

Restriction of 480/270-kD Ankyrin_G to Axon Proximal Segments Requires Multiple Ankyrin_G-specific Domains

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Abstract. Ankyrin_G (−/−) neurons fail to concentrate voltage-sensitive sodium channels and neurofascin at their axon proximal segments, suggesting that ankyrin_G is a key component of a structural pathway involved in assembly of specialized membrane domains at axon proximal segments and possibly nodes of Ranvier (Zhou, D., S. Lambert, D.L. Malen, S. Carpenter, L. Boland, and V. Bennett, manuscript submitted for publication). This paper addresses the mechanism for restriction of 270-kD ankyrin_G to axon proximal segments by evaluation of localization of GFP-tagged ankyrin_G constructs transfected into cultured dorsal root ganglion neurons, as well as measurements of fluorescence recovery after photobleaching of neurofascin–GFP-tagged ankyrin_G complexes in nonneuronal cells. A conclusion is that multiple ankyrin_G-specific domains, in addition to the conserved membrane-binding domain, contribute to restriction of ankyrin_G to the axonal plasma membrane in dorsal root ganglion neurons. The ankyrin_G-specific spectrin-binding and tail do-

main are capable of binding directly to sites on the plasma membrane of neuronal cell bodies and axon proximal segments, and presumably have yet to be identified docking sites. The serine-rich domain, which is present only in 480- and 270-kD ankyrin_G polypeptides, contributes to restriction of ankyrin_G to axon proximal segments as well as limiting lateral diffusion of ankyrin_G–neurofascin complexes. The membrane-binding, spectrin-binding, and tail domains of ankyrin_G also contribute to limiting the lateral mobility of ankyrin_G–neurofascin complexes. Ankyrin_G thus functions as an integrated mechanism involving cooperation among multiple domains heretofore regarded as modular units. This complex behavior explains ability of ankyrin_B and ankyrin_G to sort to distinct sites in neurons and the fact that these ankyrins do not compensate for each other in ankyrin gene knockouts in mice.

Key words: ankyrin • axon initial segment • cell polarity • membrane dynamics • spectrin

CLUSTERING of voltage-gated sodium channels at the axon proximal segments and nodes of Ranvier is believed to be essential for production and propagation of action potentials in myelinated axons. The mechanisms responsible for concentrating sodium channels in these specialized membrane domains in axons are not established, but are likely to involve specialized members of the ankyrin family of spectrin-binding proteins. Ankyrins are membrane-associated proteins that bind to certain ion channels, including the voltage-sensitive sodium channel, and have the potential to couple these channels to the spectrin–actin network (Srinivasan et al., 1988; Bennett and Gilligan, 1993; Bennett et al., 1997). Ankyrins also

bind through their conserved membrane-binding domains to cell adhesion molecules of the L1 cell adhesion molecule (CAM) family (Davis et al., 1993, 1996; Davis and Bennett, 1994; Garver et al., 1997), and have been proposed to form lateral complexes involving L1 CAMs and ion channels (Michaely and Bennett, 1995a,b; Lambert et al., 1997). Ankyrins are concentrated at physiological sites with a high density of voltage-sensitive sodium channels including nodes of Ranvier, axon proximal segments, and postsynaptic folds of the neuromuscular junction (Flucher and Daniels, 1989; Kordeli et al., 1990). The isoforms of ankyrin colocalized with voltage-sensitive sodium channels at nodes of Ranvier and axon proximal segments have been identified as 480/270-kD alternatively spliced variants of ankyrin_G (Kordeli et al., 1995). An ankyrin_G polypeptide also has been identified at the neuromuscular junction (Wood and Slater, 1998). In addition to ankyrin_G, isoforms of neurofascin and NrCAM, which are members of the L1 CAM family, have been identified at nodes of Ranvier and axon proximal segments (Davis et al., 1996).

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480/270-kD ankyrin_G colocalizes with sodium channel clusters in amyelinated regions of peripheral nerves of the dystrophic mouse (Deerinck et al., 1997), and in axons of cultured retinal ganglion neurons in the absence of direct contact with glial cells (Kaplan et al., 1997). These results imply targeting of channels and ankyrin to sites defined by intrinsic polarity to the axon, independent of cell-cell contact, although soluble factors released by glial cells may play a role (Kaplan et al., 1997). 480/270-kD ankyrin_G also colocalized in the developing sciatic nerve in clusters containing sodium channels, neurofascin, and NrCAM (Lambert et al., 1997). The earliest event observed in myelinating nerve, preceding expression of myelin-associated glycoprotein (MAG) by Schwann cells, was clustering of neurofascin and NrCAM, which were followed by ankyrin and sodium channels. These observations led to the proposal that extracellular signals, perhaps related to the soluble factor described by Kaplan et al. (1997), induce clustering of neurofascin and NrCAM, which in turn recruit ankyrin_G and sodium channels (Lambert et al., 1997).

Mice deficient in cerebellar ankyrin_G polypeptides have recently been developed by targeted gene disruption (Zhou, D., S. Lambert, P. Melen, S. Carpenter, L. Boland, and V. Bennett, manuscript submitted for publication). Cerebellar neurons of these ankyrin_G (−/−) mice fail to concentrate sodium channels as well as neurofascin at axon proximal segments. These findings demonstrate that 480/270-kD ankyrin_G is required for clustering of both sodium channels and neurofascin at axon proximal segments. Neurofascin and L1 CAMs therefore are not sufficient to provide the initial targeting signal for ankyrin_G at axon proximal segments, in contrast to expectations from observations of development of the node of Ranvier (Lambert et al., 1997).

This paper addresses the mechanism for restriction of 270-kD ankyrin_G to axon proximal segments of cultured dorsal root ganglion (DRG)¹ neurons. A conclusion is that multiple ankyrin_G-specific domains, in addition to the conserved membrane-binding domain, cooperate in targeting and restriction of ankyrin_G to axon proximal segments. Moreover, measurements of fluorescence recovery after photobleaching (FRAP) of green fluorescent protein (GFP)-tagged ankyrin constructs cotransfected with neurofascin in a nonneuronal cell line demonstrate that the membrane-binding, spectrin-binding, tail and serine-rich domains each contribute to immobilization of 270-kD ankyrin_G-neurofascin complexes in the plasma membrane. These results provide strong evidence that association between the ankyrin_G membrane-binding domain and neurofascin is not sufficient to restrict ankyrin_G to axon proximal segments, and that other ankyrin_G domains and yet to be defined protein interactions are also involved.

Materials and Methods

DRG Culture

The culture of embryonic DRG was modified from methods previously

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; DRG, dorsal root ganglion; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; HA, hemagglutinin.

described (Kleitman et al., 1991). In brief, DRGs were obtained from E15 rat embryos and plated on laminin-coated dishes after dissociation with 0.02% collagenase and 0.25% trypsin-EDTA. The medium was changed from normal medium (10% FBS in DME, 100 ng/ml NGF) to the defined medium (1 × N1 additive [Sigma Chemical Co., St. Louis, MO], 100 ng/ml NGF, 10 μM FdU and 10 μM uridine in DME) 24 h after plating. The culture was kept in the defined medium for 2 d before changing back to normal medium for an additional 2 d. The antimetabolic cycle was repeated once to kill most of dividing nonneuronal cells. After 8 d in culture, DRG neurons are ready for immunofluorescence and gene gun transfection.

