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## Identification of Ank<sub>G107</sub>, a Muscle-specific Ankyrin-G Isoform\*

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We previously showed that alternatively spliced ankyrins-G, the *Ank3* gene products, are expressed in skeletal muscle and localize to the postsynaptic folds and to the sarcoplasmic reticulum. Here we report the molecular cloning, tissue expression, and subcellular targeting of Ank<sub>G107</sub>, a novel ankyrin-G from rat skeletal muscle. Ank<sub>G107</sub> lacks the entire ANK repeat domain and contains a 76-residue sequence near the COOH terminus. This sequence shares homology with COOH-terminal sequences of ankyrins-R and ankyrins-B, including the muscle-specific skAnk1. Despite widespread tissue expression of *Ank3*, the 76-residue sequence is predominantly detected in transcripts of skeletal muscle and heart, including both major 8- and 5.6-kb mRNAs of skeletal muscle. In 15-day-old rat skeletal muscle, antibodies against the 76-residue sequence localized to the sarcolemma and to the postsynaptic membrane and cross-reacted with three endogenous ankyrins-G, including one 130-kDa polypeptide that comigrated with *in vitro* translated Ank<sub>G107</sub>. In adult muscle, these polypeptides appeared significantly decreased, and immunofluorescence labeling was no more detectable. Green fluorescent protein-tagged Ank<sub>G107</sub> transfected in primary cultures of rat myotubes was targeted to the plasma membrane. Deletion of the 76-residue insert resulted in additional cytoplasmic labeling suggestive of a reduced stability of Ank<sub>G107</sub> at the membrane. Recruitment of the COOH-terminal domain to the membrane was much less efficient but still possible only in the presence of the 76-residue insert. We conclude that the 76-residue sequence contributes to the localization and is essential to the stabilization of Ank<sub>G107</sub> at the membrane. These results suggest that tissue-dependent and developmentally regulated alternative processing of ankyrins generates isoforms with distinct sequences, potentially involved in specific protein-protein interactions during differentiation of the sarcolemma and, in particular, of the postsynaptic membrane.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AJ428573.

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Ankyrins, the peripheral proteins that link integral membrane proteins to spectrin, are involved in the selective accumulation and local restriction of ion channels and cell adhesion molecules in specialized membrane domains (1–3).

Ankyrins form a diverse family of modular polypeptides resulting from the expression of at least three genes, designated *Ank1*, *Ank2*, and *Ank3* in rodents (*ANK1*, *ANK2*, and *ANK3* in humans), and from tissue-specific alternative splicing of their products (ankyrins-R, ankyrins-B, and ankyrins-G, respectively) that display different subcellular localization (4). Most isoforms are composed of two highly conserved, membrane-binding (NH<sub>2</sub>-terminal) and spectrin-binding domains, and one variable COOH-terminal domain. The membrane-binding domain is composed of 24 tandem copies of 33-residue ANK repeats that provide sites of protein-protein interaction in numerous proteins (5) and bind to the cytoplasmic domains of most ankyrin-associated integral proteins (6–12). Accumulating evidence shows the existence of "truncated" ankyrins, lacking a part or the totality of the membrane-binding and/or COOH-terminal domains (13–16). Despite the lack of the membrane-binding domain, these isoforms appear associated with intracellular membrane compartments (14, 16).

Among ankyrin genes, *Ank3* shows a broad tissue expression, including kidney (14) and the nervous system (13), where it was first identified. Tissue-specific alternative processing of *Ank3* transcripts results in distinct ankyrin-G isoforms with presumably related but distinct functions. The largest 480- and 270-kDa ankyrin-G isoforms are specifically expressed in neurons, where they are targeted to the nodes of Ranvier and initial axonal segments (13, 17–19). These isoforms contain extended "tail" sequences between the spectrin-binding and COOH-terminal domains. Ankyrins-G expressed in tissues other than brain lack the tail domain, and their molecular masses range from 100 to 220 kDa. Currently, cloned ankyrins-G of the latter category include: (i) epithelial mouse *Ank3* polypeptides that display a polarized plasma membrane localization or a cytoplasmic distribution depending on the presence or lack of the ANK repeat domain (Ref. 14; Ank3-7kb and Ank3-5kb, respectively); (ii) Ank<sub>G190</sub>, a kidney- and lung-specific protein that contains ANK repeats and associates with Na,K-ATPase at the lateral plasma membrane of epithelial cells (12); (iii) Ank<sub>G119</sub>, an isoform with a truncated ANK repeat domain and a very short distinct COOH-terminal domain, that binds β spectrin and associates with the Golgi apparatus and trans-Golgi network in epithelial cells (15); (iv) two short 100- and 120-kDa isoforms that lack the ANK repeat domain and associate with lysosomes in macrophages (16).

In skeletal muscle fibers, assembly of specialized membrane domains is a functional requirement, both at the cell surface (*i.e.* the postsynaptic membrane and the costameres) and in the

TABLE I  
Expression and subcellular distribution of ankyrins in skeletal muscle

Shown are the products of the three ankyrin genes, *Ank1*, *Ank2*, and *Ank3*, that are presently identified in skeletal muscle either by Western blot analysis (WB) or by molecular cloning in this tissue (skAnk1, Ank<sub>G107</sub>). Subcellular distribution refers to immunolocalization data obtained by isoform-specific antibodies. References and corresponding isoforms, whenever known, are shown in parentheses.

Isoform	Gene	Subcellular distribution
Ank1, 210 kDa (WB) skAnk1, 20–26 kDa	<i>Ank1</i>	Sarcolemma (210 kDa) (22, 23, 27) Postsynaptic membrane (23) (isoform ?) Sarcoplasmic reticulum (skAnk1) (22)
Ankyrin-B, 150 kDa, 220 kDa (WB)	<i>Ank2</i>	Sarcolemma Sarcoplasm, A-bands (24)
Ank <sub>G</sub> , 100 kDa (WB) Ank <sub>G107</sub> , 130 kDa (this paper)	<i>Ank3</i>	Postsynaptic membrane (23, 26) (isoform ?) Sarcoplasmic reticulum (23) (isoform ?) Sarcolemma (Ank <sub>G107</sub> ; this paper)

cytoplasm, where Ca<sup>2+</sup>-regulated excitation-contraction coupling occurs. In this tissue, multiple ankyrins are expressed by all three genes (15, 20–24) and localize to several membrane sites, including the postsynaptic membrane (23, 25, 26), the costameres (27), the triads (28), and the nonjunctional sarcoplasmic reticulum (22, 23) (Table I). Interestingly, the lack of *Ank2* gene products in skeletal muscle fibers and cardiomyocytes of ankyrin-B(–/–) mice resulted in a congenital myopathy, abnormal properties of cardiac Na<sup>+</sup> channels, and dramatic alterations in intracellular localization of Ca<sup>2+</sup> homeostasis proteins, namely the Ca<sup>2+</sup>-ATPase (SERCA) and the ryanodine receptors (24, 29). *Ank1* and *Ank3* gene products still expressed in these mice cannot rescue ankyrin-B(–/–) muscle cell, indicating that ankyrins have gene-specific functions. Taken together, these data and the diversity of ankyrin gene expression and localization suggest that ankyrins play key roles in the assembly and functioning of membrane domains in skeletal muscle fibers. However, most of these isoforms have not yet been identified at the molecular level. Currently, cloned ankyrins that are expressed in skeletal muscle include the Golgi-associated Ank<sub>G119</sub> (15) and two small, membrane-bound *Ank1* gene products of 20 and 26 kDa suggested to link the sarcoplasmic reticulum to the contractile apparatus (Ref. 22; skAnk1). Our previous studies (23) identified at least two major 8- and 5.6-kb *Ank3* transcripts and one major ankyrin-G polypeptide of ~100 kDa in rat skeletal muscle. Furthermore, ankyrins-G were localized to the troughs of the postsynaptic membrane and to the sarcoplasmic reticulum of fast-twitch, SERCA1-expressing muscle fibers.

Here we report the cDNA cloning and characterization of a novel ankyrin isoform from rat skeletal muscle, which we name Ank<sub>G107</sub>, based on its predicted size of 106,911 Da and homology to the known isoforms of the ankyrin-G family. Ank<sub>G107</sub> lacks the entire membrane-binding ANK repeat domain, displays highly conserved ankyrin-G spectrin-binding and COOH-terminal domains, and contains a unique among ankyrins-G 76-residue sequence near the COOH terminus, which shows homology with corresponding ankyrin-R and ankyrin-B COOH-terminal sequences and is predominantly expressed in heart and skeletal muscle. Endogenous ankyrins-G carrying this sequence displayed developmentally regulated expression and localized to the sarcolemma and to the postsynaptic membrane. Transfection of GFP<sup>1</sup>-tagged constructs expressing either the full-length molecule or Ank<sub>G107</sub> domains in rat myotubes in culture showed that this isoform is targeted to the plasma membrane apparently via the spectrin-binding domain. Moreover, these experiments strongly suggested that the muscle-

specific 76-residue sequence is required for the stabilization of Ank<sub>G107</sub> at the membrane.

