A New Spectrin, βIV, Has a Major Truncated Isoform That Associates with Promyelocytic Leukemia Protein Nuclear Bodies and the Nuclear Matrix*

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We isolated cDNAs that encode a 77-kDa peptide similar to repeats 10–16 of β-spectrins. Its gene localizes to human chromosome 19q13.13–q13.2 and mouse chromosome 7, at 7.5 centimorgans. A 289-kDa isoform, similar to full-length β-spectrins, was partially assembled from sequences in the human genomic DNA data base and completely cloned and sequenced. RNA transcripts were seen predominantly in the brain, and Western analysis shows a major peptide that migrates as a 72-kDa band. This new gene, spectrin βIV, thus encodes a full-length minor isoform (SpβIVΔ1) and a truncated major isoform (SpβIVΔ5). Immunostaining of cells shows a micropunctate pattern in the cytoplasm and nucleus. In mesenchymal stem cells, the staining concentrates at nuclear dots that stain positively for the promyelocytic leukemia protein (PML). Expression of SpβIVΔ5 fused to green fluorescence protein in cells produces nuclear dots that include all PML bodies, which double in number in transfected cells. Deletion analysis shows that partial repeats 10 and 16 of SpβIVΔ5 are necessary for nuclear dot formation. Immunostaining of whole-mount nuclear matrices reveals diffuse positivity with accentuation at PML bodies. Spectrin βIV is the first β-spectrin associated with a subnuclear structure and may be part of a nuclear scaffold to which gene regulatory machinery binds.

Spectrin is an important component of the membrane skeleton attached to the inner leaf of the lipid bilayer of plasma membranes. First described in the erythrocyte (1), spectrins are found in all or almost all cells (2–4). In erythrocytes, an intact spectrin-based membrane skeleton is critical for the structural integrity of the plasma membrane. Defects in its components are associated with red cell fragility and premature destruction in the human diseases hereditary spherocytosis and elliptocytosis and their animal models (5). The function of a spectrin-based plasma membrane skeleton in non-erythroid tissues is less well defined, but it is hypothesized to be important in establishing and maintaining the asymmetric distribution of proteins in specialized plasma membrane domains, particularly in polarized cells (6).

Recently, components of a spectrin-based membrane skeleton have also been found in several intracellular organelles. Isoforms of spectrin and ankyrin exist in Golgi membranes (7–9), lysosomal membranes (10), and secretory vesicles (11–14). Spectrin also associates with actin-related protein 1 (c-actinin), a subunit of the dynactin complex, which associates with dynein and transports vesicles along microtubules in the secretory pathway (15). A spectrin-based membrane skeleton attached to intracellular organelles may provide a structural framework to anchor the vesicular transport machinery (16–19). The potential role of a spectrin-based membrane skeleton in the nucleus is unclear. There are interesting recent reports indicating that spectrin αII is part of a nuclear protein complex involved in repair of DNA interstrand cross-links (20–22). Whether αII-spectrin binds with a β-spectrin partner in the nucleus to form a membrane skeleton is unknown. We have previously described spectrin βIII (see footnote 1 for nomenclature),1 which associates with the Golgi and intracellular vesicles (9). We now identify another intracellular β-spectrin, spectrin βIV, which has a major truncated isoform (βIVΔ5) and a full-length isoform (βIVΔ1). Spectrin βIV resides in the cytoplasm, where it may attach to vesicles, and in the nucleus, where it associates with PML2 bodies and the nuclear matrix.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank℠/EMBL Data Bank with accession number(s) AF311855 and AF311856.

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1 Nomenclature: β1Σ1-Spectrin is the erythroid isoform (Σ1) of erythroid (β) spectrin. It is also called spectrin E. The gene name is SPTB (human) or Snpb1 (mouse). β1Σ2-Spectrin is the muscle isoform (Σ2) of erythroid spectrin. It contains a different C-terminal sequence. β1-Spectrin is “non-erythroid” β-spectrin. It has also been called fodrin, brain spectrin, spectrinα, or spectrin beta, non-erythroid type 1. The gene name is SPTB1 (human) or Snpb2 (mouse). βIII-Spectrin has the gene names SPTBN2 (human) or Snpb3 (mouse), respectively. Spectrin βV (gene name BSPECV) is the recently described mammalian equivalent of Drosophila beta heavy spectrin. Spectrin βIV has at least five isoforms, βIVΔ1 through βIVΔ5. Isoforms βIVΔ1 and βIVΔ5 are discussed in this report. The gene names of spectrin βIV are SPTBN3 (human) and Snpb4 (mouse).

2 The abbreviations used are: PML, promyelocytic leukemia; APML, acute promyelocytic leukemia; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; GST, expressed sequence tag; GFP, green fluorescence protein; MDCK, Madin-Darby canine kidney cells; MSCs, mesenchymal stem cells; N164/N155, a contig of overlapping spectrin βIV clones N164 and N155; NR, non-repeat segment of...
βIV Spectrin

While this manuscript was in revision, Berghs et al. (23) independently described spectrin βIV and four of its isoforms: βIV1, βIV2, βIV3, and βIV4. Their βIV1 isoform corresponds to our full-length spectrin βIV, also named βIV1. The 77-kDa isoform (βIV2) described here is the major isoform of spectrin βIV. It was not reported by Berghs et al. and could not have been detected with the antibodies they employed (23).