Preparation of cDNA Constructs

Assembly of the full-length cDNA for 270-kD ankyrin_G (Ank270) was achieved by ligating the first half of membrane-binding domain, which was isolated from adult rat brain 5'-stretch plus cDNA library (Clontech Laboratories Inc., Palo Alto, CA) and confirmed by DNA sequencing, with construct M-Sb-Sr-T-C (Zhang and Bennett, 1996) through the EcoRI-NsiI sites. The COOH-terminal domain of 270-kD ankyrin_G was PCR amplified and introduced into the Sall site of pEGFP-N1 vector (Clontech Laboratories Inc.), while keeping in-frame with the downstream EGFP protein (Ank-Ct). The full-length 270-kD ankyrin_G with GFP tagged at its COOH terminus (see Fig. 2, *Ank270-GFP*) was prepared by ligating the EcoRI-EcoRV fragment of construct Ank270 into the EcoRI-EcoRV sites of construct Ank-Ct. The cDNA construct for 190-kD ankyrin_G (see Fig. 2, *Ank270[ΔSR,T]-GFP*) was prepared similarly except using construct M-Sb-C (Zhang and Bennett, 1996) instead of M-Sb-Sr-T-C. The cDNA construct lacking the first half of the membrane-binding domain (corresponding to the amino acids 0–393 of human ankyrin_G [Kordeli et al., 1995]) was prepared by putting the EcoRV fragment of M-Sb-Sr-T-C (Zhang and Bennett, 1996) into the corresponding site of the construct Ank-Ct (see Fig. 2, *Ank270[ΔM1,2]-GFP*). The construct containing only the membrane-binding and the spectrin binding domain (see Fig. 2, *Ank270[ΔSR,T,Ct]-GFP*) was obtained by replacing the EcoRV-NotI fragment of construct Ank270[ΔSR,T]-GFP with a PCR-amplified EGFP sequence. The construct expressing the membrane-binding domain was made by introducing a PCR-amplified sequence into the EcoRI-Sall sites of pEGFP-N1 vector while keeping in-frame translation of the downstream EGFP (M-GFP). Constructs expressing the serine-rich (SR-GFP), spectrin-binding (SB-GFP), tail (T-GFP), and COOH-terminal (Ct-GFP) domains were prepared similarly. To make a construct with deletion of the serine-rich domain (see Fig. 2, *Ank270[ΔSR]-GFP*), a PCR-amplified fragment of the tail domain of the 270-kD ankyrin_G was introduced into the EcoRV site of construct Ank270(ΔSR,T)-GFP.

The full-length rat neurofascin cDNA with a hemagglutinin (HA) epitope at the NH₂ terminus (Garver et al., 1997) was cut out by HindIII-NotI from PBluescript KS vector (Stratagene, La Jolla, CA), and subcloned into the corresponding sites of pEGFP-N1 vector (Clontech Laboratories Inc.). The resultant construct (see HA-NF in Fig. 2) does not contain the EGFP sequence. The cytoplasmic domain-deleted neurofascin (see HA-NF[ΔCD] in Fig. 2) was made by replacing the ScaI-NotI fragment of construct HA-NF with a PCR-amplified fragment containing the sequence of the ScaI-ApaI fragment with a stop codon right after the ApaI site. The extracellular domain-truncated neurofascin (see Fig. 2, *HA-NF[ΔEC]*) was prepared through two steps. First, the 5'-untranslated region of neurofascin with the start codon, the signal peptide, and the HA tag was PCR amplified and subcloned into the BglII-HindIII sites of pEGFP-N1 vector. Then the transmembrane and cytoplasmic domains were introduced into the HindIII-NotI sites of the first-step construct. Construct with the full-length neurofascin tagged with EGFP at its COOH terminus was obtained also by two steps (see HA-NF-GFP in Fig. 2). The HindIII-ApaI fragment of neurofascin in pBluescript KS vector was transferred into the corresponding sites of pEGFP-N3 vector (Clontech Laboratories Inc.) so as to keep in-frame reading of EGFP with the introduced neurofascin fragment. Then a PCR-amplified cytoplasmic domain of neurofascin was introduced into the ApaI site of the above construct.

cDNA Transfection and Immunofluorescence

DRG cultures were transfected using Helios™ Gene Gun system following the manufacturer's instructions. 150–180 psi pressure was used to deliver cDNA-coated microcarriers into DRG cultures. 24 h after transfection, DRG cultures were fixed in 2% paraformaldehyde and then subjected to 0.5% Triton X-100 permeabilization before being stained

with a polyclonal antibody specific for GFP (Clontech Laboratories Inc.). Immunofluorescence for endogenous proteins was performed similarly.

Human kidney 293 cells were cultured in 10% FBS and DME (GIBCO BRL, Gaithersburg, MD) and transfected by Lipofectamine according to the manufacturer's protocol (GIBCO BRL). 24 h after transfection, cultures were either subject to photobleaching (see below) or fixed in 2% paraformaldehyde and then stained with a monoclonal antibody specific for the HA epitope of neurofascin (Berkeley Antibody Co., Richmond, CA).

Chicken polyclonal antibody against 270/480-kD ankyrin_G (Zhang and Bennett, 1996) and rabbit polyclonal antibodies against ankyrin_B (Chan et al., 1993), brain spectrin (Davis and Bennett, 1983), and neurofascin (Davis et al., 1996) were described previously. TRITC-conjugated goat anti-mouse and goat anti-rabbit, and FITC-conjugated goat anti-rabbit antibodies were purchased from Rockland (Gilbertsville, PA). FITC-conjugated donkey anti-chicken was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Fluorescence Recovery Measurement after Photobleaching

Photobleaching of the cytoplasm-localized Ank270-GFP was performed essentially as described, using an Odyssey confocal imaging system (Garver et al., 1997). Photobleaching of the plasma membrane-localized transfected proteins was performed in a Zeiss LSM 410 laser confocal sys-

tem coupled with an UV laser (Carl Zeiss Inc., Thornwood, NY). A selected region enclosing the plasma membrane to be studied was photobleached using the UV laser beam for 60 ms. The radii of photobleached regions range from 1 to 3 μm. The fluorescence recovery inside the photobleached area was immediately recorded by a home-designed program containing several 'Time Series' with different time intervals. The recorded data was analyzed using NIH Image software (Bethesda, MD). The recovered fraction was calculated as $(I_e - I_i)/(I_o - I_i)$. I_o , the average intensity of the selected region before photobleaching; I_i , the average intensity of the selected region immediately after photobleaching; and I_e , the average intensity of the selected region after 50 s of recovery. The recovery rate was calculated as w^2/t , where w is the radius of the photobleached region, and t is the time in seconds required for the average intensity of the selected region to recover to the value of $(I_e + I_i)/2$.

Results

Native Ankyrin_G and Neurofascin but Not Ankyrin_B and Spectrin Are Restricted to Axon Proximal Segments of DRG Neurons

480/270-kD ankyrin_G (Fig. 1, A, D, and G) and neurofascin (Fig. 1 H) are concentrated at the axolemma of axon

