INTRODUCTION

Ankyrins are a multi-gene family of peripheral proteins that link integral membrane proteins to the spectrin-based cortical skeleton in a variety of cells and tissues. The current belief is that ankyrins are involved in the biogenesis of specialized membrane domains by contributing to the selective accumulation and maintenance of distinct integral proteins at specific subcellular sites. Ankyrin-binding integral proteins belong to the ion channel and cell adhesion molecule families (reviewed in Bennett and Gilligan, 1993), including the Cl-/HCO₃⁻ anion exchanger from erythrocytes (AE1; Bennett and Stenbuck, 1980; Michaely and Bennett, 1995) and brain (AE3; Morgans and Kopito, 1993), the voltage-dependent sodium channel from brain (Srinivasan et al., 1988, 1992), the cardiac Na⁺/Ca²⁺ exchanger (Li et al., 1993), the Na⁺/K⁺-ATPase from kidney (Koob et al., 1987; Nelson and Veshnock, 1987; Devarajan et al., 1994), the amiloride-sensitive sodium channel in the kidney (Smith et al., 1991), the lymphoma ryanodine receptor (Bourguignon et al., 1995b), the neurofascin/L1/NrCAM family (Davis and Bennett, 1994), the hyaluronic acid receptor CD44 (Lokeshwar et al., 1994) and the inositol 1,4,5-triphosphate receptor (Bourguignon et al., 1995a) in lymphocytes.

Diversity within the ankyrin family is due to the expression of at least three genes, designated Ank1, Ank2 and Ank3 in rodents (ANK1, ANK2 and ANK3 in humans), as well as extensive alternative splicing and differential subcellular distribution of their products (reviewed in Bennett and Gilligan, 1993). AnkyrinG (Ank1 gene products) are present in erythrocytes, muscle and the cell bodies and dendrites of a subset of central nervous system (CNS) neurons (Lambert and Bennett, 1993).
Ankyrins \( B \) (\( Ank2 \) gene products) are the major isoforms expressed in the nervous system. 220 kDa ankyrin localizes at neuronal cell bodies and dendrites as well as glia (Kordeli et al., 1990; Kunimoto, 1995). 440 kDa ankyrin, a developmentally regulated neonatal isoform, is specifically targeted to unmethylated and premyelinated axons (Kunimoto et al., 1991; Chan et al., 1993; Kunimoto, 1995). Ankyrin 3 is expressed in many tissues, including kidney (Peters et al., 1995) and the nervous system (Kordeli et al., 1995), where it was first identified. Ankyrin 3 encodes several alternatively spliced variants, the ankynirG (also called ankyrin-3). These are modular polypeptides showing a three-domain structure shared by most ankyrins (reviewed in Lambert and Bennett, 1996). The N-terminal 89-95 kDa membrane-binding domain is composed of 24 copies of 33-residue ANK repeats and binds most integral protein ligands (reviewed in Lambent and Bennett, 1996). The central 62 kDa spectrin-binding domain binds to the fifteenth repeat of \( \beta \) spectrin and fodrin (Kennedy et al., 1991). Both domains are highly conserved among ankyrins. The C-terminal regulatory domain shows a unique primary sequence among distinct gene products and is subject to alternative splicing. Variable domains are expected to modulate binding affinities of both spectrin-binding and N-terminal domains, as demonstrated for erythrocyte ankyrin (Hall and Bennett, 1987; Davis et al., 1992). Ankyrin 3 transcripts are subjected to tissue-specific alternative processing, resulting in isoforms with presumably related but distinct functions. Two large 480- and 270-kDa ankyrin isoforms are specifically expressed in brain (Kordeli et al., 1995), and contain unique sequences inserted between the spectrin-binding and C-terminal domains; inserted sequences begin with a serine-threonine-rich, O-glycosylated stretch of about 400 residues specifically targeted to the nodes of Ranvier and initial axonal segments (Zhang and Bennett, 1996), followed by an additional extended ‘tail’ sequence that joins the C-terminal domain. These inserted sequences are spliced out from all currently known Ankyrin 3 transcripts when expressed in tissues other than brain (Peters et al., 1995; Devarajan et al., 1996; Hooch et al., 1997). Northern blot analysis suggested that some small Ankyrin 3 messages completely lack the N-terminal membrane-binding domain (Kordeli et al., 1995; Peters et al., 1995). The expression of small messages was occasionally correlated with a diffuse cytoplasmic distribution of ankyrin(s)G, further suggesting that ankyrins may associate with intracellular organelles as well as plasma membrane (Peters et al., 1995). Devarajan et al. (1996) provided evidence for the association of ankyrinG19, an ankyrin G variant with truncated N-terminal and distinct C-terminal domains, with the Golgi apparatus and trans-Golgi network in epithelial cells. Two other isoforms encoded by Ankyrin 3 and Ankyrin 1 were subsequently found to associate with lysosomes in macrophages (Hooch et al., 1997) and the Golgi apparatus in MDCK cells (Beck et al., 1997), respectively. These data broadened the proposed functions of the spectrin-ankyrin skeleton to include organization of cytoplasmic compartments and regulation of intracellular tissue (see Devarajan et al., 1997; Lippincott-Schwarz, 1998).

