurofascin and NrCAM (13, 14). Interactions of ankyrins with VGSCs (15) and other integral membrane proteins are mediated through the N-terminal 90-kDa repeat or membrane-binding (MB) domain. The interaction between the ankyrin MB domain and neurofascin/NrCAM is dependent upon a conserved FIGQGY motif in the cytoplasmic domain of these molecules (16).

Unlike neurofascin and NrCAM, the nature of the interaction between VGSCs and ankyrins remains unclear. Initial studies using uncharacterized rat brain preparations of VGSCs and purified erythrocyte ankyrin (15) did not distinguish which VGSC subunits or their isoforms were involved in ankyrin binding. Subsequent studies using a variety of techniques have suggested sites for ankyrin interaction on both the VGSC beta (17) and alpha subunits (18).

In this study, we identified a domain of the VGSC-alpha subunit that interacts with ankyrin<sub>G</sub> and demonstrated its role in the localization of VGSCs to the IS in vivo. Initially we observed that RNA<sub>I2</sub> (rat RNA<sub>I2</sub>) co-localizes with ankyrin<sub>G</sub> at the IS of hippocampal neurons and identified an ankyrin<sub>G</sub>-binding site in the cytoplasmic linker between domains II and III (loop 2), using a pull-down assay. We did not detect binding sites for ankyrin<sub>G</sub> elsewhere within the alpha subunit nor did we detect binding with the beta subunits using this assay. We further identified a 9-aa motif within the loop 2 sequence that is conserved among all vertebrate alpha subunits and demonstrated its requirement in ankyrin<sub>G</sub> binding. Finally, we demonstrated that deletion of this 9-aa motif abolishes the ability of neurofascin/loop 2 chimeric molecules to be localized to the IS of cultured hippocampal neurons. These results demonstrate that ankyrin binding is conserved among vertebrate sodium channels and is crucial to the localization of VGSCs to excitable membrane domains.

**Experimental Procedures**

Cell Culture and Transfection—Baby hamster kidney (BHK) cells were cultured in minimum essential medium alpha (alpha-MEM, Invitrogen) supplemented with 5% (v/v) fetal bovine serum (Invitrogen) at 37 °C under 5% CO<sub>2</sub>. Cells were transfected with LipofectAMINE PLUS reagent in serum-free alpha-MEM following the manufacturer’s protocol (Invitrogen). Typically, 4 µg of total DNA was used on ~10<sup>4</sup> cells in 60 mm-dishes. Primary cultures of hippocampal neurons were prepared by guest on July 23, 2018http://www.jbc.org/Downloaded from
from the hippocampi of 18-day-old fetal Wistar rats and transfected using a modified calcium phosphate protocol as described (19).

**Immunofluorescence Analysis of Primary Hippocampal Neurons**—For immunostaining of primary hippocampal neurons, coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 and processed as described (19). Primary antibodies were used rabbit anti-Na,1.2 (1:100; Upstate Biotechnology, Inc., Lake Placid, NY), or mouse anti-MyC (1:1000; Covance, Berkeley, CA), and chicken anti-ankyrinG (Molecular Probes, Inc., Eugene, OR). Fluorescent images were captured on a Leica DM IRE2 inverted microscope (Leica Microsystems, Inc., Bannockburn, IL) using an ORCA ER extended-range cooled CCD camera (Hamamatsu Photonics, Inc.) and Openlab software (Improvi-