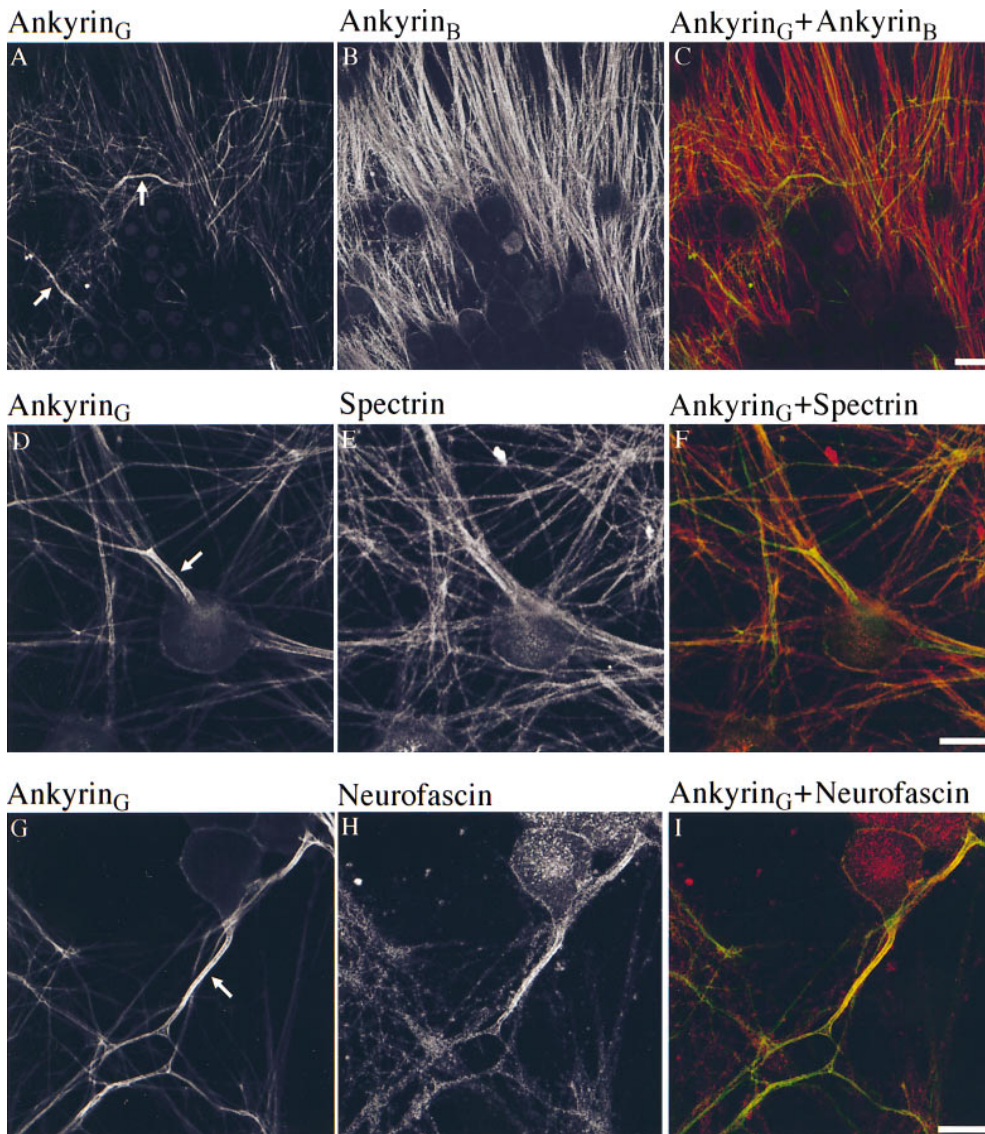


Figure 1. Distribution of 270/480-kD native ankyrin_G, ankyrin_B, spectrin, and neurofascin in cultured DRG neurons. 270/480-kD ankyrin_G (A) and ankyrin_B (B) are double labeled in 9-d-old embryonic DRG culture. (C) Composite image of A and B. Ankyrin_G is concentrated at the proximal segment (A, arrows), whereas ankyrin_B is more evenly distributed in axons (B). Spectrin (E), compared with double-labeled ankyrin_G (D, arrow, proximal segment), also lacks polarized distribution at the proximal segment. (F) Composite image of D and E. Neurofascin (H), a member of L1 CAM family of cell adhesion molecules, is concentrated and colocalized with ankyrin_G at the proximal segment (G, arrow). (I) Composite image of G and H. The green channel (FITC) corresponds to ankyrin_G stain whereas the red channel (TRITC) represents other proteins. Bar, 25 μm.

proximal segments of cultured embryonic DRG neurons. Ankyrin_G also is associated with the plasma membrane in cell bodies of neurons, although at a lower level than in the axon proximal segment (Fig. 1, *A*, *D*, and *G*). Ankyrin_B, in contrast, is distributed along the length of axons and is present in only low levels in axon proximal segments (Fig. 1 *B*). Ankyrin_B also exhibits only minimal staining in the cell body (Fig. 1 *B*). Spectrin is distributed along axons in a pattern resembling ankyrin_B (Fig. 1 *E*). These results demonstrate that cultured DRG neurons have a polarized distribution of native ankyrin_G and neurofascin in axon proximal segments similar to localization of these proteins in sections of intact brain tissue (Kordeli et al., 1995; Davis et al., 1996).

Ankyrin_G and ankyrin_B both bind to neurofascin with high affinity through their membrane-binding domains, which share 74% amino acid identity (Davis et al., 1993; Davis and Bennett, 1994; Kordeli et al., 1995; Zhang et al., 1998). Thus, enrichment of neurofascin and ankyrin_G, but not ankyrin_B, at axon proximal segments of DRG neurons (Fig. 1, *C* and *I*) suggests a mechanism independent of neurofascin for targeting ankyrin_G specifically to the axolemma of axon proximal segments. The targeting protein(s) are not currently known spectrin polypeptides, based on lack of concentration of spectrin at axon proximal segments (Fig. 1 *F*).

Unique Serine-rich and Tail Domains of 270-kD Ankyrin_G Contribute to Restriction of Ankyrin_G at Axon Proximal Segments

Ankyrin_G is distinguished from ankyrin_B by a unique O-GlcNAc-glycosylated serine-rich domain and a highly divergent tail domain (Kordeli et al., 1995; Zhang and

Bennett, 1996). In addition, ankyrin_G and ankyrin_B differ in sequence in portions of the spectrin-binding domain and in the COOH-terminal domain. To identify the roles of the serine-rich and tail domains in targeting 480/270-kD ankyrin_G to axon proximal segments, we transfected DRG neurons with cDNAs, each with a COOH-terminal GFP tag, encoding 270-kD ankyrin_G and its variants lacking the serine-rich and/or tail domains (Fig. 2). The distribution of transfected 270-kD ankyrin_G (Fig. 2, *Ank270-GFP*) is visualized by immunostaining of the COOH-terminal GFP tag, and is highly restricted to the axolemma of the proximal segment in a pattern similar to native 480/270-kD ankyrin_G (Fig. 3, *A* and *B*). The intensity of immunofluorescence attenuates abruptly beyond the proximal segment (Fig. 3 *A* and Fig. 4, *A* and *B*), which is usually tens of micrometers in length in DRG neurons (Hsieh et al., 1994). Double labeling of cultures cotransfected with GFP-tagged 270-kD ankyrin_G (Fig. 3 *D*) and HA-tagged cytoplasmic domain-truncated neurofascin, HA-NF(Δ CD) (Fig. 2 and Fig. 3 *E*), indicates the polarized distribution of the transfected ankyrin_G is not due to incomplete extension or partial optical sectioning of axons since the full length of the axon is well displayed by mutant neurofascin (Fig. 3 *E*). The relatively even distribution of cytoplasmic domain-truncated neurofascin along the length of the axon also suggests that the extracellular and transmembrane domains of neurofascin are not sufficient to achieve polarized localization at the proximal segment. Some GFP-tagged ankyrin_G is also targeted to areas of the plasma membrane of the cell body, at levels approximating those in the proximal segments (Fig. 3 *C*). This behavior is in contrast to the distribution of native ankyrin_G and could represent a consequence of high levels of expression of the transfected ankyrin_G.