MATERIALS AND METHODS

Molecular Cloning and DNA Analysis—Search of the GenBank® database was performed using the NCBI BLAST similarity search programs. Nucleotide sequence analysis was done using the University of Wisconsin Genetics Computer Group sequence analysis programs. Screening and isolation of cDNA clones were done either by standard methods or by the GeneTrapper cDNA positive-selection method according to the manufacturer’s instructions (Life Technologies, Rockville, MD). A hybridization oligonucleotide (5′-CCA AGC CGA CTG CCG CTT-3′) and human brain plasmid cDNA library (Life Technologies) were used in the GeneTrapper method. Automated nucleotide sequencing was performed in the Children’s Hospital Mental Retardation Research Center DNA Sequencing Core Facility using the dyeoxyxucleotide termination method. Polymerase chain reaction (PCR), and anchored PCR amplifications were done using the Advantage 2 polymerase kit (CLONTECH Laboratories, Palo Alto, CA). PCR templates used were Marathon-ready cDNAs prepared from retina or brain (CLONTECH). The cloning strategy and sequences of oligonucleotide primers used in the isolation of overlapping clones that constitute the full-length spectrin βIV cDNA are available upon request.

Chromosome Localization—Chromosome localization of the human spectrin βIV gene utilized the Stanford G3 radiation hybrid panel (24) (Research Genetics, Huntsville, AL). Hybrid human-hamster clones were assayed for the presence of spectrin βIV gene by PCR. Using the primers 5′-CCA TTG AGA AG-3′ (forward) and 5′-AAG TCG CAG TCG TGC GCT GC-3′ (reverse), the 107-bp product, representing the 5′-CAA GCC CAG GTG CCC CTC-3′ sequence of spectrin βIV, was analyzed by agarose gel electrophoresis (25). The 107-bp fragment, representing the 5′-CAA GCC CAG GTG CCC CTC-3′ sequence of spectrin βIV, was detected a PstI restriction fragment length polymorphism in the mouse genome corresponding to a 3.7-kb hybridization band in C57BL/6J and 4.1-kb band in SPRET/Ei. The segregation pattern of the polymorphism in progeny of the cross done using the Jackson Laboratory BSS Interspecific (C57BL/6JEi × SPRET/Ei) males backcross panel (25). A 435-bp PstI fragment of human EST clone AA054656 detected a PstI restriction fragment length polymorphism in the mouse genome that contains little homology to spectrins RPSASS, corresponding to codons 535–548 of clone N164/N155, a region that contains little homology to spectrins βI, βII, βIII, or βIV. The antibodies were affinity-purified by passing through an AminoLink Plus column (Pierce, Rockford, IL) containing a recombinant peptide of βIV-spectrin repeat 15 fused to a GST protein, and eluted with 1 μl of 0.1 M glycine (pH 2.5). A cDNA encoding βIV-spectrin repeat 15 was generated by PCR (forward primer 5′-GGC GCA TCC TCT CGG GAT CTT CAT AAC TTC-3′; reverse primer 5′-GGC GAA TTA GGC GAT CAT GAG GC-3′) and cloned into the BamHI and EcoRI sites of the pGEM7Zf (+) vector (Amersham Pharmacia Biotech, Piscataway, NJ). The recombinant GST-spectrin peptide was produced in the Escherichia coli BL21 strain and purified with glutathione-Sepharose beads according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

For Western analysis, mouse tissues were excised and homogenized on ice with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in 0.32 M sucrose, 10 mM Tris (pH 8.0), 5 mM N-ethylmaleimide, 2 mM EDTA, 5 μg/ml each of protease inhibitors leupeptin, pepstatin, aprotinin, and 0.4 μM diisopropyl fluorophosphate. Protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard. Protein samples (35 μg) were run on a 3.5–17% non-linear gradient Laemmli SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose filter. The filter was incubated with 500 ng/ml affinity-purified SpB4-R15 antibody and goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were visualized with Lumi-Light Western blotting Substrate (Roche Molecular Biochemicals, Indianapolis, IN). Molecular sizes of the positive bands were determined by comparison with the molecular weight of red cell membrane proteins (27) run in parallel. Experiments using affinity-purified antisera from two rabbit sera immunized with the βIV-spectrin peptide independently gave similar results.

Immunofluorescence Microscopic Studies—The affinity-purified rabbit polyclonal SpB4-R15 antibody was used in immunofluorescence microscopy studies. Canine kidney cells (MDCK), human neuroblastoma cells (SK-N-SH), and green monkey kidney cells (COS-7) were obtained from the American Type Culture Collection (Rockville, VA). Human embryonic kidney 293T and hepatoma Hep3B cells were kind gifts from Drs. Len Zon (Children’s Hospital, Boston, MA) and David Livingston (Dana-Farber Cancer Institute, Boston, MA), respectively. Human mesenchymal stem cells were isolated, characterized, and cultured as described (28).

For immunofluorescence studies, cells were grown in slide chambers to subconfluence. They were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at room temperature (RT) for 10 min and permeabilized with 0.1% Triton X-100 in PBS at RT for 10 min. Alternatively, cells were fixed and permeabilized in 100% methanol at −20 °C for 10 min. The cells were then incubated with affinity-purified SpB4-R15 antibody at 1:10 to 1:100 dilutions at RT for 30 min, rinsed with PBS, and incubated with Cy3-conjugated goat anti-rabbit IgG for 30 min at RT. All second stage antibodies were from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. The cells were then rinsed and mounted in ProLong antifade reagent (Molecular Probes, Inc., Eugene, OR).

Double immunofluorescence studies were performed by adding a second mouse antibody in the first incubation step and an fluorescent-tagged antiserum IgG in the second step. The primary antibodies used in double label experiments with the SpB4-R15 antibody included anti-PML (mouse monoclonal PG-M3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-SUMO-1 (mouse monoclonal 21C7; Zymed Laboratories Inc.). Additional primary antibodies used to stain SpβIV25-transfected cells included anti-nuclear porin p62 (mouse monoclonal 53; Transduction Laboratories, Lexing.

SpβIV25; NuMA, nuclear mitotic apparatus protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RARα, retinoic acid receptor alpha; RH, radiation hybrid; rp, reduced pigmentation mouse mutation; RT, room temperature; SpB4-R15, SpβIV-specific antibody; SUMO, small ubiquitin-like modifier protein; GST, glutathione S-transferase; bp, base pairs; kb, kilobase(s); Pipes, 1,4-piperazinediethanesulfonic acid.
ton, KY), anti-CBP (rabbit polyclonal A-2, Santa Cruz Biotechnology), and anti-c-myc (mouse monoclonal 9E10, Santa Cruz Biotechnology). Secondary stage antibodies were Cy3-conjugated goat anti-mouse or anti-rabbit IgG. Antibody dilutions used were empirically determined and ranged between 1:100 and 1:1000.