#### EXPERIMENTAL PROCEDURES

**Isolation of the Ank<sub>G107</sub> cDNA**—All molecular procedures were carried out using standard methods (30). An oligo(dT) and random primed rat skeletal muscle 5'-stretch plus Agt10 cDNA library (CLONTECH, Palo Alto, CA) was double screened by plaque hybridization of nylon filters (Nytran-Plus; Schleicher & Schuell) using as probes two random primed <sup>32</sup>P-labeled (Rediprime system; Amersham Biosciences, Inc.) *Ank3* cDNA fragments from the spectrin-binding (bp 229–1103) and COOH-terminal (bp 1778–2255) domains, previously amplified by RT-PCR from rat skeletal muscle total RNA (PCR A and B, respectively, in Ref. 23). Hybridization was performed at 65 °C overnight, and posthybridization washes were at a maximum stringency of 0.2× SSC, 65 °C. cDNA inserts from clones positive to both probes were subcloned into the plasmid vector pBluescript IISK(+) (Stratagene) and sequenced (Applied Biosystems), leading to identification of one full-length clone, λ21, that contained *Ank3* sequences.

Computer-assisted searches of amino acid sequence homology were performed utilizing FASTA (31) and BLAST (32) programs.

**Northern Blot Analysis**—*Ank3* domain-specific cDNA probes were prepared by standard PCRs using as template the rat skeletal muscle cDNA clone λ21 for the spectrin-binding domain (bp 62–793) and 76-aa insert (bp 2633–2860) and a rat brain *Ank3* cDNA clone kindly provided by Dr. S. Lambert (University of Massachusetts Medical School, Worcester, MA) for the membrane-binding and COOH-terminal (corresponding to bp 2255–2921 without insert) domains. The locations of the hybridization probes are illustrated in Fig. 3B. PCR products were gel-purified (Qiagen) and <sup>32</sup>P-labeled using random primed DNA synthesis (Rediprime system; Amersham Biosciences). The 228-bp PCR product encoding the 76-aa insert was sequenced to confirm its identity.

Total RNA was isolated from adult rat hind limb skeletal muscle using the guanidinium thiocyanate/phenol/chloroform method (RNA Plus; Bioprobe Systems) and enriched in poly(A<sup>+</sup>) RNA by oligo(dT) chromatography (30). 20 μg of partially purified poly(A<sup>+</sup>) RNA were fractionated in 0.8% formaldehyde/agarose gel and transferred to nylon filters (Nytran-plus; Schleicher & Schuell). After fixation by ultraviolet light (UV cross-linker; Stratagene), filters were hybridized with rat *Ank3* domain-specific cDNA probes and washed at 68 °C with 0.2× SSC, 0.1% SDS, before autoradiography.

A rat multiple tissue Northern blot (CLONTECH) was first hybridized with cDNA probe encoding the 76-aa insert and then stripped and probed with the spectrin-binding domain cDNA probe. Hybridizations were performed at 68 °C overnight, and posthybridization washes were at a maximum stringency of 0.1× SSC, 0.1% SDS, 50 °C.

**Preparation of cDNA Constructs**—cDNA fragments encoding the full-length Ank<sub>G107</sub>, the spectrin-binding domain (Ank<sub>G107</sub>Sp-b; first 590 aa), and the COOH-terminal domain (Ank<sub>G107</sub>Cter; aa 591–960) were amplified by PCR using the clone λ21 as a template and primers carrying *EcoRI* sites. A rat brain *Ank3* cDNA clone was used to PCR-amplify the COOH-terminal domain lacking the 76-aa insert (Ank<sub>G107</sub>CterΔ76aa). PCR fragments were confirmed by DNA sequencing and introduced into the *EcoRI* site of either pcDNA3 vector (Invitrogen), or pEGFP-N1 vector (CLONTECH) under the control of the cytomegalovirus promoter while keeping in-frame with the downstream enhanced green fluorescent protein (EGFP). The cDNA construct of the full-length Ank<sub>G107</sub> lacking the 76-aa insert (Ank<sub>G107</sub>Δ76aa-GFP) was obtained by replacing the COOH-terminal domain-containing *EcoRV*-

<sup>1</sup> The abbreviations used are: GFP, green fluorescent protein; EGFP, enhanced GFP; SERCA1, sarcoplasmic/endoplasmic Ca<sup>2+</sup>-ATPase isoform 1; EDL, extensor digitorum longus; aa, amino acid(s).

KpnI fragment of construct Ank<sub>G107</sub>-GFP with the corresponding fragment of construct Ank<sub>G107</sub>CterΔ76aa-GFP.

**Preparation of Antibodies**—To raise antibodies against rat skeletal muscle ankyrins-G, a cDNA fragment (bp 62–793) was amplified by PCR using the λ21 clone as template and subcloned into vector pGEX-2T (Amersham Biosciences) to generate a 53-kDa fusion protein containing the NH<sub>2</sub>-terminal portion of glutathione S-transferase and amino acids 7–250 of the Ank<sub>G107</sub> spectrin-binding domain. The recombinant fusion protein was expressed in *E. coli* BL21(DE3)pLysS cells and affinity-purified using glutathione-Sepharose beads according to the manufacturer's directions (Amersham Biosciences). To avoid proteolytic fragments, the affinity-purified polypeptides were applied to polyacrylamide SDS gels, and the band containing the full-length fusion protein was cut out of the gel and injected into rabbits. The resulting antiserum (anti-ank<sub>G</sub>Spbd) was affinity-purified using fusion protein coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences).

Antibodies against the Ank<sub>G107</sub> 76-residue insert were raised in rabbits against two peptides corresponding to amino acid residues 864–878 and 925–939 (see Fig. 1) (Eurogentec). The peptides represented sequences not included in the region of homology with the other ankyrin genes. Specific antibodies (anti-Ank<sub>G</sub>76aa) were affinity-purified against the antigenic peptides immobilized on HiTrap N-hydroxysuccinimide (NHS)-activated columns (Amersham Biosciences).

**In Vitro Transcription and Translation**—*In vitro* transcription and translation were carried out in TNT-coupled rabbit reticulocyte lysate systems (Promega) according to the manufacturer's protocols, using the pcDNA3-Ank<sub>G107</sub> construct. Products were resolved by SDS-PAGE electrophoresis and revealed by Western blot analysis. In control experiments, an aliquot was removed in the beginning of the reaction and analyzed by Western blot analysis; alternatively, *in vitro* translation was performed with antisense Ank<sub>G107</sub> cDNAs. Both controls provided identical results.

**Cell Culture and Transfection of Ank<sub>G107</sub> cDNA Constructs in Rat Myotubes**—Primary cultures of mammalian skeletal muscle cells were initiated from neonatal myogenic cells obtained by trypsinization of muscle pieces from hind limbs of 1–3-day-old rats, as previously reported (33). For 3 days following plating, cells were maintained in growth medium consisting of Ham's F-12 medium (Invitrogen) with 10% heat-inactivated horse serum (Invitrogen), 10% fetal calf serum (Invitrogen), and 1% antibiotics. Myoblasts underwent myogenesis in differentiation medium. After 48 h of culture, differentiation medium containing Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% heat-inactivated horse serum was used to promote the formation of myotubes, which occurs within 15–18 h.

The various Ank<sub>G107</sub> cDNA constructs were transfected into the myoblasts using the Effectene Reagent kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. Myoblasts were cultured for 36 h on glass coverslips (50 × 10<sup>4</sup> cells) in growth medium and then rinsed twice in serum-free medium (Opti-MEM; Invitrogen) and transfected with 1 μg of plasmid cDNA per 35-mm plastic dish. Following a 16-h incubation, the transfection medium was replaced with fresh complete growth medium.

**Western Blot Analysis**—Pieces of 15-day-old and adult rat extensor digitorum longus (EDL), soleus, and sternomastoid skeletal muscles were excised, rapidly frozen in liquid nitrogen, and ground into a powder. Tissue powder was added to boiling SDS-PAGE sample buffer containing 125 mM Tris-HCl, pH 6.8, 15% SDS, 20% glycerol, and 10% β-mercaptoethanol, homogenized, and passed through a 26-gauge needle. Samples were rapidly centrifuged, and the supernatant was used in SDS-PAGE and Western blot analysis, as previously described (23).

4-day-old myotubes in culture were washed in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 2 mM MgCl<sub>2</sub>) and lysed on ice with cold radioimmune precipitation buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 1% Tween 20, 1% Triton X-100, 0.1% SDS, 10% glycerol supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 1% protease inhibitor mixture (Sigma). Homogenates of myotubes were then sonicated and analyzed by SDS-PAGE and Western blot as previously described (23). When the same transfer membrane was probed with two antibodies, the first antibody was stripped by incubation in Tris-HCl, pH 6.8, 2% SDS, 0.1 M β-mercaptoethanol for 1 h at 57 °C.

**Immunofluorescence and Confocal Microscopy**—EDL and diaphragm skeletal muscles were removed by dissection from 15-day-old and adult Sprague-Dawley rats and immediately fixed with 3% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 °C. Fixed tissue was cut to small blocks, infused with increasing sucrose solutions (0.5–2.1 M in PBS (20 mM phosphate buffer, pH 7.5, 150 mM NaCl), and frozen in

liquid nitrogen. Semithin (0.5–1 μm) cryosections of muscle fibers were immunolabeled for indirect immunofluorescence with primary antibodies diluted at 2–5 μg/ml as previously described (23). Monoclonal antibodies to the Ca<sup>2+</sup>-ATPase (SERCA1) were from Affinity Bioreagents. Fluorescein isothiocyanate- and Cy3-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Fluorescein isothiocyanate-conjugated α-bungarotoxin (1 μg/ml; Sigma) was used to label acetylcholine receptors in the postsynaptic membrane. Micrographs were taken with a Leica DMR microscope equipped with a CCD camera (Princeton Laboratories). Images were acquired, pseudocolored, and merged using the MetaView Imaging System (Universal Imaging Corporation, West Chester, PA) and arranged using Adobe Photoshop 5.0.