The skeletal muscle fiber is specialized in the reception and transduction of the nerve signal by means of excitation-contraction coupling. These functions involve complex regulation of membrane permeability to ions and, as a consequence, precise localization of ion channels to distinct plasma and intracellular membrane domains. Ankyrins were initially localized at three membrane sites within myofibers: the costameres, where the myofibrils are laterally linked to the sarcolemma (Nelson and Lazarides, 1984), the troughs and the peripheral region of the postsynaptic membrane, where voltage-dependent sodium channels, the Na+/K+-ATPase and N-CAM accumulate (Flucher and Daniels, 1989), and the triads, the junctions between T tubules and sarcoplasmic reticulum (SR), where the ryanodine-sensitive and dihydropyridine-sensitive Ca\(^{2+}\) channels are concentrated (Flucher et al., 1990). These localization data could not distinguish among ankyrin isoforms because of the lack of isoform-specific antibodies. Yet, to address the role of ankyrins in skeletal muscle, it is important to identify the isoforms associated with distinct membrane compartments. Several ankyrin transcripts encoded by at least two genes, Ankyrin 1 and Ankyrin 3, have been previously detected in the mammalian cardiac and skeletal muscle by northern blot analysis (Moon et al., 1985; Birkenmeier et al., 1993; Peters et al., 1995; Devarajan et al., 1996; Zhou et al., 1997). In a recent report, Zhou et al. (1997) used isoform-specific antibodies to show that a novel group of small, unconventional, membrane-bound, Ankyrin 1-encoded ankyrins are associated with, and may link the SR to the contractile apparatus in mammalian skeletal muscle. The location and unusual molecular composition of these ankyrins further emphasized diversity and versatility of the ankyrin family within skeletal muscle.

In the present work, we analyzed the expression and subcellular distribution of ankyrinG, the Ankyrin 3 gene products, in the mammalian skeletal muscle. Ankyrin 3 gene products are relevant candidates for membrane domain organization in myofibers because of (1) their widespread tissue expression, (2) their tissue-specific alternative splicing and (3) their association with intracellular organelles and membrane domains rich in voltage-dependent sodium channels, the Na+/K+-ATPase and adhesion molecules. Our results indicate that alternatively spliced Ankyrin 3 transcripts are indeed expressed in skeletal muscle and encode at least one major 100 kDa ankyrin polypeptide. Furthermore, Ankyrin 3 isoforms are associated with the neuromuscular junction postsynaptic membrane and co-associate with the major SR integral protein, the Ca\(^{2+}\)-ATPase.

**MATERIALS AND METHODS**

**Antibodies**

Affinity-purified polyclonal antibody against human erythrocyte ankyrin (anti-ankyrin) was a gift from Dr V. Bennett (Bennett and Davis, 1982). Affinity-purified polyclonal antibody against the spectrin-binding domain of rat brain ankyrin was (anti-SpBand) was previously described (Kordeli et al., 1995). Monoclonal antibodies to the Ca\(^{2+}\)-ATPase (SERCA1) and \( \alpha \)-actinin were from Affinity Bioreagents and Sigma, respectively. Cy3-conjugated secondary antibodies were from Sigma. FITC-conjugated secondary antibodies F(ab')2 were from Pierce.