Fluorescence microscopy was done using a Zeiss Axioskop microscope. Microscopic images were taken with Kodak Elite ASA 400 film, digitized with a Nikon CoolScan 2000 slide scanner, and processed with the Adobe Photoshop 5.5 program on a Power Macintosh G3 computer. Confocal microscopy was performed at the Brigham and Women's Hospital Confocal Microscopy Core Facility, using a Bio-Rad MRC-1024/2P confocal microscope interfaced with a Zeiss Axiovert microscope.

Fluorescence microscopy—To make the SpßIV5-green fluorescent protein (SpßIV5-GFP) construct, a fragment of clone N164/N155 was generated by PCR and subcloned into an eukaryotic expression vector pcDNA3/HisMaxA (Invitrogen Corp., Carlsbad, CA) into which a cDNA for the enhanced GFP (CLONTECH) had first been inserted. The primers used in the PCR were DEL1 (forward: 5'-CTG ACG GCG GAT AGC CCG CGG-3') and DEL2 (reverse: 5'-TTC CAT TGA GAA GGG GCC GTC TGT-3'), and the PCR product corresponded to codons 2–678 of clone N164/N155. The resulting construct encoded a fusion protein consisting of six histidines and an Xpress epitope tag, followed by the SpßIV5 peptide fused in-frame to GFP. The myc-tagged human CREB-binding protein construct was a kind gift of Dr. David Housman (Massachusetts Institute of Technology, Boston, MA). These constructs were transfected into 293T, COS-7, and Hep3B cell lines using LipofectAMINE (Life Technologies) or FuGENE 6 (Roche Molecular Biochemicals) reagents. Expression of the heterologous protein was analyzed after 24–48 h.

Deletion constructs of SpßIV5-GFP were generated in a similar fashion, using PCR primer pairs as followed: SpßIV5.3NARN-GFP (DEL1 (forward) and DEL3 (reverse): 5'-CTG GCG GAT GC TGG TGC GCC CGG-3'), SpßIV5.2AR16-GFP (DEL1 (forward) and DEL5 (reverse: 5'-AGA GCC AGAT CAG ACC AGC TG-3') and DEL3 (reverse)) and SpßIV5.2AR15-GFP (DEL1 (forward), DEL6 (forward: 5'-CGG GCC CAG TCG CTG GCC ACA GCC GAC GCC CTC TG-3'), DEL7 (reverse: 5'-GAA GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC CTC TG-3'), and DEL3 (reverse)). The deleted domains are: SpßIV5.3NARN-GFP, codons 649–678; SpßIV5.2AR16-GFP, codons 617–678; SpßIV5.2AR10-GFP, codons 2–84; SpßIV5.2AR15-GFP, codons 509–616. The deletion constructs were transfected into mammalian cell lines, and expression of the truncated peptides was analyzed as for the SpßIV5-GFP construct.

Nuclear Matrix Analysis—Preparation of nuclear matrix was performed as published previously (29). Briefly, cells grown on chamber slides were treated sequentially with cytoskeleton buffer (10 mM Pipes (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl ribonucleoside complex, 1 mM Pefabloc) at 4 °C for 3 min, 25 units/ml DNase I in nuclease buffer (cytoskeleton buffer with 50 mM NaCl) at RT for 30 min, 0.25 mg/ml ammonium sulfate in nuclease buffer at RT for 10 min three times, high salt buffer (nuclease buffer with 2 mM NaCl) at RT for 5 min three times, and then 100 µg/ml RNase A and 40 units/ml RNase T1, in nuclease buffer at RT for 60 min. The extracted cells were fixed in methanol at −20 °C for 10 min and immunofluorescence microscopy was performed. Pefabloc, DNase I, RNase A, and RNase T1 were purchased from Roche Molecular Biochemicals; vanadyl ribonucleoside complex was from Life Technologies; all other reagents were from Sigma-Aldrich, St. Louis, MO.

RESULTS

A Spectrin-like Gene Is Localized to Human Chromosome 19q13.2 and Mouse Chromosome 7—Nucleotide sequences of αI-, αII-, βI-, and βII-spectrins were used as query sequences to search the GenBank® EST data base for clones that were similar but not identical to known spectrin chains. One EST clone from a human retinal library was identified that had sequence similarity to repeats 10 and 11 of β-spectrins (GenBank® accession number AA054636). Screening of bacteriophage cDNA libraries by conventional methods using a 453-bp PstI fragment of this clone as a probe failed to yield positive clones. Subsequently, the GeneTrapper positive selection method was used (Life Technologies). Using sequence information obtained from the clone, an oligonucleotide probe was synthesized, biotinylated, and hybridized with single-stranded DNA from a human brain cDNA library. Six unique clones that contained sequences corresponding to the oligonucleotide probe were recovered using streptavidin-coated paramagnetic beads. All of the clones were polyadenylated, but four appeared to be incomplete or partially spliced and are not described here. The complete coding sequence reported here is a composite of the two unique clones, N164 and N155 (Fig. 1A). Clone N164 extends from nucleotide 1–2172 of the composite sequence and is followed by a poly(A) tail. Clone N155 extends from nucleotide 10–2418 and is also polyadenylated. However, neither clone contains a consensus polyadenylation signal close to the polyadenylation site, and both are too short to account for the transcripts observed on Northern blots (described below). We suspect they are minor transcripts and that a significant portion of the 3′-untranslated repeat has not been cloned.