4-day-old myotubes in culture were fixed in 4% paraformaldehyde in TBS for 20 min at room temperature and either directly observed for GFP fluorescence or permeabilized with 0.1% Triton X-100/TBS for 10 min and labeled for indirect immunofluorescence with primary antibodies diluted at 2–5 μg/ml. RRX-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories. Samples were analyzed by confocal laser-scanning microscopy (Bio-Rad MRC 1024 ES equipped with an argon/krypton laser) using an inverted microscope (Olympus IX70, Tokyo, Japan).

The relative intensities of cytoplasmic and cortical GFP fluorescence were measured and scaled on 256 levels along transversal lines crossing the xy plane of the confocal optical section. Line intensity profiles were obtained with Lasershar Processing software (Bio-Rad) and analyzed by a radiometric method as follows. For each ratio, the mean cortical fluorescence intensity was obtained by averaging measurements at two different intersections of the line with the periphery of the myotube. The mean intensity of the homogeneous cytoplasmic labeling was directly calculated by the software from values measured inside a square region of interest devoid of cytoplasmic clusters. For comparison, the ratio of mean cortical over mean cytoplasmic fluorescence intensities ( $F_{\text{cortex}}/F_{\text{cytoplasm}}$ ) was calculated and reported on a graph (see Fig. 7).

## RESULTS

**Isolation and Characterization of Ank<sub>G107</sub>, a Novel Ankyrin-G Isoform from Rat Skeletal Muscle**—Ank3 cDNA sequences from the spectrin-binding and COOH-terminal domains were previously amplified by RT-PCR from rat skeletal muscle total RNA (23). Here we used the two PCR products as probes to double screen a rat skeletal muscle cDNA library. Seven out of 10 positive colonies hybridized with both probes, and the cDNA clone with the largest insert (clone λ21) was isolated and sequenced. This clone provided a 3262-bp cDNA sequence with a single open reading frame encoding a protein of 960 amino acids with a predicted molecular mass of 106,911 Da (Fig. 1). This protein, named Ank<sub>G107</sub>, is highly homologous to previously cloned ankyrin-G isoforms (12–16), with maximum amino acid identity (88.6%) to the mouse epithelial Ank3–5kb polypeptide (Fig. 2B; Ref. 14). Ank<sub>G107</sub> and the Ank3–5kb isoform share identical NH<sub>2</sub>-terminal sequences lacking the entire ANK repeat membrane-binding domain and starting with amino acids MALPHS followed by nearly identical spectrin binding (EDAIT . . . ) and COOH-terminal (ALR . . . ) domains. cDNA clone λ21 also contained 48 nucleotides of 5'-untranslated sequences showing 83.3% identity to corresponding mouse Ank3–5kb sequences (14), and 197 bp of 3'-untranslated sequences identical to corresponding sequences of rat epithelial Ank<sub>G190</sub> (12). We conclude that Ank<sub>G107</sub> is a novel isoform of the ankyrin-G family encoded by *Ank3*.

The major difference between Ank<sub>G107</sub> and the Ank3–5kb isoform occurs in the COOH-terminal domain. Ank<sub>G107</sub> contains a unique 76-residue insert near the COOH terminus that is not present in any of the currently cloned ankyrin-G isoforms. Interestingly, when compared with sequences present in available data bases, significant homology was found only between the 76-residue insert and sequences from the COOH-terminal domains of ankyrins-R and ankyrins-B, the *Ank1* and *Ank2* gene products (Fig. 2A). These sequences include the

CTGGAAGTCTCCGAAAGCATTCTCAACCAGCGTGGTGGAAATGGCTTTGCCACACAG - 60  
M A L P H S 6

TGAAGATGCCATCACAGGGGACACTGACATATCTCGGGCCAGACGACCTCAAGGAGCT - 120  
E D A I T T G D D T G Q D L Y L G P D L K E L 26  
→ SPECTRIN-BINDING

GGCGATGACTCCCTGCTCCGAGGGCTATGTGGGCTCAGTCTCGGAGCCCTTCTGC - 180  
G D D S L P A E G Y V G F S L G A R S A 46

CAGTCCCAAGATAACTGGATAGGTCCTCACCTGAACAGAGACTCCTATGCGCGGGA - 240  
S P K I S S D R S Y T L N R S S Y A R D 66

CAGCATGATGATGAGGAGCTCTGCTGCCATCAAGAGCAGCACTTCCATTCACGAG - 300  
S M M I E E L L V P S K E Q H L P F T R 86

GGAGTTGACTCCGACTCCCTCAGACACTACAGTGGGCGAGACACATGGACAATGT - 360  
E F D S D S L R H Y S W A A D T L D N V 106

GAACCTGGCTCCAGCCCTGCTGCTTCTGCTTCTGCTTATGTTGGAGCGGAG - 420  
N L V S S P V H S G F L V S F M V D A R 126

AGGGGGCTCCATCGGAGGAGCCGCCACCCGGATCGGATCATCATCCCTCCGGCAA - 480  
G G S M R G S R H G M R I I I P P R K 146

GTGTACGGCCCTCCAGCCATCTGCGCCCTGGTAAGAGACATAAATGGCCCAACC - 540  
C T A P T R I T C R L V K R H K L A N P 166

ACCCCCATGTTGGAAGGAGGATTTAGCCAGTGGCTGTAAGATGGCTTCCGGG - 600  
P P M V E G E G L A S R L V E M G P A G 186

GGCAAAATTTTAGCCGCTATGGAATCCCTCATTGGGTCATGAGAGGAAA - 660  
A Q P L G P V I V E I P H F G S M R G K 206

GGAGGGAACTCATTCTCCCGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG - 720  
E R E L I V L R S E N G E T W K E H Q P 226