**Isolation of skeletal muscle ankyrinG cDNA sequences**

All molecular procedures were carried out using standard methods (Sambrook et al., 1989). Reverse transcription and the polymerase chain reaction (RT-PCR) were used to isolate cDNA sequences corresponding to two regions of ankyrinG as expressed in rat skeletal muscle: (1) oligonucleotide primers 1, 1n and 2 flanked an 870 bp sequence from the spectrin-binding domain; (2) primers 3 and 4 flanked an alternative splicing site and were located on the spectrin-binding and C-terminal domains, respectively. Primer sequences...
were: sense primer 1: 5'-AGTCCGAGAT GTCCTACT CCTG-3';
nested primer 1 (1n): 5'-CATGGGGCAGCA GCA TGATGAT-3';
antisense primer 2: 5'-GGATATGCTGAGATCGGCTAT-3';
sense primer 3: 5'-GAGGCGGCCCCAGAGCTAC-3';
antisense primer 4: 5'-GCTGCTGGCCATAGTCGATCTACT-3'.
Primers 1, 1n, 2 and 3 were rat Ank3 cDNA sequence; primer 4
corresponded to human Ank3 sequence.

Total RNA was isolated from adult rat hindlimb skeletal muscle
using the guanidinium thiocyanate-phenol-chloroform method (RNA
PLUS; Bioprobe Systems, France). We used 1 μg purified total RNA
in a 20 μl reverse transcription reaction containing 1 mM dNTPs, 1
mM dithiothreitol, 2 U/μl RNAsin (Promega), 10 pmol of
downstream primer 2 or 4, and 200 U Superscript RT RNase H-
MLV reverse transcriptase (GIBCO BRL). After incubation at 42°C
for 1 hour, reactions were terminated by heating and the first strand
cDNA was amplified by PCR in a 100 μl reaction mixture containing
200 μM of each deoxynucleotide triphosphate (dNTP), 40 pmol of
either primers 1 and 2, 1n and 2, or 3 and 4, and 2.5 U (1 μl) pfu DNA
polymerase (Stratagene, La Jolla, CA). Reactions were carried out for
35 cycles using an annealing temperature of 55°C. Reaction products
were separated by agarose gel electrophoresis, transferred to
nylon filters (Nytran-plus; Schleicher & Schuell), hybridized with 32 P-
labeled probes from human brain ankyrinG480 and the relative positions of the three rat
cDNAs (1-3) used in the northern blots below. (b) Poly(A)+ RNA (10
μg) from rat skeletal muscle was fractionated in 0.8%
formaldehyde/agarose gels and transferred to nylon
filters. 

**Northern blot analysis**

Total RNA was isolated from adult rat hindlimb skeletal muscle using
the guanidinium thiocyanate-phenol-chloroform method (RNA
PLUS; Bioprobe Systems, France) and poly(A)+RNA prepared as
described (Sambrook et al., 1989). 10 μg of poly(A)+RNA were
fractionated in 0.8% formaldehyde/agarose gels, transferred to nylon
filters (Nytran-plus; Schleicher & Schuell), hybridized with 32P-
labeled Ank3 cDNAs and washed at 68°C with 0.2×SSC, 0.1% SDS,
before autoradiography.

**Immunoblot analysis**

Pieces of rat hindlimb and diaphragm skeletal muscle 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 used in
SDS-PAGE and immunoblot analysis. SDS-PAGE was performed
using a discontinuous and highly porous gel system with 4% stacking
and 10% separating gels as modified by Doucet et al. (1990).

**Immunofluorescence microscopy**

Adult male Sprague-Dawley rats were anesthetized by pentobarbital
and injected at the level of the diaphragm with 3%
paraformaldehyde/0.1 M phosphate buffer, pH 7.4 Following in situ
fixation for 10 minutes, the diaphragm was collected, further postfixed
cut out to small blocks from the synapse-rich medial areas,
infused with increasing sucrose solutions (0.5 to 2.1 M in PBS), and
frozen in liquid nitrogen. Semithin (0.5 to 1 μm) cross and
longitudinal cryosections of diaphragm striated muscle were
preincubated in PBS containing 1% BSA and 5% decomplemented
goat serum, immunostained for indirect immunofluorescence and
mounted in an antibleach/glycerol/PBS solution (Citifluor Ltd,
London, UK). Antibodies were diluted at 1-5 μg/ml in PBS containing
0.1% BSA and 0.5% goat serum. Fluorescein isothiocyanate-
conjugated α-bungarotoxin (1 μg/ml; Sigma) was used to label
acetylcholine receptors in the postsynaptic membrane.