Transfected 190-kD ankyrin_G (refer to Fig. 2, *Ank270*

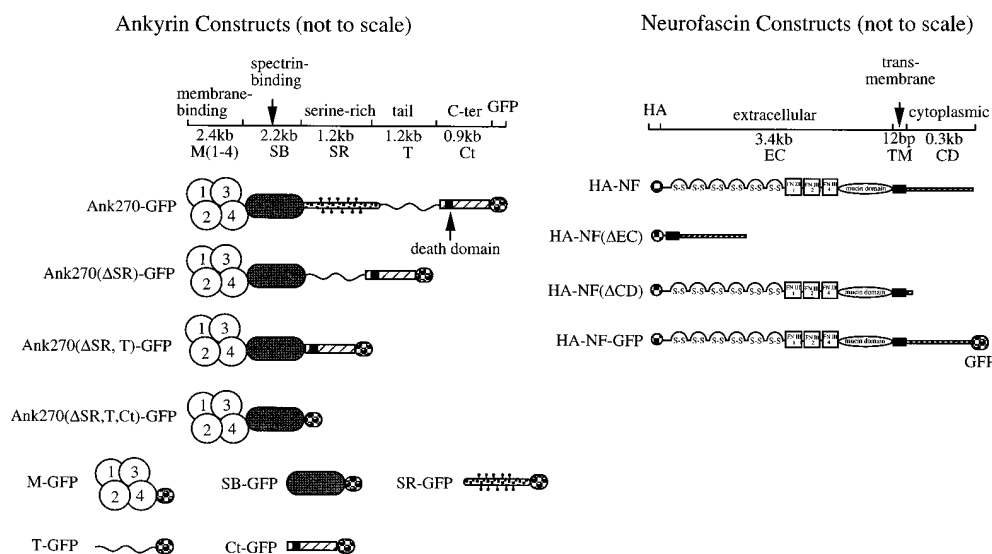


Figure 2. Schematic diagram of cDNA constructs used in transfection experiments. Preparation of cDNA constructs of ankyrin and neurofascin is described in Materials and Methods. Ank270-GFP represents the full-length 270-kD ankyrin_G with GFP tag at its COOH terminus. Construct Ank270(Δ SR)-GFP has a deleted serine-rich domain and Ank270(Δ M1,2)-GFP lacks the first half of the membrane-binding domain. Construct Ank270(Δ SR,T)-GFP is a 190-kD natural isoform of ankyrin_G without the serine-rich and tail domains. Deletion of the COOH-terminal domain from construct Ank270(Δ SR,T)-GFP results in construct Ank270(Δ SR,T,Ct)-GFP. M-GFP, SB-GFP, SR-GFP, T-GFP, and Ct-GFP represent the membrane-binding domain, the spectrin-binding domain, the serine-rich domain, the tail domain, and the COOH-terminal domain, respectively. In the neurofascin construct family, construct HA-NF contains the full-length of neurofascin with HA-epitope at the NH₂ terminus. Constructs HA-NF(Δ EC) and HA-NF(Δ CD) have the deletion of the extracellular domain and the cytoplasmic domain respectively. HA-NF-GFP contains a GFP tag at the COOH terminus of the full-length neurofascin.

results in construct Ank270(Δ SR,T,Ct)-GFP. M-GFP, SB-GFP, SR-GFP, T-GFP, and Ct-GFP represent the membrane-binding domain, the spectrin-binding domain, the serine-rich domain, the tail domain, and the COOH-terminal domain, respectively. In the neurofascin construct family, construct HA-NF contains the full-length of neurofascin with HA-epitope at the NH₂ terminus. Constructs HA-NF(Δ EC) and HA-NF(Δ CD) have the deletion of the extracellular domain and the cytoplasmic domain respectively. HA-NF-GFP contains a GFP tag at the COOH terminus of the full-length neurofascin.

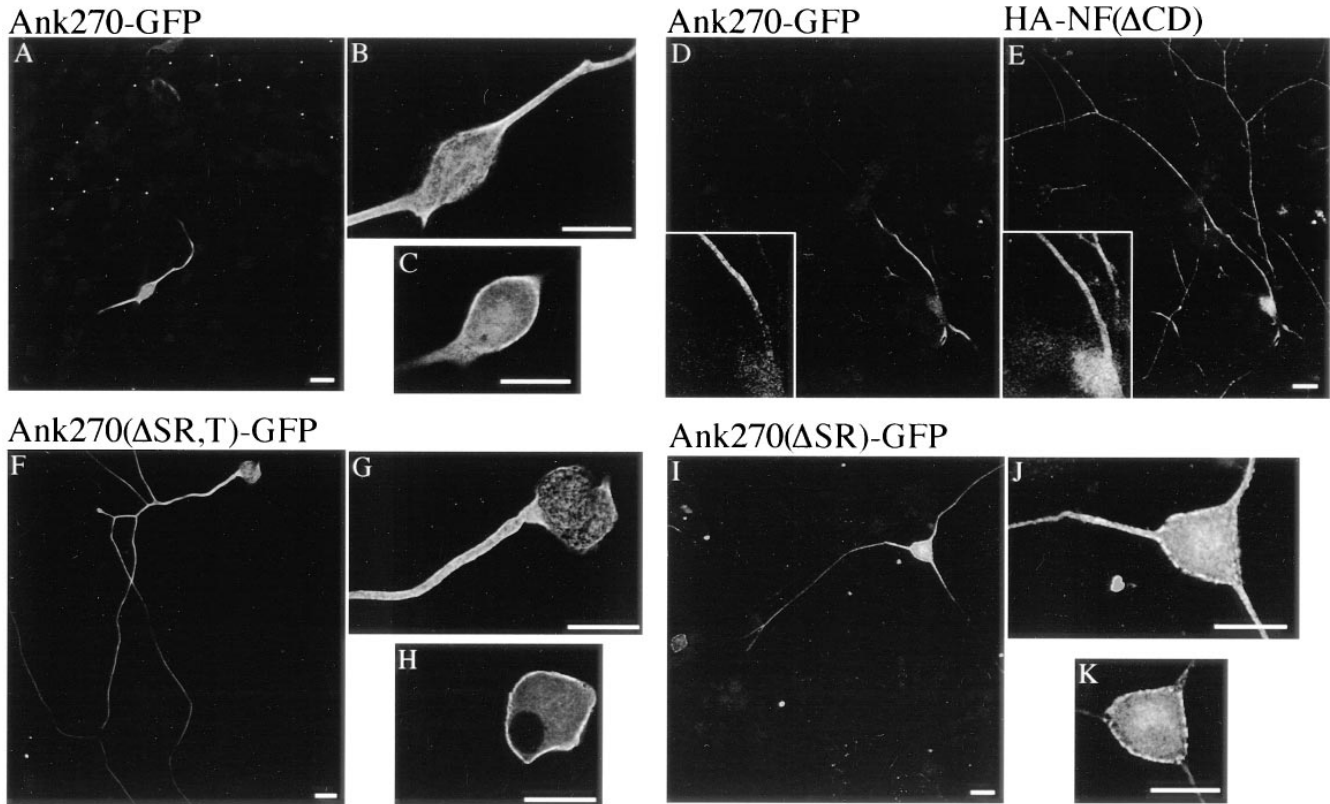


Figure 3. The unique serine-rich and tail domain of ankyrin_G contribute to restriction of 270-kD ankyrin_G at the axon proximal segment. The cDNAs of 270-kD ankyrin_G and its variants lacking the serine-rich and/or tail domains are transfected into 8-d-old DRG culture using Helios™ Gene Gun system. Expression of transfected proteins is visualized by immunostaining of the GFP tag. The transfected 270-kD ankyrin_G (*Ank270-GFP*) is highly restricted to the proximal segment (*A*). High zoom recording reveals plasma membrane stain of the transfected 270-kD ankyrin_G at the proximal segment (*B*) and the cell body (*C*). The restricted location of transfected *Ank270-GFP* (*D*) is displayed against the full length of the transfected axon which is revealed by double labeling of the cotransfected cytoplasmic domain-deleted neurofascin (*E*). The transfected 190-kD ankyrin (*Ank270[ΔSR,T]-GFP*) is also localized at the plasma membrane of the proximal segment (*G*) and the cell body (*H*). However, a significant amount of the transfected *Ank270(ΔSR,T)-GFP* (*Ank270[ΔSR]-GFP*) improves but does not totally restore restriction of the transfected ankyrin to the proximal part of the axon (*I*). *J* and *K* show membrane localization of *Ank270(ΔSR)-GFP* at the proximal segment and the cell body respectively. Bar, 25 μm.

[*ΔSR,T*]-*GFP*), which lacks the unique serine-rich and tail domains of 270-kD ankyrin_G (Kordeli et al., 1995), is also located at the axolemma of the proximal segment (Fig. 3, *F* and *G*). However, 190-kD ankyrin_G is much less restricted, and distributes well beyond the proximal segment into the axon (Fig. 3 *F* and Fig. 4, *A* and *B*). The distance required for the intensity of immunofluorescence to drop to one-third of the intensity at the proximal segment is more than twice as long for 190-kD ankyrin_G than for 270-kD ankyrin_G (Fig. 4 *B*). Insertion of the tail domain into 190-kD ankyrin_G (refer to Fig. 2, *Ank270[ΔSR-GFP]*) improves, but does not completely restore, the restriction of the transfected ankyrin_G to the proximal area of the axon (Fig. 3 *I* and Fig. 4, *A* and *B*). *Ank270(ΔSR)-GFP* distributes in a punctate pattern different from that of 270-kD ankyrin_G and 190-kD ankyrin_G (Fig. 3, *J* and *K*). These data suggest that the unique serine-rich domain and tail domain of 270/480-kD ankyrin_G contribute to restriction of ankyrin_G at axon proximal segments.