TGACAGAAAAGCAAGACTCTCGGAGCTCCTCAATGGATGGAGAAAGACTTGCAG - 780  
D S K N E D L S E L L N G M D E E L D S 246

CCCAAGAGTTGGGCAAGCGCATCTCGAAGATCATCAAGAGGATTTCCCAAGTA - 840  
P E E L G T K R I C R I I T K D F P Q Y 266

TTTCGCTGTGGTTCGGGATTAAGCAAGAAAGCAAGAGATGGTCCCGAAGTGGAT - 900  
F A V V S R I K Q E S N Q I G P E G G I 286

TCTGAGCAGCACAGCTCCCTCCCTGCTGAGCGCTCTTCCAGAGGTGCTTAACAA - 960  
L S S T T V P L V Q A S P P E G A L T K 306

AAGGATCCGTGTGGTCTGAGGCTCAGCCGATCCAGAGGAGACTGTGAAAAGATCCT - 1020  
R I R V G L Q A Q P V P E E T V K K I L 326

TGAAACAAGAGCAAGTTAGCCCAATTTGTCACGGTGGAGCCGAGGAGGAGAAATCCA - 1080  
G N K A T F S P I V T V E P R R R K F H 346

TAAGCCGATCAGTACCATCCCGGCTCCCGCCAGCTCGGGAGGCGTGTCCAATGG - 1140  
K P I T M T I P V P P P S G E G V S N G 366

GTACAAGGGGACACCCAGCCAGCTGCGACTCCTCTGCGAGCATTACAGGAGGACCT - 1200  
Y K G D T T P S L R L L C S I T G G T S 386

GCCGCTCAGTGGGAGAGCATCAGAGCAACCCCGTGAATCTCATAAAGACTGTGT - 1260  
P A Q W E D I T G T T P L T F I K D C V 406

GTCTTCAACCAATGTTCCAGCGAGTCTGCTGCGGAGTGCATCAGGTGTAGA - 1320  
S F T T N V S A R F W L A D C H Q V L E 426

AACCGTAGGCTAGCTCCGAGCTGTACAGAGGTTGATGTGTCCCTACATGGCCAA - 1380  
T V G L A S Q L Y R E L I C V P Y M A K 446

GTTTGTGTTTTGGCAAAACAAGCAGCCAGTGGAACTCCCTCAAGGTGCTCTGTAT - 1440  
F V V F A K T N D P V E S S L R C F C M 466

GACAGCAGCAGAGTGGCAAAACCTCGAGCAGCAGGAGAATTTCCAGGAGGTGCCAG - 1500  
T D D R V D K T L E Q Q E N F E E V A R 486

AAGCAAGACATCGAGTCTCGAAGAAAGCCATCTATGTTGATGCTATGGAACCT - 1560  
S K D I E V L E G K P I Y V D C Y G N L 506

GGCTCCTTGACCAAGGAGGGCAGCAGCTGTTTTAACTCTATCTTTCAAGAAAA - 1620  
A P L T K G G Q Q L V F N F Y S F K E N 526

CAGACTGCCATTTTCATCAAGTTAGAGACACAGCCAGCAAGGACCTGGCGCTCTGC - 1680  
R L P F S I K V R D T S Q E P C G R L S 546

TTTCTGAAGAGCCAAACAAAGGATTACCCAAACGGCTGTTGCAACTTAA - 1740  
F L K E P K T T K G L P Q T A V C N L N 566

TATTACTCTACCGGCGATAAAAGGCTGAGAAGCAGACAGCCAGAGCTTCACATC - 1800  
I T L P A H K K A E K A D R R Q S P T S 586

CCTAGCTTACCTCAGAGTCTTGTGAGCGGAGCGATATCAGGATGGCATATAGTAG - 1860  
L A L R P Q S P C E R T D I R M A I V A 606

CGATCACCTGGAGTATTGGAGCAGAGTGGCAAGGAACTGAATTTTTCAGTGGATGA - 1920  
D H L G L S W T E L A R E L N F S V D E 626

AATCAACSAATACGAGTGAATAATCCCAATCTTAATTTCTCAGAGCTTCATGTTATT - 1980  
I N Q I R V E N P N S L I S Q S F M L L 646

AAAAAGTGGTGACGAGAGCGGAGAAATGCCAACTGATGCTTAACTTCTGCTCT - 2040  
K K W V T R D G K N A T D A L T S V L 666

ANCAAAAATTAACGAATAGACATAGTAACCTGCTGGAAGGACCAATATTTGATATGG - 2100  
T K I N R I D I V T L L E G P I F D Y G 686

GATATTTACGAGCAGAGCTTTGAGATGAAACAATTTTTCATGACCAIVAG - 2160  
N I S G T R S F A D E N N V F H D P V D 706

TGGTGGCAGACGAGCAGCAAGTGAAGCTAGAGTCCCGGCGCAAGCGCAAGAAAT - 2220  
G W Q N E T P S G S L E S P A C K Q S 726

AACCGTGGTACTTGGACCGCTGAGCAGCAGCTGACCAAGTTCGGGATCCCATTC - 2280  
T G G L L D R L D D S S D Q V R D P I T 746

CTCATCTCAGAGGAGAGCTGGAGTTCGAAGCAAGCAAGCAAGCAAGCAAGCAAG - 2340  
S Y L T G E A G K F E A N G N H A E V I 766

TCCAGAAGCAAGGCGCAAGGCTACTTCTGATCCCAACGATATAGGAAACAGAG - 2400  
P E A K A K A Y F P E S Q N D I G K Q S 786

CATCAAGGAGAACTTGAACCAAAACACAGGATGGTGGCTGGAGAACCAAGTGC - 2460  
I K E N L K P K T H G C G R A E E P V S 806

GCCCTCAGCCCTACCAAGAACTCTAGAGAAACCAAGCAAGCTTCTCATAGAGAGCC - 2520  
P L T A Y Q K S L E E T S K L V I E D A 826

ACCTAAACCTGTGTCTGTGGCATGAAAAGATGACAGGACTCCCGCTCAGCCAA - 2580  
P K F C V P V G M K K M T R T P A D G K 846

AGCCAGGCTCAACCTCCAGGAAGAGGGGTCGCGAGCTCAGAGCTAAGCACTGAG - 2640  
A R L N L Q E E E G S A R S E P K D L R 866

ACACAGTGAAGTATTCAAGCTCAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG - 2700  
H S E S D S S S E E E R R V T T R V I R 886

AGGCGTGTATTATAAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG - 2760  
R R V I I K G E E A K T I P G E S T C A 906

AGAGCAATTTACCGATGAAGAAGCAACCTCATCACTAGAAAATCACTCGGAAGTAT - 2820  
E Q F T D E E G N L I T R K I T R K V I 926

AGACGGATTGGTCCACAAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG - 2880  
R R I G P Q E R K Q D D V Q G E G Y K V 946

GAAACAAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG - 2940  
T K K E I R N V E K K A H \* 960

GTCCAGGACCAGCGTCTGATCTGCGAGTATTGAGAACTCACCAGAGATCAGCAGGAG - 3000  
GAGAATCTCAGCAAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG - 3060

GGGTTTGGGTTTTTTTGGTTTTTTTAAATGCGAAGGATAAAGAAAACACCTTTTT - 3120  
TTTTTT - 3127

FIG. 1. Nucleotide and deduced amino acid sequence of Ank<sub>G107</sub>. The first six NH<sub>2</sub>-terminal amino acids (in *italics*) and the start of the spectrin-binding (EDAIT...) and COOH-terminal (ALR...) domains are indicated. The amino acid sequence of the 76-residue insert in the COOH-terminal domain is *underlined*. In the spectrin-binding domain, *double underlined* residues PKI show Ank<sub>G107</sub> sequence divergence from ankyrins-G. These sequence data are available from GenBank™/EMBL/DDBJ under accession number AJ428573.

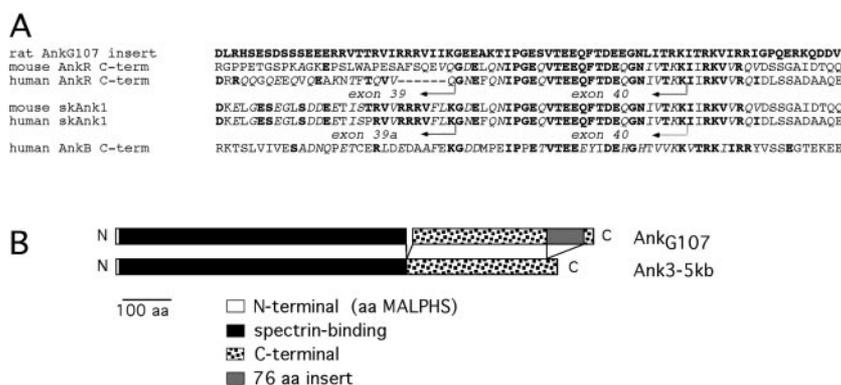
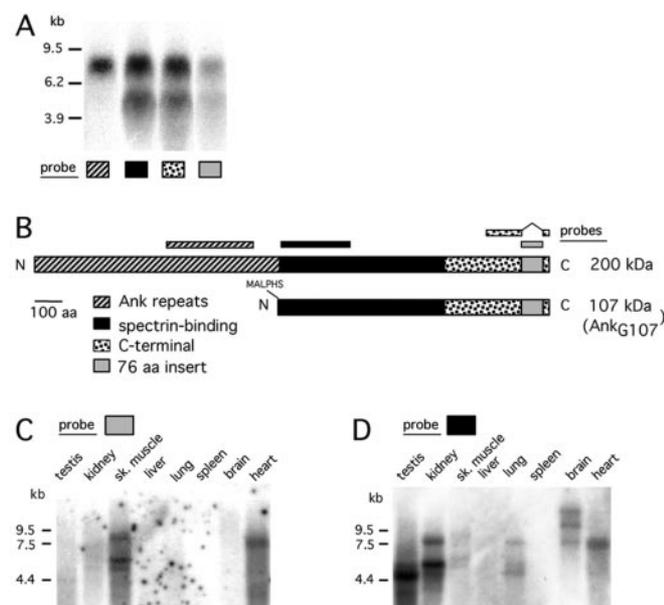


FIG. 2. Sequence analysis and domain structure of Ank<sub>G107</sub>. **A**, comparison of the 76-residue insert from Ank<sub>G107</sub> COOH-terminal domain and homologous amino acid sequences from the COOH-terminal domains of ankyrins-R (Ank<sub>R</sub>, skAnk1) and ankyrins-B (Ank<sub>B</sub>). Identical and conserved residues are in *boldface* and *italic type*, respectively. The *arrows* indicate human ankyrin-R sequences corresponding to the ends of exons 39, 39a, and 40 of the *ANK1* gene (34). **B**, schematic representation of the postulated domain structure of Ank<sub>G107</sub> and comparison with the mouse epithelial Ank3-5kb polypeptide (14), the ankyrin-G isoform that shares maximum amino acid sequence homology. The two polypeptides are almost identical with the exception of a 12-residue deletion and the 76-residue insertion (*gray box*) in the COOH-terminal domain of Ank<sub>G107</sub>, unique features of this isoform.

entire exon 40 (67.9% amino acid identity and 96.4% overall similarity) and the first 9 residues of exon 41 of human *ANK1* (34) and corresponding mouse *Ank1* (35) gene sequences (Fig.

2A). Maximum similarity (77.6%) over the entire 76-residue sequence is observed with the muscle *Ank1* gene product skAnk1 (22), where homology is extended to the last 29 resi-



**FIG. 3. Expression of Ank<sub>G107</sub> in rat tissues.** A, Northern blot analysis of *Ank3* transcripts from rat skeletal muscle using domain-specific cDNA probes as indicated in B. The two major transcripts of 8.0 and 5.6 kb contain the spectrin-binding, COOH-terminal and 76-residue insert domains. Note that the cDNA probe corresponding to the entire 76-residue insert (gray box) revealed a weak hybridization signal but an identical pattern of transcripts. The ANK repeat (membrane-binding) domain probe hybridized only with the 8.0-kb mRNA. B, schematic representation of the postulated domain structure of ankyrin-G polypeptides expressed in skeletal muscle, as deduced by the Northern blot analysis. The presence of the ANK repeat domain would result in a not yet identified ankyrin-G isoform with an estimated molecular mass around 200 kDa. C and D, hybridization of a rat multiple tissue Northern blot (CLONTECH) with cDNA probes corresponding to the entire 76-residue insert (C) and derived from the spectrin-binding domain of ankyrin-G (D), a general probe for the ankyrin-G family. Upon autoradiography, when the insert probe was used (C), the blot was exposed for a long period of time to obtain levels of signal comparable with the spectrin-binding probe (D). Insert-containing transcripts were detected in heart and skeletal muscle (C). Note that transcripts detected by both probes displayed the same pattern but different relative signal intensities.

dues (44.8% identity and 82.8% similarity) of the alternative muscle-specific exon 39a utilized by this isoform (36, 37).