**RESULTS**

**Expression of ankyrinG in the skeletal muscle fiber**

The expression and alternative splicing pattern of ankyrinG-
encoding transcripts in skeletal muscle were investigated using northern blot (Fig. 1) and RT-PCR (Fig. 2) analyses. Rat skeletal muscle poly(A\(^+\)) RNA was hybridized with domain-specific rat brain Ank3 cDNA probes (Fig. 1). Probes to sequences within the spectrin-binding (lane 1) and C-terminal (lane 3) domains each detected two transcripts of 8.0 kb and 5.6 kb. A probe from the serine-rich domain (lane 2) failed to cross-hybridize with mRNAs, indicating that this domain is not expressed in skeletal muscle.

Two sets of oligonucleotide primers corresponding to brain Ank3-specific sequences were used in PCR amplification of cDNA reverse-transcribed from rat skeletal muscle total RNA. The primers were designed to flank two distinct regions of the ankyrinG molecule: (1) amino acid sequence 2,979-3,848 from the spectrin-binding domain of human brain ankyrinG (Kordeli et al., 1995; Fig. 2a, primers 1, 1n and 2); (2) an alternatively spliced sequence inserted between the spectrin-binding and C-terminal domains; in human brain ankyrinG(480, this region includes the 7,813 bp serine-rich and tail domains (Kordeli et al., 1995; Fig. 2a, primers 3 and 4 located at the spectrin-binding and C-terminal domains, respectively). The identity of the PCR products was confirmed using Southern hybridization (Fig. 2b,c) with rat brain Ank3 cDNA probes. Positive products were subsequently cloned and sequenced.

**Fig. 2.** RT-PCR analysis of Ank3 transcripts in rat skeletal muscle. (a) Schematic diagram showing the domain structure of human brain ankyrinG(480) and the location of PCR primers 1-4 used in amplification of PCR products A and B from reverse transcribed rat skeletal muscle total RNA. Sense primer 1n is nested to primer 1. (b,c) Ethidium bromide-stained 1% agarose gel (lanes A, An, B) and Southern blot analysis (lanes A', An', B') of PCR products A (b) and B (c) probed with \(^{32}\)P-labeled rat brain Ank3 cDNA clones. PCR band A\(_n\) was amplified using the nested sense primer 1n. Open arrows indicate positive PCR products. (d) Comparison of the RT-PCR-derived first 32 amino acids sequence of PCR product B (rat skeletal muscle AnkG) and the corresponding sequences from mouse kidney Ank3, human kidney Golgi AnkG119 and human brain AnkG480. Identical amino acids are indicated by bold face letters. The residue numbers of ankyrinG(480) (published sequence; Kordeli et al., 1995) refer to the junctional areas between: spectrin-binding and serine-rich domains (aa 1,455-1,597); tail and C-terminal domains (aa 4,059-4,102). Dashes indicate a 12-residue deletion and the arrow points to the proposed splicing site between spectrin-binding and C-terminal domains of rat skeletal muscle ankyrinG.
AnkyrinG and ankyrinR are differently distributed in the skeletal muscle fiber

We used indirect immunofluorescence microscopy to compare the localization of ankyrG and ankyrin in rat diaphragm muscle. To obtain optimal resolution we used 0.5 μm-thick cryosections. Anti-SpBd antibodies were localized to the postsynaptic membrane of the neuromuscular junction (Fig. 4a). Neuronal junctions were identified by the fluorescent α-bungarotoxin (α-Bugtx), a high affinity marker for nicotinic acetylcholine receptors (AChRs) that accumulate on the crests of the postsynaptic membrane (Fig. 4a). AnkyrinG labeling was underneath AChRs, as seen on transverse sections double-labeled with anti-SpBd and the α-Bugtx (Fig. 4a,c, arrowheads). This labeling pattern is compatible with ankyrinG localization at the troughs of the postsynaptic folds. Interestingly, anti-AnkR antibodies were also localized to the postsynaptic membrane (Fig. 4d, arrowheads). These antibodies may cross-react with conserved epitopes shared by other ankyrin gene products, including ankyrG490 of the nodes of Ranvier (Kordeli et al., 1990; Kordeli and Bennett, 1991). Nevertheless, anti-AnkR mainly recognizes erythroid Ank1 gene products in several tissues and did not cross-react with the major ankyrin polypeptide in immunoblots of skeletal muscle homogenates (Fig. 3). An eventual cross-reaction with subsynaptic ankyrin might not be resolved by immunoblot analysis, because of the low relative abundance of this isoform in skeletal muscle homogenates. Taken together, these results show that ankyrG is localized and may coexist with ankyrin at the troughs of the postsynaptic membrane.