Sequence analysis showed that clones N164 and N155 contained an identical 2034-bp open reading frame that potentially encodes a peptide 678 residues in length, with a calculated molecular weight of 77,197 Da (Fig. 1B). The first methionine in this open reading frame (bp 128–130) was taken as the start codon. The amino acid sequence of this peptide is very similar to repeats 10–16 of other known β-spectrins, with 45–65% identity over the repeat domain. Analysis of the secondary structure of this spectrin-like peptide predicts the formation of multiple α-helical coils that fold into triple helical coiled-coil units, a characteristic feature found in all spectrin peptides (30). The spectrin-like peptide represented by clone N164/N155 is predicted to have five full repetitive motifs (repeats 11–15), flanked on each side by two partial repeats (repeats 10 and 16). Partial repeat 10 consists of two coils of a triple helical coiled-coil unit (helices B and C) and partial repeat 16, a single coil (helix A) (Fig. 1C). These coils may potentially allow head-to-tail interaction of multiple peptides to form concatamers (Fig. 1D). Analogs to the way β- and α-spectrins interact to form heterotetramers. However, in the latter case β-spectrin ends with two helical coils (helices A and B) and α-spectrin starts with a single coil (helix C).

The repeat domain of the spectrin-like peptide is followed by a C-terminal, non-repeat domain (NR) of 30 residues, which is rich in proline residues and dissimilar to the C termini of other β-spectrins. No similar sequence is reported in the protein database.

To show that clones N164 and N155 are transcripts of a new spectrin-like gene and not an alternative transcript of other known β-spectrins, we determined the chromosomal location of the new spectrin-like gene. PCR primers derived from the sequence of clone N164/N155 were used to screen the Stanford G3 human-hamster hybrid genomic DNA panel for the presence or absence of its gene in different cell lines (24). Tight linkage (lod score 11.25) of the gene was found to DNA marker SHGC-33106, a part of the biliverdin reductase B gene (also called NADPH-flavin reductase), which maps to chromosome 19q13.13-q13.2 (31). This chromosomal location differs from other human β-spectrin genes, which are located on chromosomes 2, 11, 14, and 15, indicating that clone N164/N155 is a transcript of a new β-spectrin-like gene. Of note, the chromosomal location of this new gene is close to that of α-actinin 4 (32). It has been previously shown that the spectrin β and βIII genes both localize close to an actinin gene (9, 33), suggesting that sequential duplications of a chromosome region containing a primordial β-spectrin gene and α-actinin gene may have given rise to the neighboring locations of these genes.

We next mapped the location of this spectrin-like gene in the mouse. Analysis of the segregation pattern of a PstI restriction fragment polymorphism in 94 progeny of The Jackson Laboratory BSS interspecific backcross panel (25) localized the gene to...
mouse chromosome 7 near the centromere (7.5 centimorgans), a region of the mouse genome homologous to human chromosome 19q13.1. The location of the gene is also different from other known β-spectrin genes, which map to chromosomes 11, 12, and 19. Mouse spectrin βV has not yet been mapped.

This gene location approximates the position of the spontaneous mouse mutation reduced pigmentation (rp) (34), in which pigment abnormalities and lysosomal dysfunction suggest an underlying defect in intracellular vesicle biogenesis (35). To determine if a mutation in the spectrin-like gene may be the cause of the rp defect, an informative subset of homozygous F2 rp animals from an intercross between C57Bl/6J-rp/rp and M. musculus castaneus (Cast/Ei) were analyzed for recombination between the rp locus and part of the spectrin-like gene detected with a clone N155-derived probe. Of the 70 meioses analyzed, four recombination events were identified, indicating that the rp locus and the spectrin-like gene are distinct genes separated by 5.7 centimorgans on chromosome 7.

**The Spectrin-like Gene Is a Bona Fide β-Spectrin, Designated Spectrin βIV**—We then searched the GenBank® high through-
put genomic sequence (htgs) data base for genomic DNA clones that contain this spectrin-like gene. Two genomic clones (accession numbers AC021625 and AC020929), derived from chromosome 19, contained sequences that matched clone N164/N155. Analysis of these clones revealed additional sequences similar to β-spectrins that were not represented in clones N164/N155, indicating that a longer isoform of the spectrin-like gene exists.

Using the putative exon sequences deduced from the genomic DNA clones, PCR and anchored PCR primers were designed and used to amplify human retinal and brain cDNA templates to obtain overlapping fragments of this long isoform, which were completely sequenced. Clones were obtained that together constitute an 8069-bp cDNA, with an open reading frame that encodes a 289.0-kDa peptide similar in structure to other β-spectrins. The predicted peptide contains a highly conserved actin/protein 4.1-binding domain, followed sequentially by 17 spectrin-repetitive motifs, a unique domain of 307 amino acids, a pleckstrin homology domain, and a conserved C terminus (Fig. 2).

Compared with the corresponding repeats of other β-spectrins, repeat 4 of this peptide has two small insertions, 15 and 8 amino acids in length. Repeat 5 has a 3-residue insertion, repeat 7 has a 6-residue deletion, repeat 8 has two 2-residue insertions, and repeat 15 has a 2-residue insertion. All of these insertions and deletions are positioned near the predicted turns of the triple helical coiled-coil unit of spectrin repeats, thus preserving their overall conformational structure.

The unique domain after repeat 17 is longer than the corresponding domain of other β-spectrins and contains, near its beginning, three tandem repeats, which are underlined by arrows in Fig. 2. The consensus sequence of these repeats, which includes amino acids present in at least two of the three repeats, is RRRPERQESADXXEXXX and is unlike any other known motifs found in data bases. Some of these insertions, particular those in repeat 4, and the unique domain are potential interaction sites for binding partners of the spectrin-like peptides. Of note, repeat 4 lies within the region (repeats 2–7) previously defined as a membrane binding site in SpβI (36).