In addition to the COOH-terminal insert, Ank<sub>G107</sub> sequence diverges from other ankyrins-G in two points: (i) within the spectrin-binding domain, residues PKI (Fig. 1a, bp 48–50, *double underlined*) replace residues LRSF; (ii) the present cloning data confirm a 12-residue deletion in the beginning of the COOH-terminal domain (bp 590) previously identified in skeletal muscle *Ank3* sequences amplified by RT-PCR (23). These discrepancies are unique features of Ank<sub>G107</sub>, since both the 12-residue stretch of sequence and residues LRSF are present in all currently cloned ankyrins-G with the exception of Ank<sub>G119</sub> (15), where residues LRSF are deleted and not replaced.

**Domain Structure of Skeletal Muscle Ankyrins-G and Tissue Expression of Ank<sub>G107</sub>**—RNA isolated from rat skeletal muscle was hybridized with *Ank3* domain-specific cDNA probes derived from the membrane-binding (ANK repeat), spectrin-binding and COOH-terminal domains, and the 76-residue insert. (Fig. 3A). The two major 8- and 5.6-kb *Ank3* transcripts previously reported in skeletal muscle (23) were detected by all probes with the exception of the ANK repeat probe, which hybridized only with the 8-kb mRNA. The 76-residue cDNA probe repeatedly revealed a fainter signal but an identical pattern of transcripts when compared with the other probes (Fig. 3A). Cross-hybridization with *Ank1* and *Ank2*, also ex-

pressed in skeletal muscle, would have resulted in a different pattern of transcripts and therefore is unlikely. These data indicate that (i) the smaller 5.6-kb transcript does not contain ANK repeats and therefore could encode the Ank<sub>G107</sub> isoform and (ii) the spectrin-binding and COOH-terminal domains, including the novel 76-residue sequence, are present in both transcripts. Therefore, at least two ankyrin-G polypeptides are expressed in skeletal muscle, characterized by the 76-residue insert near the COOH terminus and differing in the presence or lack of the repeat domain (Fig. 3B).

High stringency hybridization of poly(A<sup>+</sup>) RNA from several rat tissues with the 76-residue cDNA probe identified a limited tissue expression pattern (Fig. 3C). Interestingly, positive transcripts were detected in skeletal muscle (8–9 kb and 5.6–6.2 kb) and heart (7.5 kb). This pattern differed from that of the ankyrin-G family in general, as revealed by a cDNA probe from the spectrin-binding domain (Fig. 3D). This probe contains sequences present in all currently identified ankyrins-G and detected a variety of *Ank3* transcripts in most tissues, as previously reported (13, 14). Transcripts positive to both probes displayed the same pattern but different relative hybridization signal intensities. The spectrin-binding domain probe provided relatively low hybridization signals in skeletal muscle and heart when compared with other tissues. Inversely, the only nonmuscle tissues showing barely detectable hybridization signals with the 76-residue probe were kidney and testis, where *Ank3* transcripts display their highest levels of expression (Fig. 3D). In addition, brain, spleen, lung, and liver were totally negative to 76-residue probe (Fig. 3C), despite the presence of *Ank3* transcripts in brain and lung. These observations further support the specificity of the 76-residue probe.

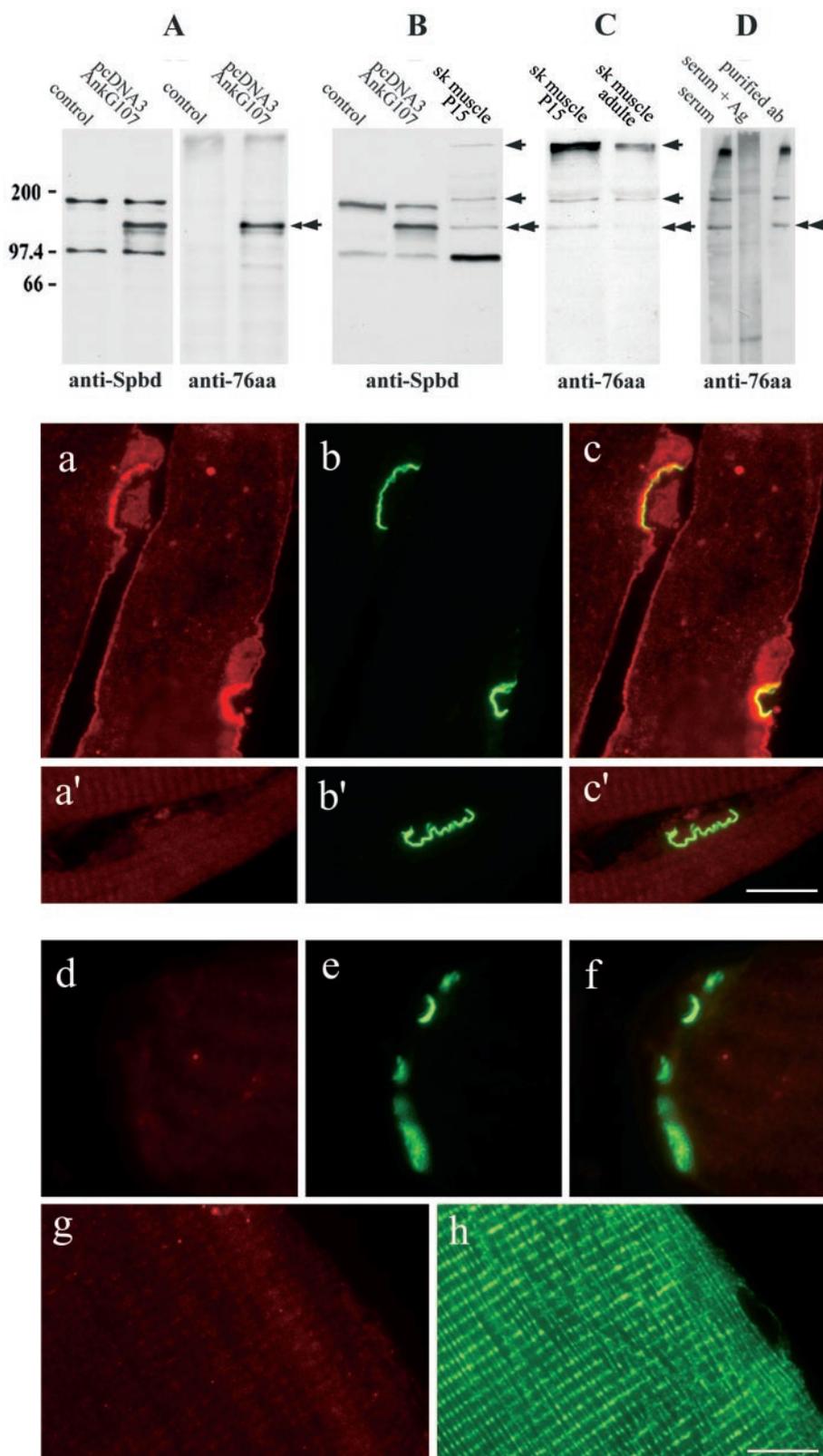
When muscle tissues were compared, relative signal intensities of the two probes suggested that transcripts carrying the 76-residue insert are predominant in skeletal muscle. Moreover, small insert-positive transcripts likely to encode Ank<sub>G107</sub> are detected only in skeletal muscle. Collectively, these data indicate that ankyrin-G isoforms containing the 76-residue insert, and therefore Ank<sub>G107</sub>, are expressed in muscle tissues, and in particular in skeletal muscle.

**Expression and Subcellular Localization of Endogenous Ankyrin-G Carrying the 76-Residue Insert in Skeletal Muscle**—The weak hybridization signal of the 76-residue probe with *Ank3* transcripts in muscle (Fig. 3A) could indicate that only a minor fraction of ankyrins-G contain these sequences. Indeed, a 228-nt difference in transcript size may not be detectable in the present Northern blots, and therefore comigration of transcripts with and without the 76-residue insert is not to be excluded. To elucidate this hypothesis, we raised domain-specific antibodies against muscle ankyrins-G. Antibodies to the 76-residue insert (anti-ank<sub>G</sub>76aa) were raised against two peptides not sharing homology with *Ank1* and *Ank2* sequences. Antibodies to the spectrin-binding domain (anti-ank<sub>G</sub>Spbd) were raised against the NH<sub>2</sub>-terminal portion of the Ank<sub>G107</sub> spectrin-binding domain. This region is present in all currently cloned ankyrins-G and contains unique sequences previously used to generate ankyrin-G-specific peptide antibodies (13, 23); anti-ank<sub>G</sub>Spbd and the previous peptide antibodies provided identical Western blot and immunolocalization patterns in rat skeletal muscle (not shown).

Selective cross-reaction of endogenous Ank<sub>G107</sub> with either of the two antibodies was not expected, because this alternatively spliced isoform can not be distinguished from other muscle ankyrins-G on the basis of primary amino acid sequence. To get information on endogenous Ank<sub>G107</sub>, a full-length cDNA construct was *in vitro* translated in rabbit reticulocyte lysate (Fig. 4A). One band of 130-kDa apparent molecular mass was re-

FIG. 4. Expression and *in situ* localization of endogenous ankyrins-G containing the 76-residue insert in skeletal muscle.