The major ankyrinG labeling in rat skeletal fibers was associated with intracellular structures. Anti-SpBd labeling displayed a cytoplasmic reticular distribution on rat diaphragm transverse sections (Fig. 5a). On the contrary, the major anti-AnkR localization was on the sarcolemma (Fig. 5b, arrows).
agreement with previous observations (Nelson and Lazarides, 1984). Erythrocytes in the capillaries between the unperfused skeletal muscle fibers were intensely labeled with anti-AnkR but not with anti-SpBd antibodies (Fig. 5a,b, open arrow), further demonstrating that these are isoform-specific antibodies. With the notable exception of the postsynaptic membrane, anti-SpBd did not significantly label the sarcolemma. Therefore, the major ankyrin isoform associated with the sarcolemma is AnkR. Intracellular and subsynaptic labelings were abolished when anti-SpBd was incubated with a 20-fold molar excess of the immunogenic peptides prior to incubation with cryosections (Fig. 4b’). Preincubation of anti-AnkR antibody with the same peptides had no effect (not shown).

In longitudinal sections, two distinct labeling patterns were observed when using anti-SpBd: a transverse, striated, Z-line-like repetitive pattern (Fig. 5c,d, arrowheads), and a longitudinal component (small arrows). Anti-AnkR labeling was distinct from anti-SpBd. This antibody labeled transverse repetitive structures at the level of Z- and M-lines (Fig. 5e,f); no labeling was observed along longitudinal sarcoplasmic structures (Fig. 5f). The anti-AnkR punctate repetitive labeling (Fig. 5f, arrowheads) was reminiscent of previous electron microscopic localization of ankryins at the A-I interface flanking the Z-lines (Flucher et al., 1990), and similar to the distribution of the recently identified small, membrane-bound polypeptides that contain part of ankyrin C-terminal domain (Zhou et al., 1997). The anti-AnkR antibody was raised against the total molecule of human erythrocyte ankyrin and most probably cross-reacts with epitopes from the C-terminal domain. Small polypeptides were occasionally detected by this antibody in immunoblots of skeletal muscle homogenates, when low molecular mass bands were present on the polyacrylamide gels (not shown).

To further identify the sarcoplasmic structures labeled with anti-SpBd and anti-AnkR around Z- and M-lines, we compared localizations of the two ankyrin isoforms and α-actinin, a Z-line component, in double immunofluorescence experiments (Fig. 6). Interestingly, labeling patterns were clearly distinct: ankyrinG transverse repetitive labeling is in register with Z-lines (Fig. 6a-d), whereas the major ankyrin R labeling is at the A-band level. A thinner ankyrin R-containing band codistributes with α-actinin at Z-lines. These results demonstrate that ankyrin(s)R and ankyrin(s)G are localized differently in the sarcoplasm, and strongly suggest that several ankyrin isoforms coexist at adjacent subcellular sites.

**DISCUSSION**

In the present study we show that alternatively spliced Ank3...
transcripts are expressed in skeletal muscle and their products, ankyrin(s)G, are selectively localized at the postsynaptic membrane of the neuromuscular junction, and the SR. With the exception of the postsynaptic folds, ankyrin(s)G did not accumulate at the sarcolemma. Instead, the major sarcolemmal isoform was the Ank1 gene product, ankyrin R, as previously suggested (Nelson and Lazarides, 1984) and recently demonstrated using isoform-specific antibodies (Zhou et al., 1997). These new data extend the notion that multiple ankyrin isoforms are expressed in the skeletal muscle fiber and localized at distinct membrane compartments.

Diversity of Ank3 transcripts in rat skeletal muscle
Ankyrin transcripts encoded by at least two distinct genes, AnkI and Ank3, have been previously detected in cardiac and striated muscle, based on northern blot analysis. Two classes of messages, large (9.0 kb and 7.5 kb) and small (1.6 kb to 3.5 kb), cross-hybridized with AnkI cDNA probes in avian and mammalian skeletal muscle (Moon et al., 1985; Birkenmeier et al., 1993; Zhou et al., 1997). Small messages were missing large portions of coding sequence from the membrane- and spectrin-binding domains (Birkenmeier et al., 1993; Zhou et al., 1997). Similar results were obtained regarding Ank3 transcripts. cDNA clones from the Ank3 spectrin-binding domain cross-hybridized with multiple transcripts in the mouse heart (7, 6.3, 6.0 and 5.6 kb; Peters et al., 1995), human heart (8 and 9 kb) and human skeletal muscle (8.0 and 6.0 kb; Devarajan et al., 1996). Interestingly, by analogy to AnkI, the smaller Ank3 transcripts lacked the membrane-binding domain, at least when expressed in kidney (Peters et al., 1995) and macrophages (Hoock et al., 1997). These data suggested a complex expression pattern of the Ank3 gene in skeletal muscle, involving multiple alternative processing of messages. To better identify this expression pattern, we used ankyrinG domain-specific hybridization probes