Spectrin-binding and Tail Domains of Ankyrin_G Contain Specific Plasma Membrane-binding Sites

To examine which domain(s) are sufficient for restriction of ankyrin_G at the axolemma of axon proximal segments, different domains of 270-kD ankyrin_G were individually introduced into DRG neurons. The membrane-binding domain (Fig. 5, *E* and *F*), the serine-rich domain (Fig. 5, *G* and *H*) and the COOH-terminal domain (Fig. 5, *I* and *J*) are predominantly distributed throughout the cytoplasm of the cell body and the axon, but are not concentrated at the axolemma. The failure in recruiting the membrane-binding domain to the plasma membrane might be a consequence of over-expression and does not exclude the possibility of a limited number of binding sites. The spectrin-binding domain (Fig. 5, *A* and *B*) and the tail domain (Fig. 5, *K* and *L*), on the other hand, were targeted to the axolemma of the proximal segment as well as the plasma membrane of the cell body. The spectrin-binding domain

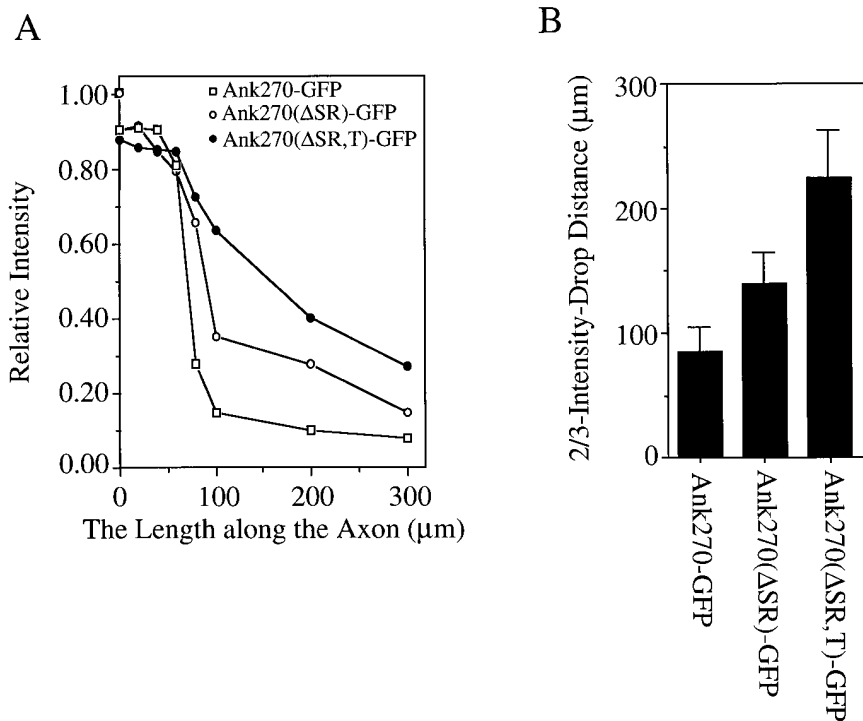


Figure 4. The immunofluorescence intensity profile of transfected ankyrins along the length of axons. The immunofluorescence intensity of the GFP tag of transfected ankyrins is measured using Carl Zeiss LSM measurement program. The distance is calculated from the starting region of the proximal segment. The fluorescence intensity is normalized to the intensity at the plasma membrane of the cell body. (A) Measurement of the intensity drop along the length of the axon. (B) Two-thirds of the intensity-drop distance (the distance for the intensity of immunofluorescence to drop to one-third of the intensity at the starting region of the proximal segment). Data in B represent the mean \pm SEM for five transfected neurons.

also distributes in a punctate pattern similar to that of Ank270(Δ SR)-GFP (Fig. 5, A and B). However, neither the spectrin-binding nor tail domains are restricted to the proximal segment as the case with transfected 270-kD ankyrin_G, and their intensity-drop profiles along axons are similar to that of 190-kD ankyrin_G (Fig. 6, A and B). These data suggest an important role for plasma membrane-binding sites in the spectrin-binding and the tail domain for restriction of ankyrin_G at axon proximal segments. The restrictive effect of the serine-rich domain (refer to Fig. 3 I and see Fig. 5, G and H) seems unrelated to targeting to the plasma membrane, at least as resolved by these techniques.

The transfected spectrin-binding domain of ankyrin_B is not targeted to the axolemma and is distributed in the axoplasm and the cytoplasm of the cell body (Fig. 5, C and D). The spectrin-binding domain of ankyrin_G thus contains targeting information lacking in the spectrin-binding domain of ankyrin_B, and could contribute to specific restriction of ankyrin_G to axon proximal segments.

Ankyrin_G Domains Cooperate in Restricting the Lateral Mobility of Ankyrin_G-Neurofascin Complexes

The serine-rich domain and possibly other domains of 270-kD ankyrin_G could contribute to restriction of ankyrin_G to axon proximal segments through interactions limiting lateral mobility in addition to binding to membrane-targeting sites. Activity of ankyrin_G domains in immobilizing ankyrin_G-neurofascin complexes was evaluated in a nonneuronal cell line (293 cells from human embryonic kidney) cotransfected with GFP-tagged ankyrin_G and HA-tagged neurofascin (Fig. 7). 270-kD ankyrin_G transfected alone is located throughout the cytoplasm of 293 cells (Fig. 7 A). However, 270-kD ankyrin cotransfected with neurofascin

is recruited to the plasma membrane (Fig. 7, B and C). Targeting of transfected ankyrin_G in 293 cells to the plasma membrane is solely governed by interactions between the membrane-binding domain and cotransfected neurofascin, since the membrane-binding domain of ankyrin_G alone is sufficient for membrane recruitment by cotransfected neurofascin (Fig. 7, D and E). Moreover, deletion of the first half of the membrane-binding domain abolishes recruitment of ankyrin_G to the plasma membrane even though the COOH-terminal, tail, serine-rich, and spectrin-binding domains are still present (Fig. 7, F and G). The structural requirements for activity of neurofascin in recruiting ankyrin_G are explored in a separate study (Zhang et al., 1998).

Dynamic behavior of GFP-tagged ankyrin_G-neurofascin complexes were examined by measurements of FRAP of the GFP signal (Fig. 8). Dynamic parameters were first determined for GFP-tagged 270-kD ankyrin_G (Ank270-GFP) alone, which was localized in the cytoplasm, and for GFP-tagged neurofascin (HA-NF-GFP) alone. Cytoplasmic 270-kD ankyrin_G exhibits rapid and nearly complete recovery after photobleaching (91% recovery at a rate of 1.1×10^{-7} cm²/s), consistent with the diffusion rate of a protein in solution (Fig. 8, A and B). GFP-tagged neurofascin (HA-NF-GFP) transfected in the absence of cotransfected ankyrin_G exhibited a 72% recovery at a rate of 5.6×10 cm²/s (Fig. 8, A and C). This rate is comparable to other membrane proteins that are freely diffusing in plasma membranes (Jacobson et al., 1987; Zhang et al., 1991). In contrast, plasma membrane-associated 270-kD ankyrin_G (Ank270-GFP cotransfected with HA-NF) displays a greatly reduced rate (4×10^{-10} cm²/s) as well as extent (10%) of FRAP (Fig. 8, A, B, and C). This dramatic decrease of membrane lateral mobility is not due to lateral association of the extracellular domain of neurofascin with