**A**, pcDNA3-Ank<sub>G107</sub> cDNAs were *in vitro* translated in rabbit reticulocyte lysates, and the product was revealed by Western blot using either anti-ank<sub>G</sub>Spbd (left) or anti-ank<sub>G</sub>76aa (right) antibodies. The cross-reacting polypeptide migrated at 130 kDa (double arrow). Control experiments were carried out as described under "Experimental Procedures." **B**, Western blot analysis of total homogenates of 15 day-old rat skeletal muscle (sternomastoid; right lane) using anti-ank<sub>G</sub>Spbd revealed several cross-reacting bands including one polypeptide comigrating with *in vitro* translated Ank<sub>G107</sub> (middle lane; double arrow). **C**, Western blot analysis of equal amounts of total homogenates of 15 day-old (left lane) and adult (right lane) rat skeletal muscle (EDL) using anti-ank<sub>G</sub>76aa revealed three cross-reacting bands of >300, 190, and 130 kDa (double arrow). These polypeptides were also revealed by anti-ank<sub>G</sub>Spbd (**B**) and significantly decreased in the adult. The arrows in **B** and **C** indicate polypeptides cross-reacting with both antibodies. **D**, in control experiments, cross-reaction of anti-serum against the 76-residue insert with the three polypeptides (left lane) in total homogenates of 15-day-old rat skeletal muscle (soleus) was totally abolished after preincubation with an excess of antigenic peptides (middle lane). Right lane, Western blot analysis of the skeletal muscle homogenates using anti-ank<sub>G</sub>76aa antibodies after affinity purification of the antiserum. Semithin cryosections from 15-day-old (*a-c*, *a'-c'*) and adult (*d-h*) rat EDL (*a-c*, *a'-c'*, *g*, *h*; longitudinal sections) and diaphragm (*d-f*; cross-sections) skeletal muscle were labeled with anti-ank<sub>G</sub>76aa antibodies revealed by Cy3-conjugated goat anti-rabbit IgG. In 15-day-old tissue, labeling was in the sarcolemma and the postsynaptic membrane (*a*) and was completely abolished in control experiments (*a'*) following preincubation of the purified antibody with an excess of antigenic peptides. In adult tissue, no labeling was detected either in the sarcolemma (*g*) or in the postsynaptic membrane (*d*); the adult muscle fiber in *g* was visualized by double labeling with anti-SERCA1 antibody (*h*). Postsynaptic membranes were identified by double-labeling with fluorescein isothiocyanate-conjugated  $\alpha$ -bungarotoxin (*b*, *b'*, and *e*). *c*, *c'*, and *f*, merge of the two fluorochromes. Bars, 10  $\mu$ m.



vealed by Western blot with both anti-ank<sub>G</sub>Spbd and anti-ank<sub>G</sub>76aa antibodies and was occasionally accompanied by a minor lower polypeptide that may indicate posttranslational modifications. Western blot analysis using anti-ank<sub>G</sub>Spbd showed that *in vitro* translated Ank<sub>G107</sub> comigrated with one endogenous polypeptide in 15-day-old rat skeletal muscle homogenates (Fig. 4B), suggesting that Ank<sub>G107</sub> is expressed in this tissue. In these homogenates, anti-ank<sub>G</sub>Spbd cross-reacted

with several polypeptides including the major 100-kDa ankyrin-G previously reported in the adult tissue (23). Among those ankyrins-G, only three minor polypeptides of 130, 190, and >300 kDa cross-reacted with anti-ank<sub>G</sub>76aa. Interestingly, these polypeptides appeared significantly decreased in the adult tissue (Fig. 4C). Specificity of the anti-ank<sub>G</sub>76aa antibody is shown in Fig. 4D, where preincubation of the antiserum with the antigenic peptides completely abolished cross-

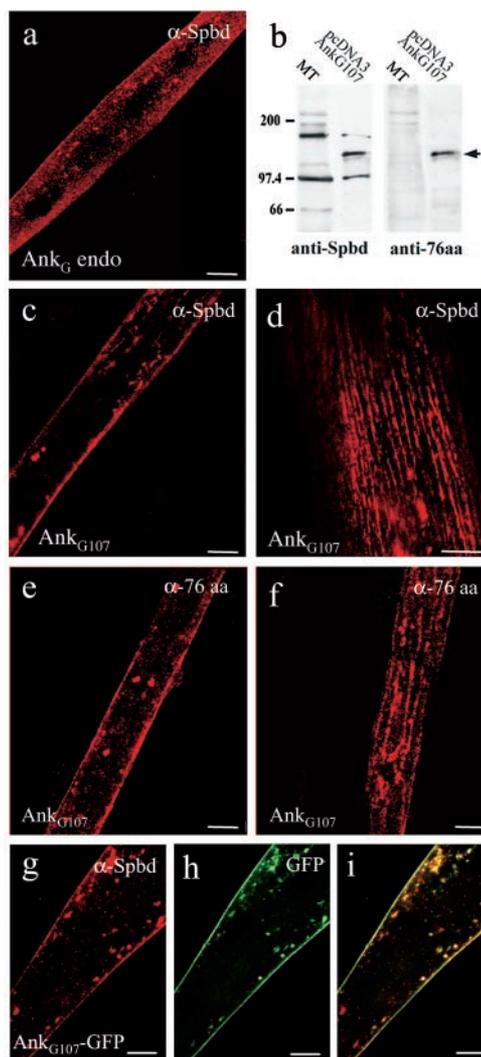
reaction with the three polypeptides. These results showed that only a minor fraction of ankyrins-G contains the 76-residue insert, in agreement with Northern blot analysis, and their expression appears developmentally regulated.

In longitudinal semithin cryosections of 15-day-old rat EDL skeletal muscle, ankyrins-G containing the 76-residue insert localized to the sarcolemma (Fig. 4, *a-c*). Major accumulation was observed on the postsynaptic membrane of the neuromuscular junction. Labeling was abolished following preincubation of anti-ank<sub>G76aa</sub> with antigens (Fig. 4, *a'-c'*). Interestingly, no labeling was detectable in adult EDL and diaphragm muscle fibers (Fig. 4, *d-h*), further supporting a developmentally regulated decrease in the expression of these polypeptides.

**Subcellular Targeting of Ank<sub>G107</sub> in Transfected Myotubes**—We used primary cultures of rat myotubes to study the subcellular distribution of transfected Ank<sub>G107</sub>, using confocal immunofluorescence microscopy (Fig. 5). In nontransfected myotubes, ankyrins-G are present on the sarcolemma as well as in the cytoplasm (Fig. 5*a*), as revealed by anti-ank<sub>GSpbd</sub>. Western blot analysis of total extracts of nontransfected myotubes using anti-ank<sub>GSpbd</sub> confirmed the expression of several ankyrin-G isoforms (Fig. 4*b*, left), including the major 100-kDa protein of adult muscle (23), a major 160-kDa band, and minor 220-, 190-, and 65-kDa bands. Ank<sub>G107</sub> is not expressed at this stage of cell differentiation, since no polypeptide comigrating with *in vitro* translated Ank<sub>G107</sub> was revealed by either anti-ank<sub>GSpbd</sub> or anti-ank<sub>G76aa</sub> (Fig. 5*b*, right) antibodies.

Transfected Ank<sub>G107</sub> was mainly targeted to the sarcolemma (Fig. 5, *c* and *e*), in agreement with *in situ* localization experiments (Fig. 4), and was also detected in cytoplasmic clusters. In optical sections tangential to the surface of transfected myotubes, Ank<sub>G107</sub> appeared organized in parallel, longitudinal strands (Fig. 5, *d* and *f*). The transfected polypeptides were revealed by both domain-specific antibodies, which provided identical results. When anti-ank<sub>GSpbd</sub> was used, the high level expression of Ank<sub>G107</sub> resulted in a higher fluorescence signal that allowed distinction between transfected and endogenous ankyrins-G.

To further evaluate the role of Ank<sub>G107</sub> domains, and in particular of the 76-residue insert, in subcellular targeting, myotubes were transfected with GFP constructs. Subcellular distribution of the full-length GFP fusion protein was identical to Ank<sub>G107</sub> (Fig. 5, *c* and *e*), as revealed by both GFP fluorescent signal (Fig. 5*g*) and anti-ank<sub>GSpbd</sub> immunolabeling (Fig. 5*h*), showing that the presence of GFP in the COOH terminus did not alter the targeting properties of Ank<sub>G107</sub>. GFP constructs (Fig. 6*A*) contained the spectrin-binding domain (Ank<sub>G107</sub>Spb-GFP), the COOH-terminal domain with and without the 76-residue insert (Ank<sub>G107</sub>Cter-GFP and Ank<sub>G107</sub>CterΔ76aa-GFP, respectively), and full-length Ank<sub>G107</sub> without the 76-residue insert (Ank<sub>G107</sub>Δ76aa-GFP). Interestingly, deletion of the 76-residue insert did not prevent localization of Ank<sub>G107</sub> to the sarcolemma but resulted in an additional cytoplasmic distribution (Fig. 6*b*) not observed with the full-length molecule (Fig. 6*a*). A similar pattern was obtained with the spectrin-binding domain (Fig. 6*c*). Localization of the COOH-terminal domain to the sarcolemma was partial and less frequently observed (Fig. 6*d*). Moreover, similarly to the other Ank<sub>G107</sub> truncated polypeptides, a significant fraction of this domain distributed in the cytoplasm. Interestingly, deletion of the 76-residue insert totally abolished membrane distribution of the COOH-terminal domain and resulted in a predominantly diffuse cytoplasmic labeling (Fig. 5*e*) distinct from that of the GFP alone (Fig. 6*f*). These observations were further analyzed by representing the subcellular distribution of expressed Ank<sub>G107</sub> do-