The predicted amino acid sequence of clone N164/N155 is identical to cDNA-1325–1972 of the long isoform. The non-repeat (NR) segment in the C terminus of the N164/N155 peptide corresponds to a translated portion of intron 27, similar to the way the truncated C terminus of the erythrocyte isoform (SpβIγ1) of spectrin βI originates (37). The 5′-terminal portion of the N164/N155 cDNA is not present in the DNA sequence of the long isoform and represents an alternatively spliced 5′ exon (exon 18a) located between exons 18 and 19 of the gene. Comparison between the cDNA and genomic sequence of the spectrin-like gene reveals an exon-intron structure identical to that of spectrin βI gene (38), with 36 exons that span >145 kb, except that exon 31, which contains most of the unique three-repeat domain, is longer in size.

These results indicate that the new spectrin-like gene is a bona fide β-spectrin gene, which we designate as spectrin βIV (human gene SPTBN3 and mouse gene Spnb4) and which has both a full-length isoform (SpβIVγ1; GenBank accession number AF311855) and a truncated isoform represented by clone N164/N155 (SpβIVγ5; GenBank accession number AF311856). Data on the exon-intron structure of the two spectrin βIV isoforms are available in the annotations of the GenBank entries, and are shown schematically in Fig. 1E. A search of the GenBank data bases for other clones derived from SpβIV revealed only three additional clones: BE107551, which matches sequences in the 5′-end of SpβIVγ1 (bp 440–900), D81941, which corresponds to sequences near the 3′-end of SpβIVγ1 (bp 7778–8023), and AL133093, which matches bp 838–2174 of SpβIVγ5. After this work was completed, a partial, uncharacterized cDNA clone, KIAA1642, was deposited in the GenBank data base (accession number AB046862) (39). Clone KIAA1642 is identical to SpβIVγ1 from bases 1258 to 7249, except for three single nucleotide substitutions at positions 2238, 4093, and 5523. The substitution at base 4093 results in a Ser → Gly change at codon 1331, whereas the other two substitutions do not affect the amino acid sequence.

**Spectrin βIV Is Expressed Predominantly as the Truncated βIVγ5 Isoform**—To analyze the expression pattern of spectrin βIV, a 1.96-kb fragment of clone N164/N155 that encodes spectrin repeats 10–16 (nucleotides 114–2071) was generated by PCR and subcloned into a pBluescript vector. The fragment was used as a probe in Northern analysis of a blot of poly-(A') RNA isolated from different mouse tissues (Fig. 3A). A major 5-kb hybridizing band and minor 9- and 3-kb bands are found in brain. Expression of spectrin βIV in other tissues was at a level too low to be detected by the analysis.

Whole-mount *in situ* hybridization was also performed using mouse embryos at post-coital day 9.5. Spectrin βIV-specific riboprobes in both the sense and antisense orientations were generated using as a template the 1.96-kb fragment of N164/N155 cloned in the pBluescript vector. Hybridization using riboprobes in the antisense orientation revealed positive signals mainly in the forebrain and hindbrain and in the developing eye (optic vesicle) (Fig. 3B). No other tissues were positive at this stage of development at the level of detection of the method. Control hybridization using riboprobes in the sense orientation showed no signals, indicating that the signals seen with the antisense riboprobes were specific.

PCR analysis of cDNAs prepared from multiple mouse tissues using primers designed to amplify the 90-bp segment encoding the unique 30 amino acids at the C terminus of the truncated isoform (SpβIVγ5) showed prominent expression in post-coital day 7 mouse embryos (data not shown).

These results indicate that spectrin βIV transcripts are found predominantly in the brain, especially in the developing embryo, although there must be a low level of spectrin βIV expression in other tissues undetectable by the techniques used, because, as shown below, the protein is detectable in multiple tissues.

A polyclonal antiserum against spectrin βIV (SpB4-R15) was generated by immunizing rabbits with a 14-residue synthetic peptide derived from a segment of repeat 15 that has limited homology to other β-spectrins. The human sequence matches the corresponding mouse sequence in 13 out of 14 residues (data not shown), indicating that the polyclonal antiserum against the human spectrin βIV peptide should also recognize the mouse peptide. The antiserum was affinity-purified with a recombinant protein containing repeat 15 of spectrin βIV fused to glutathione S-transferase (GST) and used in Western analysis of proteins from mouse tissues. A major 72-kDa band was detected in all tissues (Fig. 3C). Spleen also contained a major band at 30-kDa, and there was a minor 68-kDa band in the heart. The major 72-kDa band is slightly smaller than the expected size of the truncated isoform SpβIVγ5, probably due to aberrant migration of the isoform in SDS-polyacrylamide gels. It is notable that other spectrins also migrate below their actual molecular weight on SDS gels. At the sensitivity where the 72-kDa band was easily detected, no band was seen at 289 kDa, the size of the full-length isoform SpβIVγ1 (Fig. 3C). However, with prolonged exposure faint bands were detected at 272, 240, 166, and 146 kDa (Fig. 3D). These could correspond to βIVγ1 (289 kDa) and the βIVγ3 (149 kDa) and βIVγ4 (242 kDa) isoforms described by Berghs and his colleagues (23),...
FIG. 2. Amino acid sequence of the full-length spectrin βIV isoform. The sequence of full-length spectrin βIV (SpβIVΣ1) is aligned with the sequences of spectrins βI, βII, and βIII (SpβI, SpβII, and SpβIII, respectively). Residues that are identical in more than two of the four spectrins are boxed in black. The first residues of the actin/4.1-binding domain, each of the 17 spectrin repeats, and the pleckstrin homology domain are indicated above the sequences with arrowheads. The beginning of the 77-kDa isoform (N164/N155) is also indicated in repeat 10. The amino acids in repeat 15 that are marked by dots were used to generate the SpB4-R15 peptide antibody. The three tandem repeats in the spectrin βIV non-homologous domain near the C terminus are indicated beneath the sequence by three arrows. The muscle isoform of spectrin βI (SpβIΣ2) is used in the alignment, because it is the full-length isoform. Spectrin βV (91) is not included, because its structure is different from the other β spectrins in several aspects.
along with an unknown isoform (166 kDa). The βIV-Σ2 isofom would not be detected with the SpB4-R15 antisera. The results indicate that the major isofom of spectrin βIV is the truncated isofom SpβIVΣ5.