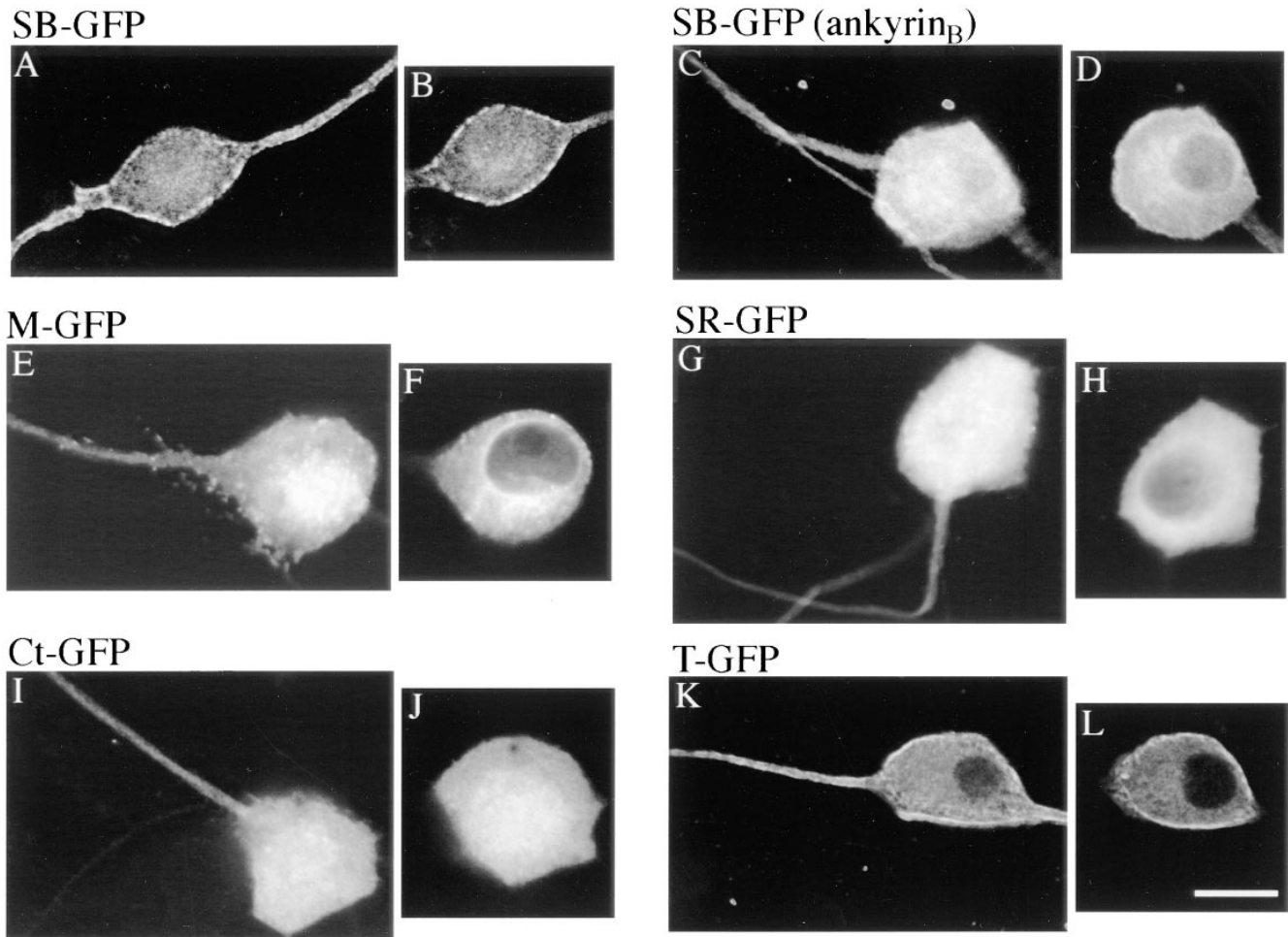


Figure 5. The spectrin-binding domain and the tail domain of ankyrin_G contain binding sites to the axolemma of axon proximal segments. Different domains of 270-kD ankyrin_G tagged with GFP at their COOH termini were individually transfected into DRG neurons. Expression of transfected proteins is examined by immunostaining of the GFP tag. The spectrin-binding domain SB-GFP (*A* and *B*) and the tail domain T-GFP (*K* and *L*) are targeted to the plasma membrane of the proximal segment and the cell body. The membrane-binding domain M-GFP (*E* and *F*), COOH-terminal domain Ct-GFP (*I* and *J*), and the serine-rich domain SR-GFP (*G* and *H*), however, are predominantly localized in the cytoplasm of the axon and the cell body. The spectrin-binding domain of ankyrin_B (SB-GFP [ankyrin_B]) is not targeted to the axolemma of the proximal segment (*C*), although a small fraction is distributed at the membrane of the cell body (*D*). Bar, 25 μ m.

itself or with other membrane-spanning proteins, since deletion of the extracellular domain of neurofascin has little effect on membrane dynamic behavior of cotransfected 270-kD ankyrin_G (Ank270-GFP with HA-NF[Δ EC] in Fig. 8, *A* and *B*). Thus, 270-kD ankyrin_G provides the major contribution to immobilization of ankyrin-neurofascin complexes in the plane of the plasma membrane.

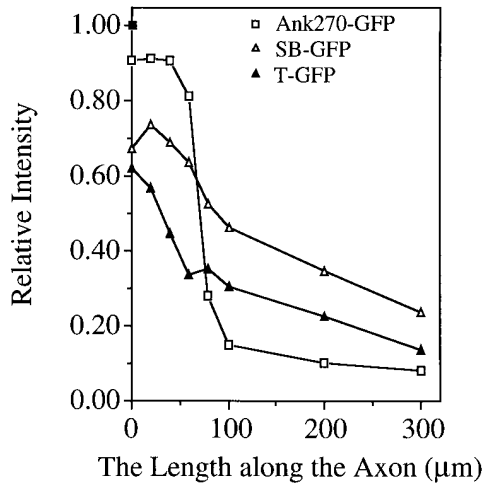
The role of ankyrin_G domains in immobilizing neurofascin were evaluated using a range of ankyrin_G constructs (refer to Fig. 2, and see Fig. 8, *A* and *C*). The lateral mobility of the membrane-localized complexes formed by cotransfected neurofascin (HA-NF) and the membrane-binding domain of ankyrin_G (M-GFP) exhibits more than a 50% reduction of rate of recovery (2.6×10^{-9} cm²/s) and a 30% decrease of extent of recovery (51%) compared with behavior of GFP neurofascin (HA-NF-GFP) alone. Addition of the spectrin-binding domain to the mem-

brane-binding domain (Ank270[Δ SR,T,Ct]-GFP) results in another 50% reduction in the rate of recovery (1.4×10^{-9} cm²/s) and a decrease in extent of recovery to \sim 25% (refer to Fig. 4). Subsequent addition of the COOH-terminal domain (Ank270[Δ SR,T]-GFP), however, had no apparent effect. Addition of the tail domain (Ank270[Δ SR]-GFP) further decreased the rate (7×10^{-10} cm²/s) and the extent (16%) of recovery. Finally, addition of the serine-rich domain (Ank270-GFP) had another reduction in rate to 4×10^{-10} cm²/s and extent of recovery to 10 percent.

Discussion

The current paradigm for organization of ankyrin-membrane protein complexes derives from the erythrocyte membrane, and envisions monomeric ankyrin forming lateral complexes between integral proteins via the multi-

A



B

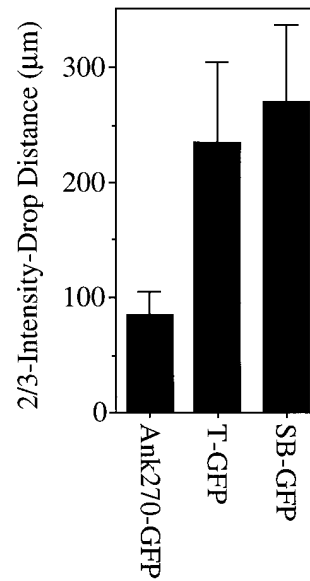


Figure 6. The immunofluorescence intensity profile of transfected spectrin-binding and tail domains of ankyrin_G in axons. (A) Immunofluorescence profile. (B) the two-thirds intensity-drop distance of transfected spectrin-binding and tail domains. Data represent the mean \pm SEM for five transfected neurons.

valent membrane-binding domain and spectrin via the spectrin-binding domain (Bennett, 1990; Michaely and Bennett, 1995a,b). This study provides evidence that a simple erythrocyte-based model is not adequate to explain ankyrin_G organization at axon proximal segments. The spectrin-binding, serine-rich, and tail domains all contrib-

ute to restriction of ankyrin_G to the axonal plasma membrane in DRG neurons. Multiple ankyrin_G domains also contribute to limiting the lateral mobility of ankyrin-neurofascin complexes in a nonneuronal cell. The ankyrin_G-specific spectrin-binding and tail domains are capable of binding directly to sites on the plasma membrane of axon