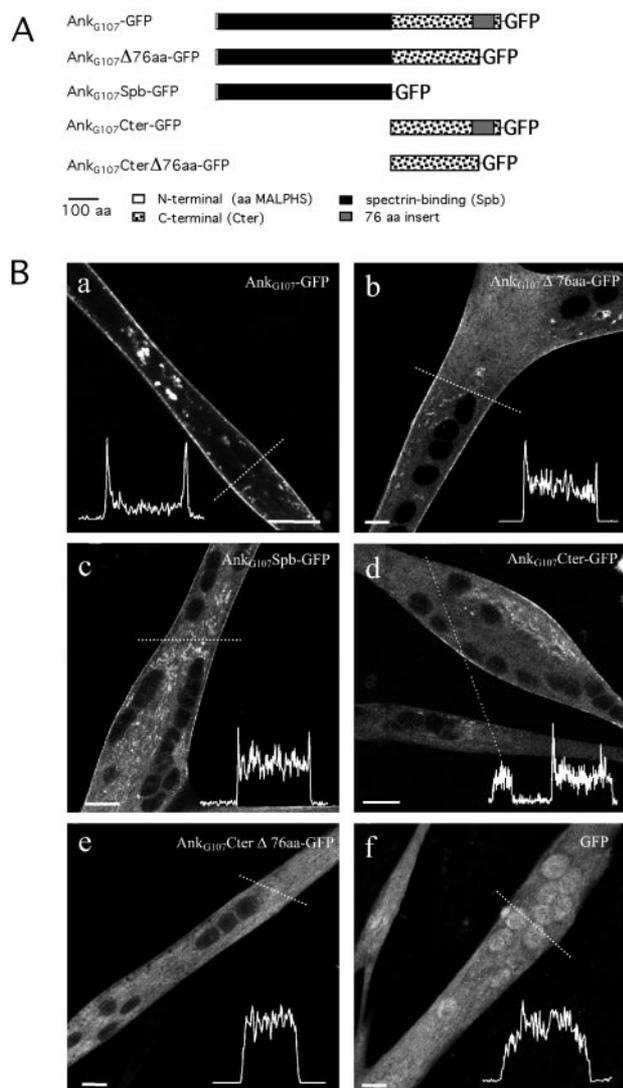


**FIG. 5. Subcellular targeting of Ank<sub>G107</sub> to the plasma membrane of transfected rat myotubes.** Primary cultures of neonatal rat myoblasts were used to study the subcellular distribution of endogenous ankyrin-G (*a*) and recombinant Ank<sub>G107</sub> (*c-i*) polypeptides. Endogenous ankyrins-G are present in the sarcolemma and in the cytoplasm (*a*). Western blot analysis (*b*) of extracts of myotubes (*MT*) using first the anti-ank<sub>G76aa</sub> (*right*) and then the anti-ank<sub>GSpbd</sub> antibodies (*left*) (see “Experimental Procedures” for antibody stripping conditions) showed that although several ankyrins-G are expressed at this stage of cell differentiation, Ank<sub>G107</sub> is not yet present, as deduced by comparison with the *in vitro* translated protein (*right lanes*, pcDNA3-Ank<sub>G107</sub>). In transfection experiments, myoblasts were transiently transfected with recombinant pcDNA3 (*c-f*) and pEGFP-N1 (*g-i*) vectors expressing full-length Ank<sub>G107</sub>. The recombinant polypeptides were localized in myotubes 4–5 days following fusion of myoblasts by confocal microscopy using either anti-ank<sub>GSpbd</sub> (*c*, *d*, *g*, and *i*) or anti-ank<sub>G76aa</sub> labeling (*e* and *f*) or direct detection of the GFP fluorescence (*h* and *i*). Ank<sub>G107</sub> was targeted to the sarcolemma and was also present in cytoplasmic clusters (*c* and *e*). In *d* and *f*, confocal superficial optical sections of pcDNA3-Ank<sub>G107</sub>-transfected myotubes show a labeling pattern of parallel strands running along the longitudinal axis of the cell. GFP-tagged Ank<sub>G107</sub> was localized with either GFP fluorescence (*h*) or anti-ank<sub>GSpbd</sub> antibody (*g*) and displayed identical distribution to Ank<sub>G107</sub> (*c* and *e*), indicating that the GFP tag did not alter subcellular targeting. Overlapping (*i*) of immunolabeling and GFP fluorescence confirmed that the GFP fusion protein contains Ank<sub>G107</sub>. Bars, 10  $\mu$ m.

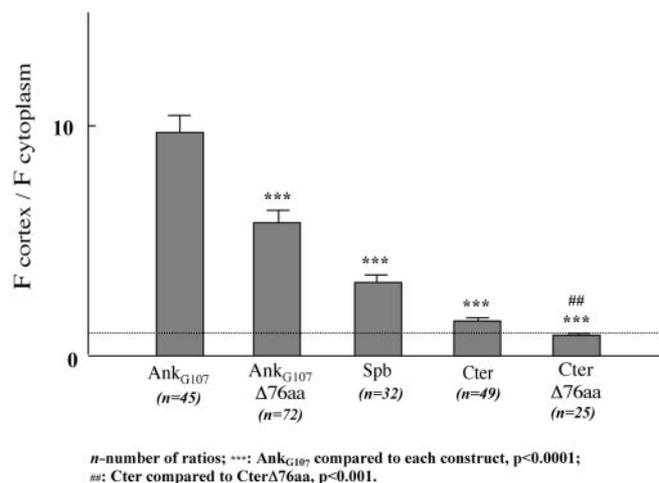
mains as the ratio of cortical over cytoplasmic GFP fluorescence intensities across the transfected myotubes (Fig. 7).

#### DISCUSSION

In this study, we report the molecular cloning and characterization of a novel ankyrin-G polypeptide that is expressed in skeletal muscle. We named this isoform Ank<sub>G107</sub> based on its



**FIG. 6. Contribution of distinct Ank<sub>G107</sub> domains to the subcellular targeting of Ank<sub>G107</sub> in transfected myotubes.** A, schematic representation of GFP-tagged cDNA constructs encoding distinct domains of Ank<sub>G107</sub>. Construct Ank<sub>G107</sub>-GFP contains the full-length Ank<sub>G107</sub> molecule. Construct Ank<sub>G107</sub>Δ76aa-GFP lacks the 76-residue insert. Deletion of the COOH-terminal domain results in construct Ank<sub>G107</sub>-Spb-GFP containing NH<sub>2</sub>-terminal amino acids MALPHS and the spectrin-binding domain. Constructs Ank<sub>G107</sub>-Cter-GFP and Ank<sub>G107</sub>-CterΔ76aa-GFP represent the COOH-terminal domain with and without the 76-residue insert, respectively. B, primary cultures of neonatal rat myoblasts were transiently transfected with the cDNA constructs shown in A, and the expressed polypeptides were detected by the GFP fluorescence in myotubes 4 days following fusion of myoblasts. Full-length Ank<sub>G107</sub> localized to the plasma membrane and to cytoplasmic clusters (a, Ank<sub>G107</sub>-GFP). Deletion of either the 76-residue insert (b, Ank<sub>G107</sub>Δ76aa-GFP) or the total COOH-terminal domain in construct Ank<sub>G107</sub>-Spb-GFP did not abolish sarcolemmal labeling but resulted in an additional homogeneous cytoplasmic distribution. A significant fraction of the COOH-terminal domain was detected in the cytoplasm (d, Ank<sub>G107</sub>-Cter-GFP). This domain also displayed partial and less frequent localization to the plasma membrane, as illustrated in d, where two myotubes with and without membrane labeling are present in the same optical field. Deletion of the 76-residue insert prevented the plasma membrane localization of the COOH-terminal domain and resulted in a predominantly diffuse cytoplasmic labeling (e, Ank<sub>G107</sub>-CterΔ76aa-GFP). The GFP alone distributed in the cytoplasm and the nuclei (f, GFP). Although the spectrin-binding domain is sufficient to address Ank<sub>G107</sub> to the membrane, the 76-residue insert appears to confer stabilization of Ank<sub>G107</sub> at the membrane. White traces in all images show relative cortical and cytoplasmic fluorescence intensity profiles along a straight line (white dotted lines) crossing the labeled myotubes (see "Experimental Procedures"). Bars, 10 μm.



**FIG. 7. Comparison of cortical over cytoplasmic distribution of Ank<sub>G107</sub> domains in transfected rat myotubes.** GFP fluorescence intensity profiles of transfected GFP-tagged Ank<sub>G107</sub> domains as shown in Fig. 6 were measured and analyzed using a ratiometric method as described under "Experimental Procedures." The mean ratios ± S.E. of cortical over cytoplasmic fluorescence intensities ( $F_{\text{cortex}}/F_{\text{cytoplasm}}$ ) obtained from two different experiments are shown for each construct. Two ratios were calculated per myotube to take into account heterogeneous distribution within one cell. Statistical significance was determined using a two-tailed student's *t* test. A decrease in ratio values indicates increased cytoplasmic labeling. Values of CterΔ76aa at the dotted line set at 1 indicate no difference between cortical and cytoplasmic intensities.

sequence homology to Ank3-encoded ankyrins-G and its predicted molecular mass of 106,911 Da. Sequence analysis of Ank<sub>G107</sub> led to the identification of a unique 76-residue sequence within the COOH-terminal domain that is present in a subset of ankyrins-G predominantly expressed in muscle and transiently localized to the sarcolemma and to the postsynaptic membrane during development.