**Construction of Plasmids for Protein Expression**—Rat cDNAs for the β1 and β2 subunits of the voltage-gated sodium channels (a gift from Dr. L. L. Isom, University of Michigan) were subcloned into pEGFP-N1 (BD Biosciences) (not in frame with the downstream GFP), and a fragment coding for the HA epitope (YPDYVPIA) was inserted by PCR at a position corresponding to aa 26 for β1 and 34 for β2 (see Fig. 1). PCR products were verified by sequencing. cDNA constructs encode nucleotides representing different domains of the rNav1.2a molecule (full-length cDNA for rNav1.2a was a gift from Dr. A. L. Goldin, University of California, Irvine, CA) fused to the C terminus of GFP (see Figs. 1 and 2) and were obtained using PCR and standard molecular biology techniques. Similarly, PCR was used to generate a series of chimeric constructs (see Fig. 3) encompassing areas of loop 1 from rNav1.2a (aa 599–694, construct II-1-II) and loop 2 from rNav1.1 (aa 1033–1131, construct II-III (1.1)), rNav1.6 (aa 1014–1108, construct II-III (1.6)), rNav1.4 (aa 837–933, construct II-III (1.4)), and rNav1.5 (aa 953–1102, construct II-III (1.5)). These substitutions were based on a sequence alignment of the whole loop 2 region of these isoforms (see Table 2). These products were verified by sequencing. PCR products were then substituted for the neurofascin-cytoplasmic domain fragments (Fig. 2) in constructs II-1-II and II-3-II (1.5) to obtain efficiently fused BHK cells transfected with GFP-Naα or neurofascinNaα chimeric constructs as described above and incubated for 24 h at 37 °C. Cells were then lysed as above in 600 μl/60-mm dish of lysis buffer with protease inhibitors. 300 μl of cell lysate was then added to either 20 μl of MB-GST beads or 20 μl of GST beads and rocked overnight at 4 °C. The concentration of MB-GST or GST used in each pull-down was typically 2 μl. After incubation, the beads were washed three times with ice-cold lysis buffer, and bound proteins were eluted with twice 1 bed volume of 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0. Cell lysates and proteins associated with MB-GST and GST were probed by immunoblotting using antibodies to GST (Covance) or the HA tag (Covance). Similarly, cell lysates were analyzed to ensure expression of the tagged protein, and batches of beads used in the assays were analyzed using antibodies to MB to ensure the presence of the MB-GST and GST proteins (not shown).

**RESULTS**

**Naα,1.2 Co-localizes with AnkyrinG in Vivo and Binds to Its Repeat Domain in Vitro**—Rat hippocampal neurons in culture (11 days in vitro) were double-labeled with an antibody to the conserved tail region of ankyrinG (480/270 kDa (Fig. 1a, ankyG, 480/270 kDa)) and with a specific antibody to the C-terminal end of rNav1.2 (Fig. 1a, rNav1.2). Neurons exhibited localized concentrations of rNav1.2 at their IS coincident with similar concentrations of ankyrinG (480/270 kDa).

We developed a pull-down assay to determine ankyrinG-binding sites within defined subunits and domains of rNav1.2a using a GST fusion of the repeat domain (MB) of ankyrinG (MB-GST). This construct was expressed at high levels in BHK cells using the Sindbis viral expression system and immobilized on glutathione-Sepharose beads. Similar beads coupled to GST alone were used as controls for each assay. To test for interactions with β subunits, beads were added to cell lysates from BHK cells expressing HA-tagged β1 and β2 subunits (Fig. 1b). As shown in Fig. 1b, these subunits did not exhibit interactions with MB-GST.

To test for interactions with the rNav1.2a α subunit, we incubated MB-GST and GST beads with BHK cell lysates expressing full-length rNav1.2a. However, we were unable to obtain efficient solubilization of the full-length rNav1.2a protein under the non-denaturing conditions used in our binding assay (data not shown). We therefore generated a series of GFP-tagged constructs that spanned rNav1.2a (Fig. 1c) in...
which we tried to maintain the structure of the cytoplasmic loops while preserving the integrity of the transmembrane domains (Fig. 1c). Constructs spanning the rNa\textsubscript{1.2a} subunits III-IV, II-III, and the III domain all precipitated with MB-GST beads and not with GST beads alone (Fig. 1c). This interaction did not appear to involve the C terminus of rNa\textsubscript{1.2a} as deletion of this region of the molecule from construct III-IV (construct III-IV\textsubscript{C}) did not interfere with its ability to interact with MB-GST. In contrast, domain I-II of rNa\textsubscript{1.2a} and a GFP fusion of the cytoplasmic C terminus alone (not shown) did not exhibit interactions with MB-GST.

These results suggested the presence of a site of ankyrin\textsubscript{G} interaction between aa 1044 and 1474 of rNa\textsubscript{1.2a}. Further studies in which individual cytoplasmic loops of the III-IV construct were replaced sequentially with the nonbinding N terminus of rNa\textsubscript{1.2a} confirmed that the binding site was restricted to loop II-III (not shown). These results are in contrast to the findings of Bouzidi et al. (18) who recently reported interactions between ankyrin\textsubscript{G} and fusion proteins representing the isolated intracellular loops between domains I-II and III-IV but not between the II-III loop and ankyrin\textsubscript{G}.