Fig. 5. Comparative immunofluorescence localization of ankyrinG and ankyrin in skeletal muscle fibers. Transverse (a,b) and longitudinal (c-f) rat diaphragm semi-thin cryosections were labeled with polyclonal antibodies anti-SpBd to ankyrinG (AnkG; a,c,d) or anti-AnkR to erythroid ankyrin (AnkR; b,e,f) followed by Cy3-conjugated goat-anti-rabbit IgG. Note the reticular cytoplasmic distribution of ankyrinG (a) and the punctate sarcolemmal localization (b, arrows) of ankyrin in transversely oriented muscle fibers. Anti-AnkR, but not anti-SpBd, also labeled erythrocytes (b, open arrow), as expected. In longitudinal sections, ankyrinG labeling (c,d) was on punctate, transverse structures (small arrowheads) and longitudinal strands (small arrows); ankyrin punctate labeling (e) was around Z- and M-lines, as well as sarcolemma. Bars, 10 μm.
to detect Ank3 transcripts in rat muscle (Fig. 1). Two Ank3 transcripts of 8.0 kb and 5.6 kb carrying the spectrin-binding and C-terminal, but not the serine-rich, domains were observed. The size of these transcripts was similar to human skeletal muscle Ank3 transcripts (Devarajan et al., 1996), and consistent with the splicing out of serine-rich and tail domains (Kordeli et al., 1995; Peters et al., 1995). However, all evidence supporting identification of muscle Ank3 transcripts was limited to high stringency northern blot analysis. A more direct insight into this question was provided by reverse transcriptase PCR analysis (Fig. 2). Two PCR products were amplified from total rat skeletal muscle RNA. PCR product A corresponded to a highly conserved region from the spectrin-binding domain that contains short segments of ankyrinG-specific sequences. PCR product A was identical to corresponding rat brain Ank3 cDNA sequences, demonstrating the expression of Ank3 gene in skeletal muscle. PCR product B brought additional information on the alternative processing of muscle Ank3 transcripts. Primers were located in the spectrin-binding and C-terminal domains, and were flanking a major splicing site observed in brain transcripts (Kordeli et al., 1995). This region includes a 7,813 bp insert corresponding to the serine-rich and tail domains of brain ankyrinG480 (Fig. 2a, primers 3,4). To date, this insert has been found only in two brain-specific 15 kb and 10 kb Ank3 transcripts (Kordeli et al., 1995), and it is spliced out from transcripts encoding ankyrinG in tissues other than brain (Peters et al., 1995; Devarajan et al., 1996; Hoock et al., 1997). These primers amplified a single positive PCR product B of 478 bp. Sequencing data showed that (1) the spectrin-binding domain directly joined the C-terminal domain, suggesting that at least one major transcript encoding ankyrinG in rat skeletal muscle has the serine-rich and tail domains spliced out; and (2) confirmed the splicing site (Fig. 2d, arrow) by comparison with corresponding cDNA sequences from brain (Kordeli et al., 1995) and epithelial transcripts (Peters et al., 1995; Devarajan et al., 1996). These results were in agreement with northern blot data (Fig. 1). Yet, they do not rule out the occurrence of additional, minor Ank3 transcripts carrying the total or part of the 7,813 bp insert. For instance, alternatively spliced minor transcripts may encode ankyrin(s)G localized at restricted subcellular sites of the myofiber (see below). Extensive alternative processing has been reported for other domains of the ankyrinG molecule, including total or partial lack of the membrane-binding repeats, two alternatively-spliced sequences between repeats and spectrin-binding domain, an acidic insert within the C-terminal domain, and a short totally distinct C-terminal domain (Peters et al., 1995; Devarajan et al., 1996).

Taken together, the present data suggest that the major Ank3 transcripts in skeletal muscle contain the spectrin-binding and C-terminal domains, but not the serine-rich and tail-inserted sequences, as is the case in other non-brain tissues. This alternative splicing pattern is also consistent with the size of the single major 100 kDa polypeptide detected by ankyrinG-specific antibodies in skeletal muscle immunoblots (Fig. 3). Cloning of muscle ankyrin(s)G, currently in progress in our laboratory, will provide further information on the domain structure of these polypeptides.