Spectrin βIV Is Present in the Nucleus and Associates with the PML Nuclear Bodies—The affinity-purified spectrin βIV antibody SpB4-R15 was used in indirect immunofluorescence microscopy of mammalian cells. Staining of human neuroblastoma SK-N-SH cells and canine kidney MDCK cells showed diffuse, micropunctate signals in the cytoplasm and the nucleus with no involvement of the plasma membrane (Fig. 4, upper panel). The prominent nuclear staining suggested that spectrin βIV might actually be present inside the nucleus. To better delineate the nuclear distribution of spectrin βIV, we examined its distribution in human mesenchymal stem cells (MSCs), multipotential stromal cells derived from bone marrow that appear as large and flat fibroblast-like cells in culture (28). Staining of the MSCs with the SpB4-R15 antibodies showed a similar micropunctate pattern in the cytoplasm and the nucleus. In addition, 10–30 prominent foci of spectrin βIV staining were clearly seen in the nuclei of these cells above the background of diffuse nucleoplasmic staining (Fig. 4, middle panel, left).

The pattern of nuclear foci seen in the MSCs is reminiscent of that seen with antibodies against PML, a protein that is involved in the pathogenesis of acute promyelocytic leukemia and that associates with a specific nuclear structure, the PML bodies (40–43). We therefore performed double immunofluorescent staining of MSCs using antibodies against both PML and spectrin βIV. Staining of MSCs with an antibody against PML protein also showed 10–30 distinct nuclear dots in each cell, which coincided perfectly with the nuclear dots revealed by the spectrin βIV antibody (Fig. 4, middle panel). These results indicate that spectrin βIV is present in the nucleus and colocalizes with PML nuclear bodies.

PML protein has been shown to translocate into the nucleus to form nuclear bodies only after post-translational modification by a small ubiquitin-like modifier, SUMO-1 (also called PIC1 or GMP1) (44, 45). We co-stained MSCs with antibodies against SUMO and spectrin βIV. The SUMO antibody revealed a faint, diffuse staining of both the cytoplasm and the nucleus, with marked accentuation of signals in 10–30 nuclear dots (Fig. 4, lower panel). The diffuse staining pattern and the nuclear dots revealed with the SUMO antibody coincides very well with the staining pattern of the spectrin βIV antibody, indicating that spectrin βIV colocalizes in cells with SUMO-modified proteins, such as PML and Sp100 proteins, or that spectrin βIV is itself modified by SUMO-1.

Expression of a Spectrin βIV-GFP Fusion Protein Forms Nuclear Dots—To demonstrate a specific association of spectrin βIV with nuclear bodies, a plasmid construct was made that encoded the SpβIVΣ5 isofom fused to the green fluorescence protein (SpβIVΣ5-GFP). When the construct was transfected into COS-7 monkey kidney cells, 40–60 prominent, spherical, GFP-positive dots were evident inside the cell nuclei (Fig. 5). The nuclei were outlined in this experiment with an antibody against p62 nucleoporin, a component of the nuclear pore complex on the nuclear membrane (46). The possibility that these dots lie in the cytoplasm on top of the nuclei was excluded by
confocal microscopy. In a small number of cells, however, a few GFP-positive dots were also found in the cytoplasm, mainly in the perinuclear region (Fig. 5, lower panel, arrows). The SpβIVΣ5-GFP nuclear dots were relatively uniform in size within each transfected cell but varied in size in relation to the level of expression of the fusion protein. Cells with bright fluorescence signals had fewer and larger dots, whereas cells with dim fluorescence had smaller and more numerous dots.

We next investigated whether the SpβIVΣ5-GFP fusion protein colocalized with PML nuclear bodies. Because the PML antibody used in our experiments does not stain nuclear dots in COS-7 cells, we performed the expression experiments using an antibody against nucleoporin, a component of the nuclear pore complex. Right, this indicates that the GFP-positive dots are almost all located within the nucleus. Lower panel, occasionally a few GFP-positive dots are also found in the cytoplasm, especially in the perinuclear region (arrows).

To demonstrate a quantitative relationship between expression of SpβIVΣ5-GFP and PML, the number of PML- and GFP-positive dots in transfected cells is roughly proportional to the number of GFP-positive dots in these cells (dashed line). This indicates that overexpression of the SpβIVΣ5-GFP fusion protein leads to a corresponding increase in the number of PML bodies in cells, from either enhanced formation or dispersal of these structures, suggesting that spectrin βIV may be involved in the genesis of PML bodies.

CREB-binding Protein Concentrates in Spectrin βIV-containing Nuclear Dots—Because both COS-7 and 293T cells contain the SV40 T antigen, which increases the expression of the transfected plasmid but may interfere with the function of the PML bodies (47), we transfected the same SpβIVΣ5-GFP construct into human hepatoma Hep3B cells, which do not contain T antigen. Co-staining of the transfected Hep3B cells with PML and spectrin βIV antibodies shows the same nuclear dot relationship (Fig. 7, upper panel), indicating that colocalization of the spectrin βIV and PML nuclear dots is not an artifact or a unique feature of the T antigen-expressing cell lines.