**Ankyrin\textsubscript{G} Associates with a Conserved 9-aa Motif in Loop 2 of VGSC-\alpha Subunits**—To further define the ankyrin\textsubscript{G}-binding site within loop 2, we used deletion analyses. An internal deletion of aa 1024–1121 in loop 2 or its substitution with a portion of loop 1 (aa 599–694) (Fig. 2a, constructs II-\textDelta-III and I-II-III, respectively) abolishes the interaction with MB-GST restricting the binding site to aa 1044–1121 of rNa\textsubscript{1.2a}.

Fig. 2c shows an alignment of the sequence of loop 2 between rat VGSCs. A high degree of homology is observed between aa 1024–1121 of rNa\textsubscript{1.2a} and the analogous region of other neuronal isoforms (87, 76, 71, and 65% identity with rNa\textsubscript{1.1}, -1.3, -1.6, and -1.7, respectively) with the exception of rNa\textsubscript{1.8} and rNa\textsubscript{1.9} (only 15 and 26% identity, respectively). This region is less well conserved between rNa\textsubscript{1.2a} and the skeletal muscle and cardiac isoforms rNa\textsubscript{1.4} (41% identity) and rNa\textsubscript{1.5} (16% identity), respectively.

We decided to exploit the range of conservation within various loop 2 sequences to further map the site of ankyrin\textsubscript{G} interaction. We substituted aa 1024–1121 in construct II-III with the nonbinding N subunits. Bound proteins (left) or 5% of the total lysate used in each pull-down (right) was analyzed by immunoblotting using an antibody to the HA epitope (arrowheads) with respect to the extracellular Ig domain. Right panel, HA-tagged \( \beta_1 \) or \( \beta_2 \) subunits were expressed in BHK cells, and lysates were incubated with glutathione-Sepharose beads conjugated to MB-GST (+ lanes) or GST (= lanes). Bound proteins (left) or 5% of the total lysate used for each pull-down (right) was analyzed by immunoblotting with a GFP antibody. Higher molecular weight denaturation-dependent aggregation artifacts of the expressed rNa\textsubscript{1.2a} subdomains were also observed. Similar artifacts have been reported previously for transiently expressed VGSC-\( \alpha \) subunits in hu-

To test whether loop 2 and the conserved 9-aa motif were sufficient and necessary for ankyrin\textsubscript{G} association, we constructed chimeric molecules consisting of HA-tagged neurofascin extracellular domain fused to the loop 2 sequence (Fig. 3a). Loop 2 sequences from rNa\textsubscript{1.2a} and a variant of rNa\textsubscript{1.2a}
lacking the 9-aa motif (rNav1.1Δ) were utilized (Fig. 3a). These chimeric constructs along with wild type neurofascin 186 kDa were used in pull-down assays with MB-GST. As a negative control, we mutated the tyrosine residue within the FIGQY motif of wild type neurofascin to an alanine (NF Y81A). Phosphorylation of this tyrosine residue has been previously shown to abolish ankyrin binding (20).

Fig. 3a shows that chimeric molecules containing loop 2 sequences from rNav1.1a (NF 1.2) exhibited interactions with MB-GST suggesting that loop 2 alone was sufficient to promote association with ankyrin. However, deletion of the conserved 9-aa motif (NF 1.2Δ) led to a loss of MB-GST interaction. As expected, wild type neurofascin associated strongly with MB-GST, whereas mutation of the tyrosine residue to alanine (NF Y81A) abolished this interaction despite comparable expression levels (Fig. 3a). The observation that a single aa change can abolish ankyrin binding illustrated the sensitivity and specificity of our pull-down assay using MB-GST. Our data demonstrate that loop 2 and the conserved 9-aa motif are necessary for ankyrin binding.

To determine whether the loop 2/ankyrinΔ interaction involved proteins specific to BHK cell lysates, we carried out a yeast two-hybrid reaction. As shown in Fig. 3b, the loop 2 sequence from rNav1.5 fused to the LexA DNA-binding domain (pBTM Nav1.5) was able to activate expression of the L40 HIS3 reporter gene when co-transfected with the MB sequence fused to the Gal4 activation domain (pGAD MB). His3 was not expressed when pBTM Nav1.5 was co-transfected with the Gal4 activation domain alone nor when a mutated version of rNav1.5 lacking the 9-aa motif (pBTM Nav1.5Δ) was used. The wild type neurofascin cytoplasmic domain and the Y81A variant of this domain were used as positive and negative controls, respectively (Fig. 3b). Similar studies involving loop 2 sequences from Na1.2 and -1.6 could not be carried out as these constructs were observed to transactivate reporter genes in the absence of pGAD MB.