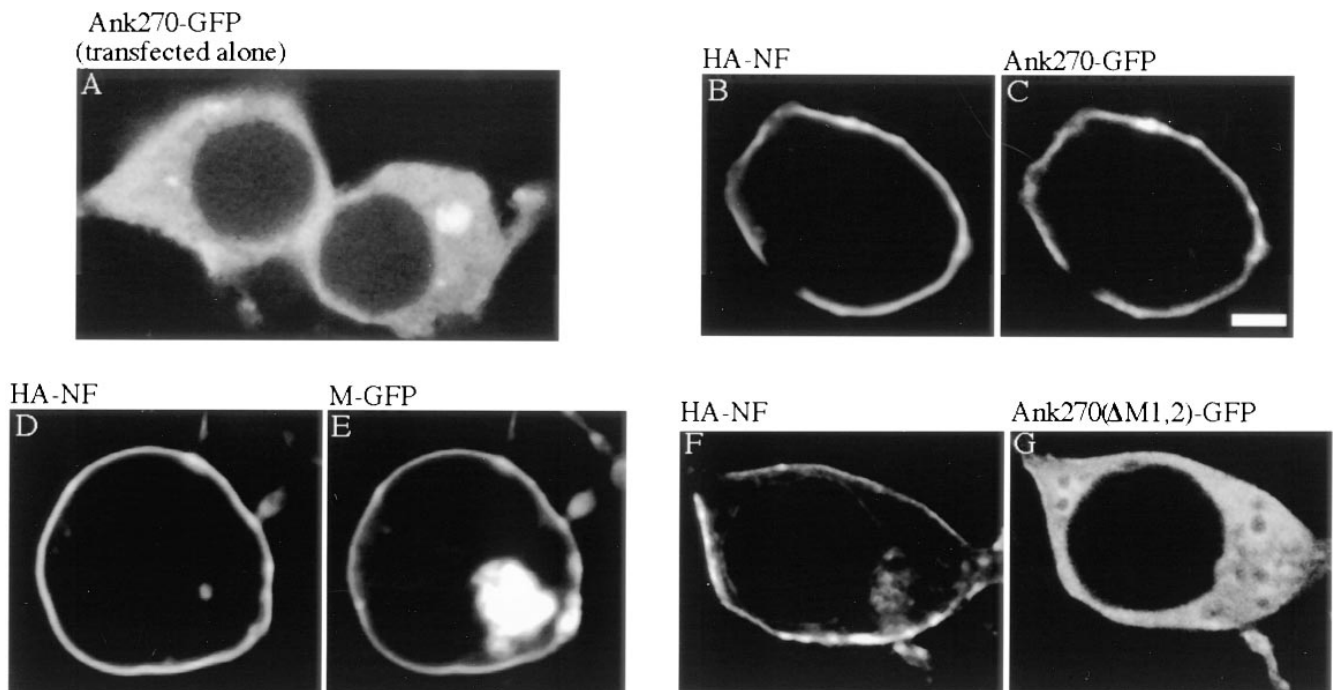


Figure 7. Association of 270-kD ankyrin_G to the plasma membrane of human kidney 293 cells requires the membrane-binding domain and cotransfected neurofascin. Transfected 270-kD ankyrin_G (*Ank270-GFP*) alone visualized by the GFP fluorescence distributes throughout the cytoplasm of 293 cells (A). Cotransfection with neurofascin (*HA-NF*) recruits *Ank270-GFP* to the plasma membrane (C). (B) Double labeling of cotransfected neurofascin by immunostaining of the HA epitope. The membrane-binding domain of ankyrin (*M-GFP*) is sufficient to be recruited to the plasma membrane (E) by cotransfected neurofascin (D). Deletion of the first half of the membrane-binding domain (*Ank270(ΔM1,2)-GFP*) abolishes the membrane-recruitment by cotransfected neurofascin (F and G). Bar, 3 μm.

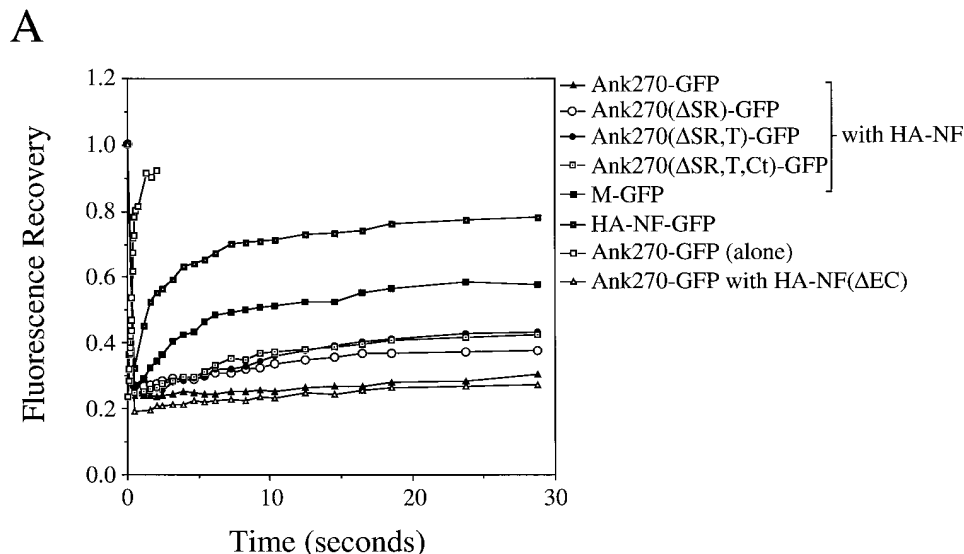
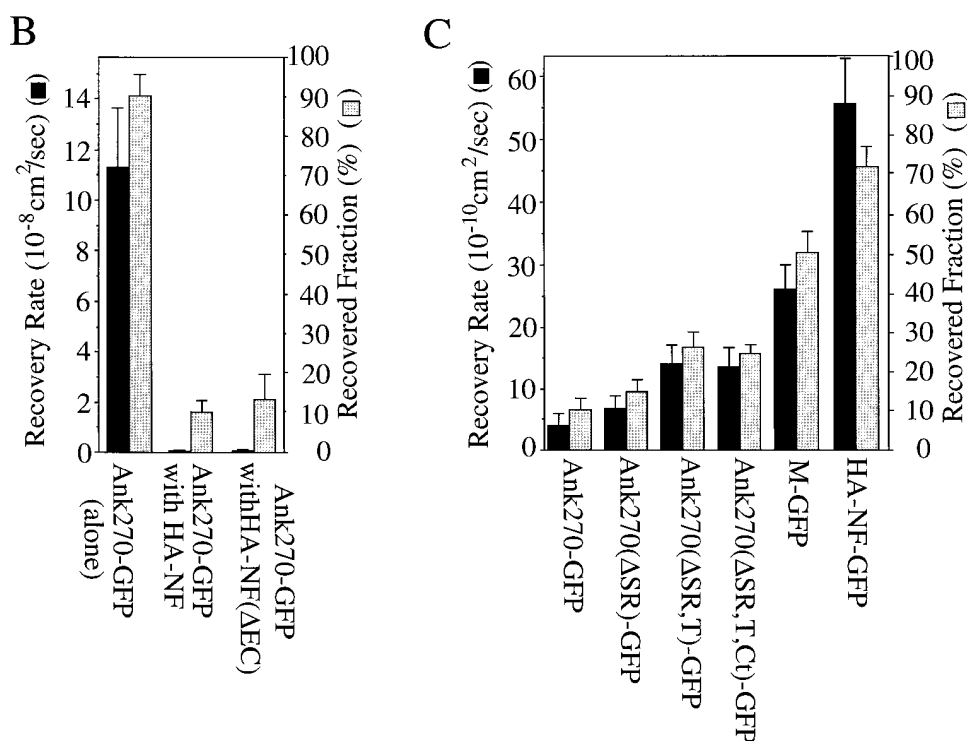