**AnkyrinG is a component of the postsynaptic membrane**

We used an ankyrinG-specific antibody to show that Ank3 gene products are selectively associated with two membrane...
domains of the skeletal myofiber, the postsynaptic membrane and the SR. In mammals, the postsynaptic membrane is organized in two domains with distinct molecular composition: (1) the crests, with local accumulation of AChRs and (2) the folds, where voltage-dependant sodium channels, N-CAM and the Na+/K+-ATPase are concentrated. This membrane organization favors optimal functioning of the mammalian synapse. Distinct cortical skeletons are associated with the two domains: AChRs codistribute with rapsyn- and the utrophin-based skeleton (reviewed in Cartaud and Changeux, 1993), whereas a spectrin-based skeleton underlines the folds (Flucher and Daniels, 1989; Vybiral et al., 1992). In addition to in situ codistribution at the postsynaptic folds, ankyrins bind in vitro to the brain voltage-dependant sodium channel (Srinivasan et al., 1988; 1992), the epithelial Na+/K+-ATPase (Koob et al., 1987; Nelson and Veshnock, 1987; Devarajan et al., 1994) and the neurofascin/L1/NrCAM-related adhesion molecules (Davis and Bennett, 1994). The molecular composition of the postsynaptic folds is very similar to the axolemma of the nodes of Ranvier, where ankyrinsG specifically accumulate (Kordeli et al., 1995; Zhang and Bennett, 1996; Davis et al., 1996). In both cases, the voltage-dependent sodium channel would be a relevant candidate to bind ankyrinsG. Sodium channels in the postsynaptic membrane are immobilized (Angelides, 1986) presumably via interaction with the spectrin skeleton. Because of low relative abundance in the skeletal muscle fiber, the precise domain organization of the postsynaptic ankyrinG cannot be deduced from the present immunoblot and RT-PCR experiments. An alternative approach would be the use of several domain-specific antibodies in localization experiments. However, in situ immunological detection of postsynaptic ankyrinG by anti-SpBd antibody indicates the presence of a spectrin-binding domain. Both α-fodrin and the muscle isoform of erythroid β-spectrin (Winkelmann et al., 1990) were previously localized at the neuromuscular junction (Vybiral et al., 1992). Another muscle-specific β-spectrin isoform is present at the neuromuscular junction and codistributes with AChR clusters of myocytes in vitro (Bloch and Morrow, 1989). Therefore, ankyrinG is likely to link integral postsynaptic proteins, i.e. sodium channels, to the spectrin-skeleton.

Fig. 7. Comparative immunofluorescence localization of ankyrinG and SERCA1 ATPase in skeletal muscle fibers. Longitudinal (a-c) and transverse (d-f) rat diaphragm semi-thin cryosections were double-labeled with antibodies to ankyrinG (anti-SpBd) (AnkG; a,d) and to the Ca2+-ATPase of SR (SERCA; b,e) followed by FITC-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG, respectively. Simultaneous visualization of the two fluorochromes (c,f) showed extensive overlap (yellow signal), indicating codistribution of the two polypeptides in the SR. Insets, higher magnification of the indicated areas (c,d rectangles). Arrows in c point to the longitudinal structures presumably corresponding to nonjunctional SR. Bars, 10 μm; inset bars, 6.5 μm.
Interestingly, timing and regulation of AChR and sodium channel accumulations at the postsynaptic membrane during development are different. At variance with AChR clustering occurring at the earliest stages of synaptic differentiation, the sodium channel aggregation takes place gradually over the first 5-6 weeks after birth, coincides with the formation of the postsynaptic folds, and is a key event for the late stage of synaptogenesis (Lupa et al., 1993). Selective targeting of an ankyrin isoform at the postsynaptic folds may be crucial to the assembly of sodium channels during synaptogenesis.