CREB-binding protein (CBP), a transcription coactivator important in regulation of expression of many genes (48), also concentrates in PML nuclear bodies (49). We investigated whether expression of spectrin βIV would recruit CBP into the same nuclear dots. We expressed the SpβIVΣ5-GFP construct in Hep3B cells and stained the cells with A22, an antibody against an N-terminal epitope of CBP that reveals nuclear dots in cells (49). CBP staining in these cells was mostly diffuse and nucleoplasmic, but in occasional cells distinct dots were seen above the background. These CBP-positive nuclear dots coincided with the GFP-positive dots containing the spectrin βIVΣ5-GFP fusion protein, indicating that CBP and spectrin
βIVΣ5 resided in the same nuclear bodies (data not shown). To more clearly demonstrate this association, an expression vector containing a human CBP cDNA fused to an myc tag was transfected into Hep3B cells together with the SpβIVΣ5-GFP construct. Staining of the transfected cells with an antibody against the myc tag revealed multiple nuclear dots that coincided with GFP-positive signals derived from the SpβIVΣ5-GFP peptide (Fig. 7, lower panel), showing that CBP is recruited into the same nuclear bodies that contain SpβIVΣ5. The N- and C-terminal Helical Coils of SpβIVΣ5 Are Needed to Form Nuclear Dots—The C- and N-terminal helical coils of the SpβIVΣ5 peptide can potentially interact to allow these peptides to form multimers (Fig. 1D). To determine whether these structures are essential for nuclear body formation, we made DNA constructs that encode different truncated forms of SpβIVΣ5 fused to a GFP tag (Fig. 8). A full-length SpβIVΣ5-GFP construct expressed in 293T or COS-7 cells generated prominent nuclear bodies (Fig. 8, images A). In the case of 293T cells these coincided with PML bodies, as described before (Fig. 6). A construct with the C-terminal non-repeat (NR) domain deleted gave the same expression pattern (images B). In contrast, a construct with both coil A of repeat 16 and the NR domain deleted formed a diffuse pattern in both the cytoplasm and nucleus (images C). Similarly, deletion of coils B and C of the N-terminal partial repeat 10 resulted in faint intranuclear blobs and nucleus (images D). Similarly, deletion of coils B and C of the N-terminal partial repeat 10 similarly eradicates nuclear dots. E, an internal deletion that removes repeat 15, in contrast, does not alter formation of nuclear dots. Upper micrograph panel, transfected 293T cells. Lower panel, transfected COS-7 cells.

Staining of the nuclear matrices with anti-spectrin βIV antibody (Fig. 9A) revealed a well-demarcated, finely reticular pattern throughout the nuclear matrix, with accentuation of signals at 10–30 distinct nuclear dots, similar to the nuclear staining pattern seen in non-extracted MSCs (Fig. 4), but with the cytoplasmic staining almost completely eliminated. Co-staining of the extracted MSCs with the PML antibody (Fig. 9B) showed 10–30 bright nuclear dots that align perfectly with the dots revealed with the spectrin βIV antibody (Fig. 9C). Co-staining with the SUMO-1 antibody showed the same colocalization of nuclear dots positive for spectrin βIV and SUMO-1 (data not shown). In contrast, a control antibody against p62 nucleoporin showed no signals, because nucleoporin does not associate with the nuclear matrix (53). The resistance of spectrin βIV to well established extraction procedures used for preparation of nuclear matrix indicates that it is a nuclear matrix protein.

To confirm that spectrin βIV-positive nuclear bodies associate with the nuclear matrix, we also prepared whole-mount nuclear matrices from 293T cells transfected with the SpβIVΣ5-GFP constructs. The post-extraction nuclear matrices of the transfected cells retained the prominent GFP-positive
the potential of forming end-to-end multimers. Spectrin isoform that is truncated at the N- and C-terminal ends and has nuclear matrix. E and F, nuclear matrix preparations of 293T cells transfected with the SpβIV-S5-GFP construct (Spectrin βIV-GFP) show that the GFP-positive nuclear dots resist extraction. E and G, phase contrast micrographs showing the extracted nuclei.

nuclear dots (Fig. 9, lower panel), indicating the nuclear bodies formed from expression of SpβIV-S5-GFP also tightly associate with the nuclear matrix. These results show that both endogenous spectrin βIV and exogenously expressed spectrin βIV retain their association with the nuclear matrix and PML bodies.

**DISCUSSION**

We have identified a new β-spectrin, which has a major isoform that is truncated at the N- and C-terminal ends and has the potential of forming end-to-end multimers. Spectrin βIV associates with the nuclear matrix and PML nuclear bodies and localizes to cytoplasmic vesicles.

The finding of spectrin βIV in the nucleus of cells was unexpected. There have been reports of reactivity in mammalian cell nuclei to antibodies against α- or β-spectrin (54, 55), but the findings have not been fully explored. There is intriguing evidence that suggests spectrin αII is present in the nucleus and is deficient in cells derived from Fanconi anemia patients (20–22), but whether it binds with a β-spectrin partner in the nucleus has not been investigated. The finding of a new β-spectrin in the nucleus, as reported here, suggests that a spectrin-based skeleton may be an important component of the nuclear structure.

Immunostaining of spectrin βIV in mesenchymal stem cells (MSCs) reveals nuclear dots that coincide perfectly with PML nuclear bodies (Fig. 4). PML nuclear bodies, alternatively known as Kr-bodies, nuclear domains 10, or PML oncogenic domains (41, 42), are 0.1- to 1-μm spherical bodies in the nucleus that associate with the Sp100 protein, an antoantigen in patients with primary biliary cirrhosis (56), and the PML protein, a RING finger protein disrupted in acute promyelocytic leukemia (APML) that carries the t (15;17) translocation (57–59). The PML protein has been shown to negatively regulate growth and suppress tumor formation (60), mediate apoptosis through caspase-dependent and independent pathways (61), repress or activate gene transcription (49, 62), and mediate ras-induced premature senescence (63, 64). Whether these functions of PML require that it be located in nuclear bodies has not been resolved.

PML nuclear bodies are highly dynamic structures that vary in size and number under different conditions. Their distribution is cell-cycle-dependent (65) and is affected by viral proteins (66) and interferon treatment (67). The aberrant PML-RARα chimeric peptide in APML with t (15;17) disrupts PML bodies, which are restored when the cells are treated with retinoic acid or arsenic, two agents useful in APML therapy (57–59). Conjugation of the PML protein by the small ubiquitin-like peptide SUMO-1 is necessary for its translocation into the nucleus to form nuclear bodies (44, 45). Beyond this, the structural basis for the formation and dynamic distribution of PML bodies in the nucleus is still unclear.