**The Conserved 9-aa Motif Affects Localization to the IS of Cultured Hippocampal Neurons**—To determine whether loop 2 and the 9-aa motif affected localization of membrane proteins at the IS, we utilized the neurofascin/loop 2 chimeric constructs. These molecules were transfected into 3 days in vitro hippocampal cells, and these cells were fixed and stained with HA antibodies at various time points after transfection to determine when and where these proteins could be first detected on the cell surface. Cells were then permeabilized and stained with antibodies to ankyrin, 480/270 kDa to delineate the IS.
shown in Fig. 4, ~75% of neurons 6 h after transfection with neurofascin/loop 2 chimeric molecules (NF 1.2) exhibited concentrated HA staining at their IS (Fig. 4A) as delineated by ankyrinG staining (Fig. 4A, inset). Staining was also observed on the cell body of transfected neurons. 24 h after transfection, ~90% of the neurons now exhibited HA concentrations at the IS, and neuronal cell body staining was diminished compared with the signal at the IS (Fig. 4E). In contrast to wild type loop 2 molecules, chimeric molecules lacking the 9-aa motif (NF 1.2A) were observed over the surface of the entire neuron and did not exhibit concentration at the IS even at later time points (Fig. 4, B and F). Indeed, at earlier time points NF 1.2A showed increased concentrations at the distal end of the axon (Fig. 4B).

We also introduced wild type and Y81A neurofascin constructs into hippocampal neurons. Unlike NF 1.2, wild type neurofascin did not immediately exhibit concentrations at the IS except in a small population of neurons (<10%). Instead, HA staining was distributed throughout the cell (Fig. 4G) in a similar fashion to that observed for the Y81A mutant (Fig. 4D). However, NF was observed to accumulate over time at the IS, such that between 45 and 65% of transfected neurons showed IS accumulations of neurofascin at 24 h after transfection (Fig. 4G). Similar concentrations were not observed for the Y81A mutation (Fig. 4H) supporting the idea that concentration of chimeric constructs at the IS is independent of the neurofascin extracellular domain. These results indicate that the 9-aa motif is necessary for the specific localization of neurofascin/loop 2 chimeric constructs at the IS.

**DISCUSSION**

In this study, we describe for the first time the identification of a conserved motif in the family of VGSC-α subunits necessary for their binding to ankyrinG. Initially, we observe that ankyrinG co-localizes with rNa1.2 at the IS of cultured hippocampal neurons and interacts with the domain II-III cytoplasmic linker (loop 2) of rNa1.2a. This represents the first specific function that has been ascribed to this area of VGSC-α subunits. We provide evidence to show that ankyrinG binding may be a common feature of the VGSC-α subunit family and further identifies a conserved 9-aa motif within loop 2 sequences that is necessary for ankyrinG binding. Finally, we demonstrate that the 9-aa motif is required for the localization of neurofascin/loop 2 chimeric molecules to the IS of cultured neurons. Based on these observations, we propose that the 9-aa motif functions in the concentration of VGSCs within excitable membrane domains through interactions with ankyrin.

Recent studies have yielded conflicting data on the nature of the interaction between ankyrin and VGSCs. Regulated interactions between ankyrin and VGSC-β subunits have been detected using a cell-based recruitment assay (17, 21). In contrast to these results, Bouzidi et al. (18) did not detect interactions between ankyrinG and the cytoplasmic domains of the β subunits using bacterially expressed proteins in an in vitro binding assay. However, they did detect interactions with recombinant fusion proteins representing the I-II (loop 1) and the III-IV (loop 3) cytoplasmic linkers. They detected no interactions with loop 2. In our study, we also did not detect interactions with the VGSC-β subunits using ankyrinG, but detected an interaction with the loop 2 cytoplasmic linker of the α subunit. We further confirmed this interaction using yeast two-hybrid and demonstrated that loop 2 was sufficient for the co-localization of chimeric molecules at the IS with ankyrinG. Differences between our findings and those of Bouzidi et al. (18) might reflect their use of bacterially expressed polypeptides. In support of this idea, we have been unable to detect interactions between bacterially expressed ankyrinG and loop 2 in an in vitro assay.

To further examine the effects of loop 2 on VGSC-α subunit localization we have used chimeric neurofascin/Na1,2 molecules and observed that these molecules localize at the IS in the presence of the 9-aa motif. The observation that chimeric molecules are also initially detected on the neuronal cell body but are down-regulated there over time (Fig. 4) suggests that the retention of chimeric molecules at the IS is important for their concentration. However, ankyrin binding could also play a role in the trafficking of these chimeric molecules to the initial segment as has been suggested for the intracellular sorting of Ca2+-homeostasis proteins in cardiomyocytes (22).