Anti-AnkR antibody was localized to the postsynaptic folds as well. Although a cross-reaction between this antibody and subsynaptic ankyrinG cannot be totally excluded (see Results), these data strongly suggest that both ankyrin isoforms coexist at the postsynaptic membrane. Coexistence of Ank1 and Ank3 gene products has been recently discovered in other membrane compartments, i.e. the Golgi apparatus (Devarajan et al., 1996; Beck et al., 1997). AnkyrinR being associated with both junctional and extra-junctional sarcosome, the presence of an additional ankyrinG isoform at the postsynaptic membrane further implies a precise function for ankyrinG in synaptic differentiation. In most in vitro binding studies between ankyrin and integral membrane proteins, the erythrocyte ankyrin was used mainly because of well established purification procedures. However, in situ codistribution data strongly suggest that distinct ankyrins preferentially bind to distinct integral polypeptides. Accordingly, erythrocyte ankyrin binds with much higher affinity to the cerebral than to the axonal isoform of Na+/K+-ATPase (Shibayama et al., 1993). Erythrocyte ankyrin coimmunoprecipitates with the brain anion exchanger AE3, but not with the closely related homologue AE2 (Morgans and Kopito, 1993). Subsynaptic ankyrinG and ankyrinR may have different membrane ligands, and therefore related but distinct functions during synaptogenesis.

AnkyrinG codistributes with the Ca²⁺-ATPase in the sarcoplasmic reticulum

The major localization of ankyrin(s)G in rat myofibers was intracellular and consisted of two components: a transverse, repetitive, Z-line-like one and a longitudinal one. This reticular labeling was reminiscent of the SR. This hypothesis was confirmed by the extensive codistribution of ankyrinG with Ca²⁺-ATPase, the major integral protein of SR, in double-labeling experiments. AnkyrinG also colocalized with α-actinin at the Z-line, as expected. It is reasonable to assume that the major 100 kDa polypeptide detected in immunoblots of total muscle homogenates, as well as the splicing out of serine-rich and tail domains resolved by RT-PCR, correspond to the SR-associated ankyrin. In addition to the cortical skeleton(s) of plasma membrane, ankyrins are known to associate with intracellular compartments, apparently in a dynamic manner (Beck et al., 1997; Devarajan et al., 1997). Ank3 gene products were the first ankyrins to be found in association with the Golgi apparatus in MDCK cells (Devarajan et al., 1996) and the lysosomes in macrophages (Hoock et al., 1997). The Golgi protein, Ankg119, is missing half of the N-terminal repeated sequences, and contains a short, distinct C-terminal domain. This polypeptide of apparent mass 116 kDa has been detected by immunoblotting in the myoblastic cell line C2C12 (Devarajan et al., 1996). Cloning of the muscle ankyrin(s)G will indicate to what extent the 100 kDa ankyrinG, probably associated to the SR, and the Golgi AnkG119 are related. The cross-reacting epitopes in our localization experiments indicate that the SR ankyrinG, and the presumably related 100 kDa polypeptide, contain at least the spectrin-binding domain. This polypeptide, therefore, represents a more conventional variant of ankyrin, likely to interact with both spectrin and integral protein(s) of the SR. The muscle isoform of β-spectrin has been observed in the sarcoplasm of a subpopulation of myofibers (Porter et al., 1997). A plausible candidate to interact with ankyrinG in the SR membrane is the Ca²⁺-ATPase, as suggested by their in situ codistribution, and the fact that ankyrins in general associate with ion pumps. Interestingly, ankyrin(s) may be involved in the regulation of internal Ca²⁺ release through interaction with ryanodine receptors and IP₃ receptors (Bourguignon and Jin, 1995a,b). According to two recent reports, Ank1 gene products also associate with the Golgi apparatus (Beck et al., 1997) and the SR (Zhou et al., 1997). In the latter case, small membrane-bound polypeptides containing only the last 82 residues of ankyrinG surround the myofibrils at the M- and mainly Z-lines, and are supposed to link SR to the contractile apparatus. We also observed ankyrinG immunoreactivity at the level of Z- and M-lines. In contrast to the small membrane-bound ankyrins (Zhou et al., 1997), ankyrinG detected in the present study were mainly associated with A bands flanking M-lines, as compared to α-actinin distribution. A plausible explanation would be that more than one AnkG gene product, as well as ankyrinG, are present in this sarcoplasmic compartment.

The multiplicity of ankyrin isoforms in the muscle fiber as well as the diversity of ankyrin functions in cells (see Lipincott-Schwartz, 1998) are beginning to unravel. Of particular interest with respect to the function of ankyrins in muscle is that isoforms encoded by the same gene are targeted to distinct domains, whereas distinct isoforms totally or partially coexist in the same subcellular compartment. This places muscle fibers in a privileged position as a model system for use in further identifying the structure/function relationship of ankyrins in cells.

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