A large number of proteins associate with PML nuclear bodies (40, 41, 43). A sampling includes: CREB-binding protein (49), Rb (62), p53 (63, 64), HP1 (68), p17Kip1 (69), Daxx (70), and the BLM (Bloom syndrome) DNA helicase (71). These proteins are involved in nuclear processes such as gene expression, DNA damage repair, and apoptosis but are unlikely to constitute a structural framework for PML nuclear bodies. In contrast, spectrin βIV belongs to a well characterized family of structural proteins that could help form these nuclear bodies.

Spectrin βIV is also found throughout the nuclear matrix or nuclear skeleton, a structure of filamentous proteins that remains in the nucleus after removal of soluble proteins and chromatin by nuclease treatment and high salt extraction (Fig. 9A) (72). The nuclear matrix is a network of highly branched 10-nm filaments that connects to the inside of the nuclear lamina and extends throughout the nuclear interior (29, 73). Although some researchers consider the nuclear matrix an artifact of the extraction process (74), there is growing evidence that it is a subnuclear organelle involved in regulation of mRNA processing, DNA replication, and gene transcription (75–77).

Our understanding of the nuclear matrix is hindered by a paucity of data about its molecular composition. Most of the associated proteins are involved in RNA processing. These probably attach to the nuclear matrix but do not form the underlying structural skeleton (78). Several structural proteins have been described in the nucleus, but they are not good candidates to be one of the major filamentous components of the nuclear skeleton. Actin is present in the nucleus and is part of a chromatin remodeling complex (79, 80), but it is likely to be present in the nucleus only as monomers or short filaments (81). The Tpr nuclear pore complex-associated protein forms filaments extending from the nuclear envelope inward (82), but its presence is likely to be confined to the outer nuclear perimeter. The nuclear envelope lamins are also present in the interior of the nucleus (83), but whether lamins polymerize to form filaments throughout the nucleus is unclear. The nuclear mitotic apparatus protein NuMA may self-assemble into a lattice and serve as a scaffold protein in interphase nuclei (84), but it also does not form filaments. Protein 4.1 isoforms are present in the nucleus and specifically interact with NuMA (85–89) but, likewise, protein 4.1 does not polymerize to form filaments.

Spectrin βIV, for several reasons, is a good candidate to be a major component of the filamentous nuclear matrix seen in electron microscopy (29, 73). It is a member of a well characterized family of structural proteins that form a membrane skeleton network; it has an extensive helical coiled-coil structure that is frequently found in filamentous proteins; and the full-length isoform, SpβIVΣ1, has a well-conserved actin/4.1-binding domain that potentially binds nuclear actin and protein 4.1, both of which are known to be in the nuclear matrix. Endogenous spectrin βIV, stained with the SpB4-R15 antibody, diffusely distributes throughout the nuclear matrix and concentrates at PML nuclear bodies (Fig. 9, upper panel). Exogenously expressed SpβIVΣ5-GFP peptide, in contrast, resides only in concentrated foci in the nucleus in the form of nuclear dots (Fig. 9, lower panel). Because the SpB4-R15 antibody does
not distinguish between the SpβIV1 and SpβIV2 isoforms, this result is consistent with the hypothesis that the SpβIV1 isoform (or one of the other isoforms identified by Berghs et al. (23)) contributes to formation of the nuclear skeleton throughout the nucleus, whereas SpβIV5 contributes to formation of the nuclear bodies. Other evidence supports this concept. Over-expression of SpβIV5 in cells leads to a proportional increase in the number of PML bodies (Fig. 6). Expression of different deletion constructs of SpβIV5 shows that the requirement for nuclear body formation is quite specific and may involve the ability of the peptide to form multimers (Fig. 8). In our view, then, a dynamic spectrin βIV skeleton in the nucleus formed by SpβIV1 and SpβIV5 peptides may serve to gather and organize nuclear proteins (transcription factors, RNA splicing factors, DNA damage repair enzymes) into specific domains to fulfill their function, much like cytoplasmic spectrin is proposed to work in cargo selection and loading during vesicular trafficking (17, 18).

The other structural proteins that interact with spectrin βIV in the nuclear matrix remain to be defined. In both erythroid and non-erythroid cells, β-spectrin binds to α-spectrin, protein 4.1, adducin, and F-actin to form the well-characterized membrane skeleton. Whether spectrin βIV binds to similar proteins in the nuclear matrix is not yet known. Evidence suggests it may. Protein 4.1 isoforms are a component of the nuclear matrix and associate with mRNA splicing factors and the NuMA protein (85–89). Nuclear actin is a subunit of the BAF (Brahma associated factor) chromatin remodeling complex that stably associates with the nuclear matrix (80, 81). As mentioned above, α-spectrin is also present in the nucleus and may act as a scaffold to align proteins involved in DNA damage repair (20–22). It will be important to investigate whether spectrin βIV interacts with these proteins to form a structural framework in the nucleus. A recent paper noted that spectrin also forms distinct spots in plant nuclei and is located in the nuclear matrix (90). It appears the association of spectrin with the nuclear matrix is preserved from plants to higher mammals, which suggests it has an essential role in nuclear function.

In addition to its presence in the nucleus, spectrin βIV is also found in a diffuse, micropunctate pattern in the cytoplasm, suggesting it also associates with cytoplasmic vesicles. Because little SpβIV5 is expressed in the cytoplasm (Fig. 5), full-length SpβIV1 or one of the other SpβIV isoforms (23) is probably the vesicular form. There is good evidence that spectrin βIII contributes to vesicular trafficking, even though the mechanism has not been worked out (9, 17–19). It will be important to identify the vesicles that spectrin βIV associates with, and whether spectrins βIII and βIV are involved in distinct vesicular trafficking pathways.

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