Figure 8. Multiple domains of 270-kD ankyrin_G contribute to immobilization of ankyrin_G at the plasma membrane of human kidney 293 cells. (A) FRAP plasma membrane-localized 270-kD ankyrin_G and its variants with deleted domains. Ankyrins with the membrane-binding domain are capable of being recruited to the plasma membrane by cotransfected neurofascin HA-NF or the extracellular domain-truncated neurofascin HA-NF(ΔEC) (Zhang et al., 1998). Dynamics of membrane-associated ankyrins are evaluated by fluorescence recovery measurement after photobleaching the GFP signals. Also included are the FRAP of cytoplasm-located 270-kD ankyrin_G and membrane-associated GFP-neurofascin (HA-NF-GFP). (B) Comparison of recovery rate (the time taken to achieve half of the 50-s recovery) and recovered fraction (the fraction recovered after 50 s) between the cytoplasm-localized ankyrin and the membrane-associated ankyrin. (C) Recovery rate and recovered fraction of membrane-associated ankyrins. HA-NF-GFP is measured as a reference for freely diffusing membrane proteins. Data in B and C represent the mean ± SEM for six experiments.



proximal segments and neuronal cell bodies, and presumably have yet to be identified docking sites. The serine-rich domain, which is present only in 480- and 270-kD ankyrin_G polypeptides, contributes to restriction of ankyrin_G to axon proximal segments as well as limiting lateral diffusion of ankyrin_G-neurofascin complexes. Ankyrin_G thus functions as an integrated mechanism involving cooperation among multiple domains heretofore regarded as modular units. This complex behavior explains the ability of ankyrin_B and ankyrin_G to sort to distinct sites in neurons and the fact that these ankyrins do not compensate for each other in ankyrin gene knockouts in mice (Zhou, D., S. Lambert, P. Malen, S. Carpenter, L. Boland, and V. Bennett, manuscript submitted for publication) (Scotland,

P., D. Zhou, H. Benveniste, and V. Bennett, manuscript submitted for publication).

The mechanism for restriction of dynamic behavior of neurofascin by ankyrin_G could result from self-association of ankyrin_G domains and/or low affinity interaction with membrane or cytoskeletal proteins. For example, ankyrin associates with micromolar affinity with microtubules (Bennett and Davis, 1981). Association of transfected ankyrin_G with endogenous spectrin does not seem likely, since ankyrin_G remained in the cytoplasm in the absence of cotransfected neurofascin, whereas spectrin in these cells is primarily localized at the plasma membrane (Zhang et al., 1998). As an alternative to specific protein interactions, ankyrin_G could be nonspecifically trapped in

the membrane-attached cytoskeletal meshwork, according to the membrane-skeleton fence model (Kusumi et al., 1993; Jacobson et al., 1995). Further analysis will be required to differentiate these possibilities.

Functions attributed so far to the spectrin-binding domain of ankyrin have been association with spectrin (Bennett, 1978; Davis and Bennett, 1984a,b), and contact with the Na⁺/K⁺ ATPase (Davis and Bennett, 1990). Disruption of the spectrin network abolishes the polarized distribution of ankyrin as well as Na⁺/K⁺-ATPase at cell-cell contact sites in cultured cells (Hu et al., 1995). However, antibody against total brain spectrin revealed no enrichment of spectrin at axon proximal segments of DRG neurons and thus, known spectrins are unlikely to provide a sufficient mechanism to specifically enrich ankyrin_G at the sites. A similar lack of correlation between spectrin and ankyrin_G has been reported at nodes of Ranvier in sciatic nerves, where spectrin is continuously distributed along plasma membranes of axons and the myelinating Schwann cell (Lambert et al., 1997). Since different spectrin isoforms have different affinities for ankyrins (Howe et al., 1985; Davis and Bennett, 1984a), one explanation of these results is the existence of a novel isoform of spectrin which is targeted to axon proximal segments and nodes of Ranvier, but is not identified by our current antibody. Alternatively, a novel ankyrin_G-binding protein unrelated to spectrin could be targeted to axon proximal segments. In addition, a yet to be identified protein at the axolemma of axon proximal segments provides a binding site for the tail domain of 270-kD ankyrin_G.

The traditional interactions between membrane proteins and ankyrin mediated by the membrane-binding domain of ankyrin may not provide the initial step in restriction of ankyrin at the axolemma of axon proximal segments. Instead, enrichment of ankyrin, which could be achieved through combined influences of the spectrin-binding, tail, and serine-rich domains, may precede and direct assembly of membrane proteins at the sites. Failure of transfected mutant neurofascin with a deleted cytoplasmic domain to be concentrated at axon proximal segments (Fig. 3 E) favors the possibility that the ankyrin-binding activity, which is located in the cytoplasmic domain of neurofascin (Davis and Bennett, 1994) (Zhang, X., J.D. Davis, S. Carpenter, and V. Bennett, manuscript in preparation), is required for restriction of neurofascin at these sites. Moreover, the disrupted assembly of neurofascin as well as sodium channels at axon proximal segments in ankyrin_G (−/−) mice further supports the role of ankyrin in directing membrane proteins to axon proximal segments (Zhou, D., S. Lambert, P. Malen, S. Carpenter, L. Boland, and V. Bennett, manuscript submitted for publication).

The potential defining role of ankyrin in clustering of membrane proteins at axon proximal segments is inconsistent with conclusions from analysis of morphogenesis of nodes of Ranvier, suggesting that clusters of neurofascin/NrCAM provide sites for subsequent assembly of ankyrin and sodium channels (Lambert et al., 1997). The disparity could be attributed to the potentially different mechanisms used in establishment of axon proximal segments and nodes of Ranvier, despite similarities in their molecular organization (Kordeli et al., 1995; Davis et al., 1996; Bennett et al., 1997) and ultrastructure (Waxman, 1984).

Clustering of ankyrin or sodium channels at axon proximal segments does not require the presence of glial cells and thus is most likely determined by an intrinsic polarity of neurons (Fig. 3, A, D, and G) (Kaplan et al., 1997). In contrast, clustering of ankyrin and sodium channels in the axonal region beyond the proximal segment, which is regarded as an early event for assembly of nodes of Ranvier, requires signals from glial cells, although whether these signals are diffusible or require direct glia-axon contact has been debated (Salzer, 1997; Dugandzija-Novakovic et al., 1995; Vabnick et al., 1996, 1997; Deerinck et al., 1997; Kaplan et al., 1997). Clustering of neurofascin/NrCAM at the initial sites of nodes of Ranvier provides a potential mechanism to bridge glial cell signals to intracellular determinants, such as clustering of ankyrin, during the early development of nodes of Ranvier. On the other hand, the unique position of axon proximal segments might be sufficient to provide a cue to polarize the molecular composition at this region characterized by concentration of ankyrin and subsequent recruitment of ankyrin-binding membrane proteins.

In carefully timed neuronal Schwann cell cocultures, we have observed that clustering of ankyrin precedes clustering of sodium channels. In addition, each MAG-positive myelinating Schwann cell has two ankyrin clusters at each end of this Schwann cell. These ankyrin clusters are covered by processes of the myelinating Schwann cell, suggesting axon-Schwann cell contact defines clustering as well as the clustering sites of ankyrin (Zhang, 1998). These results indicate that ankyrin, as at axon proximal segments, is a candidate to direct assembly of sodium channels at the precursor sites of nodes of Ranvier, although the role of glial cells may differ between nodes and proximal segments.

This paper suggests a complicated and cooperative scenario for establishment of the specialized membrane domain of axonal proximal segments, which may involve not only interactions between membrane proteins and ankyrin but also ankyrin and other yet to be identified proteins which precede ankyrin at these sites. Isoforms of ankyrin_G also are targeted to sites of cell-cell contact in epithelial tissues (Peters et al., 1995), and proteins similar to those of axon proximal segments could also be required for epithelial polarity. A challenge for future work will be to fully elucidate the structural pathway leading to assembly of ankyrin-based membrane domains.

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