The finding of a conserved motif in VGSCs involved in ankyrinG interaction is supported by increasing evidence for spatial and temporal diversity in VGSC isoforms enriched at ankyrinG-defined excitable membrane domains. Both Na1.1 and Na1.2 have been observed at the nodes of Ranvier in young mice and are gradually replaced with Na1.6 during development (23, 24). Na1.6 is the VGSC isoform found at most adult nodes in the peripheral nervous system and the central nervous system (25); however, Na1.2, -1.8, and -1.9 have also been detected at adult nodes (24, 26, 27). A similar diversity is observed at the IS where Na1.2, -1.6, and -1.8 have been localized (11, 26). In other tissues, ankyrinG is also localized to the neuromuscular junction (10) where it is a candidate to interact with skeletal isoforms of the sodium channel such as Na1.4. The observation that ankyrinG can also interact with cardiac Na1.5 suggests that isoforms of ankyrinG might also be linked with heart disease as has been shown for ankyrinG in Long QT syndrome (28). What are the mechanisms responsible for the segregation of specific VGSC isoforms with ankyrinG in excitable membrane domains? The distinct spatial and temporal pattern of expres-
sion of VGSC isoforms in nervous tissue (29) is clearly a factor in determining the molecular identity of the VGSC present at the nodes of Ranvier and the IS. Indeed, the gradual isoform switch from Nav1.2 to Nav1.6 at developing nodes is paralleled by a down-regulation of Nav1.2 and an up-regulation of Nav1.6 proteins (24). The selective segregation of VGSCs with ankyrinG could also result from isoform-dependent differences in the affinities of interaction that are not detectable by the methods used in this study. In addition, post-translational modifications similar to those reported for neurofascin (20) could regulate the ankyrin/VGSC interaction and play a role in determining VGSC specificity.

Additional sequences within the α subunit could also influence localization of specific VGSC isoforms within neurons. For example, the C terminus of rNa1.2 targets CD4 chimeric molecules to the axon of hippocampal neurons, whereas distribution of a similar construct derived from the C terminus of rNa1.6 is restricted to the somatodendritic region (30). The role of accessory subunits in VGSC localization should also be considered. β subunits appear to influence the surface expression of VGSC isoforms (31) and are suspected to exhibit selective associations with different α subunits (24). Similarly, the association of annexin II light chain with the N terminus of Na1.8 increases its translocation to the plasma membrane (32). Thus, accessory subunits may participate in the functional localization of VGSC isoforms by promoting their specific insertion into neuronal membranes.

Given the high degree of homology between the repeat domains of different ankyrin molecules, we might also expect VGSC-α subunits to interact with other ankyrins such as ankyrinα or ankyrinβ. Indeed, ankyrinβ has been shown to bind to uncharacterized native VGSC preparations from the rat brain (8). However, it is yet to be determined whether the affinities for a specific VGSC isoform vary between different ankyrins.

Data base searches with a consensus sequence for the 9-aa ankyrin-binding motif found this sequence to be present only in members of the VGSC-α subunit family, suggesting that this particular ankyrin-binding motif is unique to these proteins. This is in agreement with current models of ankyrin interactions proposing that different families of ankyrin-binding proteins independently evolve the ability to bind ankyrin (16). These searches also found the motif to be present in VGSC-α subunits from non-mammalian vertebrates such as the puffer fish, Takifugu pardalis, and the newt. Intriguingly, this motif was not found in the loop 2 α-subunit sequences of invertebrate sodium channels such as those observed in Drosophila melanogaster. It would therefore be of some interest to determine...
whether or not VGSC/ankyrin-enriched excitable domains can be detected in Drosophila.

Observations in knockout animals have demonstrated the role of the cytoskeletal elements ankyrin, and βIV spectrin in establishing and/or maintaining a functional axon IS required for initiation of an action potential (11, 12, 33). The 9-aa motif described in this study links VGSCs to these cytoskeletal elements and as such is necessary to confer the excitable dimension to this domain. The highly conserved nature of the motif suggests a complex regulation of VGSC/ankyrin association to achieve isoform specificity but provides a universal code for the establishment of VGSC-enriched membrane domains through ankyrin interaction that is likely to be common to a variety of tissues and organisms.

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Identification of a Conserved Ankyrin-binding Motif in the Family of Sodium Channel α Subunits
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