**Ank<sub>G107</sub> Is a Novel Muscle-specific Ankyrin<sub>G</sub> Isoform**—Molecular cloning and sequencing analysis of a full-length cDNA clone showed that Ank<sub>G107</sub> (i) lacks the entire ANK repeat, membrane-binding domain, (ii) starts with the spectrin-binding domain preceded by amino acids MALPHS, and (iii) possesses a 76-residue insert near the COOH terminus, a novel sequence not found in the currently identified ankyrins-G. Northern blot analysis using domain-specific probes showed that the two major 8- and 5.6-kb Ank3 transcripts previously detected in skeletal muscle (23) contain the spectrin-binding and COOH-terminal domains, including the 76-residue insert. In contrast, the repeat, membrane-binding domain is present only in the 8-kb transcript. We conclude that in adult skeletal muscle at least two ankyrin-G polypeptides are expressed, one with estimated molecular mass around 200 kDa carrying the ANK repeat domain and the cloned Ank<sub>G107</sub> that lacks the repeat domain and is apparently encoded by the 5.6-kb transcript. This pattern of Ank3 expression is similar to that in mouse kidney epithelial cells (14), where two classes of transcripts were identified (6.9–7.5 kb and 4.6–6.3 kb) with smaller transcripts lacking the repeat domain. With the exception of the 76-residue insert, Ank<sub>G107</sub> is nearly identical to the Ank3-5kb polypeptides encoded by the small epithelial Ank3 transcripts (Fig. 2B). Some of these polypeptides contain an alternatively spliced acidic insert in the COOH-terminal domain that is not found in Ank<sub>G107</sub>. A 5.7-kb epithelial mRNA, similar in size to the skeletal muscle transcript, lacks the acidic insert and could encode the Ank3-5kb polypeptide that shows maximum homology to Ank<sub>G107</sub>. Alternative expression of distinct insert sequences could provide ankyrin isoforms encoded by the same gene with tissue-specific functions.

The 76-residue insertion is a unique feature of Ank<sub>G107</sub> among the presently cloned ankyrins-G. Surprisingly, the only sequences showing extensive homology with this insert are from the COOH-terminal domains of ankyrins-R and ankyrins-B, the *Ank1* and *Ank2* gene products, and include the entire exon 40 of *Ank1* gene. The COOH-terminal sequences vary among ankyrin genes and apparently regulate the protein interactions of the two other conserved domains (38, 39). Consequently, they could provide specificity with regard to the particular functions of the different isoforms. The only sequences conserved to all three ankyrin genes within the COOH-terminal domain were a 12-kDa stretch of residues, also present in Ank<sub>G107</sub>, showing homology with the "death domain" of proteins involved in apoptosis pathways (40). The 76-residue sequence constitutes an additional region of homology among ankyrins in this domain. Moreover, despite extensive alternative splicing (21) and variation among species (35) occurring in ankyrin-R COOH-terminal sequences, exon 40 and the first part of exon 41 are present in most human *ANK1* (and rodent *Ank1*) gene transcripts (34), suggesting that these sequences are essential to the molecular structure of ankyrins-R. Interestingly, maximum homology is observed between the 76-residue insert and skAnk1, the muscle-specific, truncated, membrane-bound *Ank1* gene product (36, 37). Homology is extended to the nonhydrophobic portion of the alternative exon 39a, which is specifically expressed in muscle tissues presumably under the control of an alternate muscle promoter of the *Ank1* gene (36). These observations raise the question whether the presence of the 76-residue insert provides Ank<sub>G107</sub> with muscle-specific functions. In favor of this hypothesis, expression of the 76-residue insert is predominantly detected in skeletal muscle and heart, by contrast with the widespread tissue expression of the ankyrin-G family (Fig. 3; Refs. 13 and 14). With regard to muscle tissues, insert-containing transcripts are predominant in the skeletal muscle. Moreover, this tissue contains the only small insert-positive transcripts likely to encode Ank<sub>G107</sub>. These observations suggest that Ank<sub>G107</sub> is a skeletal muscle ankyrin-G isoform.

*Endogenous 76-Residue Insert-containing Ankyrins<sub>G</sub> Are Transiently Expressed and Associate with the Postsynaptic Membrane in Developing Muscle Fibers*—The weak hybridization signal of the insert probe in Northern blots of mRNAs from adult skeletal muscle could reflect the presence of the 76-residue sequence only in a minor subset of *Ank3* transcripts. An interesting hypothesis would be that polypeptides carrying this sequence are assigned to specific sites within muscle fibers. To elucidate this question, we immunolocalized these polypeptides in rat skeletal muscle fibers using antibodies raised against the 76-residue insert. Interestingly, labeling was on the sarcolemma and accumulated in the troughs of the postsynaptic folds in 15-day-old muscle fibers, a period of time coincident with formation of the postsynaptic folds and synaptic maturation. A surprising observation was that this labeling was completely lost in adult muscle fibers, suggesting that expression of these ankyrin-G isoforms is developmentally regulated. Ankyrins-G lacking the 76-residue insert remain localized to the adult postsynaptic membrane (23, 26). In agreement with immunolocalization results, in 15-day-old skeletal muscle homogenates anti-ank<sub>G76aa</sub> antibodies cross-reacted with three 130-, 190-, and >300-kDa polypeptides that appeared significantly decreased in the adult tissue. Moreover, the three polypeptides corresponded to a subset of minor proteins when compared with the general pattern of ankyrins-G detected in 15-day-old skeletal muscle by antibodies against the spectrin-binding domain. These results confirmed the hypothesis that the 76-residue sequence is expressed in a subset of muscle

ankyrins apparently involved in temporally restricted events during postnatal maturation of the postsynaptic apparatus. Regarding the >300-kDa polypeptide, it is of interest that minor high molecular weight neuronal ankyrins-G, Ank<sub>G480</sub> and Ank<sub>G270</sub>, are specifically associated with the axolemma of nodes of Ranvier and initial axonal segments (13, 17–19), membrane domains with similar molecular composition and functional properties to the troughs of the postsynaptic membrane of muscle fibers. Further studies are on the way to better identify the isoforms carrying the 76-residue sequence and their function in developing muscle fibers.

*Role of the 76-Residue Sequence in Targeting of Ankyrins<sub>G</sub> to Membrane Domains of Muscle Cells*—To get an insight into the potential function of the muscle-specific 76-residue insert, we transfected Ank<sub>G107</sub> in primary cultures of rat muscle cells. *In vitro* translated Ank<sub>G107</sub> comigrated with the 130-kDa endogenous polypeptide in rat skeletal muscle homogenates, strongly suggesting that the cloned polypeptide is present in muscle and participates in the labeling of the sarcolemma. Several ankyrins-G are expressed in myotubes and localize to the sarcolemma and in the cytoplasm. Interestingly, no 130-kDa polypeptide cross-reacting with any of the two domain-specific antibodies was detected in extracts of myotubes, suggesting that this isoform is not yet expressed at this early stage of muscle cell differentiation.

Full-length Ank<sub>G107</sub> was mainly targeted to the sarcolemma. An interesting observation was that subsarcolemmal Ank<sub>G107</sub> organized in parallel strands running along the longitudinal axis of myotubes. Other cytoskeletal proteins, including dystrophin and spectrin, have been observed in myofibers to form a discontinuous subsarcolemmal lattice including longitudinal strands (41). The periodic pattern of Ank<sub>G107</sub> distribution may thus indicate that this isoform associates with the cortical cytoskeleton. The lack of ANK repeats does not prevent ankyrins from interacting with membrane sites. Such interactions could involve the spectrin-binding domain as well, as was shown for the binding of kidney Ank<sub>G190</sub> to the Na<sup>+</sup>,K<sup>+</sup>-ATPase (6, 12). Ankyrins-G with a truncated or totally deleted ANK repeat domain are still capable of localizing to membrane compartments (15, 16).

The contribution of the different domains of Ank<sub>G107</sub>, and in particular of the 76-residue insert, to the sarcolemmal localization was evaluated by studying their subcellular targeting in transfected rat myotubes. The Ank<sub>G107</sub> spectrin-binding domain was recruited at the plasma membrane, as was previously shown for other ankyrin-G isoforms in neurons (19). However, and at variance with the full-length molecule, a significant amount of the spectrin-binding domain was detected in the cytoplasm. A likely interpretation of the cytoplasmic distribution would be that the truncated Ank<sub>G107</sub> molecules are not stabilized at the membrane. Targeting of the COOH-terminal domain of Ank<sub>G107</sub> to the sarcolemma was much less efficient but still possible, at variance with the neuronal ankyrin-G COOH-terminal domain that remained in the cytoplasm of transfected neurons (19). The COOH-terminal domains of these two ankyrin-G isoforms differ only in the presence of the 76-residue insert. Interestingly, deletion of the 76-residue insert totally abolished membrane localization of the Ank<sub>G107</sub> COOH-terminal domain in transfected myotubes. Accordingly, deletion of the 76-residue insert from the full-length Ank<sub>G107</sub> molecule did not prevent recruitment at the plasma membrane but resulted in increased cytoplasmic distribution, similar to the spectrin-binding domain. Collectively, transfection experiments suggested that the 76-residue insert partially contributes to the targeting and is essential to the stabilization of Ank<sub>G107</sub> at the plasma membrane.

Taken together, these results show that fine tuning of distinct functions of ankyrins encoded by the same gene may be achieved by tissue-dependent and developmentally regulated alternative processing, leading to the expression of distinct sequences. A working hypothesis is that a subset of ankyrins-G playing a key role in the assembly of distinct membrane domains during postnatal differentiation of muscle fibers are stabilized into a membrane-associated multiprotein complex via interactions of the 76-residue insert with other muscle proteins.

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