

Phosphorylation of a Threonine Unique to the Short C-terminal Isoform of β II-Spectrin Links Regulation of α - β Spectrin Interaction to Neuritogenesis^{*,§}

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Spectrin tetramers are cytoskeletal proteins required in the formation of complex animal tissues. Mammalian α II- and β II-spectrin subunits form dimers that associate head to head with high affinity to form tetramers, but it is not known if this interaction is regulated. We show here that the short C-terminal splice variant of β II-spectrin (β II Σ 2) is a substrate for phosphorylation. *In vitro*, protein kinase CK2 phosphorylates Ser-2110 and Thr-2159; protein kinase A phosphorylates Thr-2159. Anti-phospho-Thr-2159 peptide antibody detected phosphorylated β II Σ 2 in Cos-1 cells. Immunoreactivity was increased in Cos-1 cells by treatment with forskolin, indicating that phosphorylation is promoted by elevated cAMP. The effect of forskolin was counteracted by the cAMP-dependent kinase inhibitor, H89. *In vitro*, protein kinase A phosphorylation of an active fragment of β II Σ 2 greatly reduced its interaction with α II-spectrin at the tetramerization site. Mutation of Thr-2159 to alanine eliminated inhibition by phosphorylation. Among the processes that require spectrin in mammals is the formation of neurites (incipient nerve axons). We tested the relationship of spectrin phosphorylation to neuritogenesis by transfecting the neuronal cell line, PC12, with enhanced green fluorescent protein-coupled fragments of β II Σ 2-spectrin predicted to act as inhibitors of spectrin tetramer formation. Both wild-type and T2159E mutant fragments allowed normal neuritogenesis in PC12 cells in response to nerve growth factor. The mutant T2159A inhibited neuritogenesis. Because the T2159A mutant represents a high affinity inhibitor of tetramer formation, we conclude that tetramers are requisite for neuritogenesis. Furthermore, because both the T2159E mutant and the wild-type allow neuritogenesis, we conclude that the short C-terminal β II-spectrin is phosphorylated during this process.

Spectrin is a cytoskeletal protein that appeared in evolution at the same time as the metazoa. It is essential for the development and function of most tissues (1). In particular, spectrin is required by both vertebrates and invertebrates for development of the nervous system, where it fulfils a role in the production and guidance of nerve axons (2–5). Erythrocytes require spectrin for their stability in circulation (6). It is also essential in the development and resilience to the forces of contraction of muscle systems (2, 3).

Spectrin is normally considered to be a tetramer capable of cross-linking cytoplasmic actin filaments to a number of transmembrane proteins, either directly or via adaptor proteins (1). Each tetramer comprises two types of subunit, α and β . α and β chains are elongated polypeptides largely made up of consecutive triple helical repeats (7, 8). Dimers form by the side-to-side interaction of one α with one β chain (9, 10). To form a tetramer, two dimers come together, head to head, such that the interaction occurs between the N-terminal region of an α chain and the C-terminal region of a β chain (11, 12). At the N terminus of α is a single α -helix that represents helix C of a triple helix, and in the C-terminal region of β are two helices that represent the A and B helices of a triple helix. These partial helices interact to recapitulate a complete triple helical domain.

Mammals have two genes encoding α -spectrin (α I and α II), four encoding “conventional” β subunits (β I–IV) that have 16 full triple helices, and one β_{Heavy} subunit that has 30 triple helices (β V-spectrin) (1). Of these, α II and β II are widespread in most tissues. β II is essential for the normal formation of nervous and cardiac tissue; knock-out mice die *in utero* with major malformations of both organs (5).

β II has several splice variants, including “long” and “short” C-terminal regions that are expressed in both brain and heart (13). The “long form” has a pleckstrin homology domain close to the C terminus, and this is joined to the helices that bind α -spectrin by a linker of \sim 100 amino acids. In the short form, the linker is spliced after \sim 50 residues so that the pleckstrin homology domain is absent, and a region of \sim 28 residues is substituted in its place. Little is known of the function of this short unique region. It makes no substantial difference to the affinity of interaction with α subunit; both the long and short forms bind α II with nanomolar affinity (14).

We noted that the alternatively spliced sequence present in the short β II subunit contains a number of predicted phosphorylation sites, potential regulators of protein-protein interaction. In this article we show that one such residue is a substrate

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1S.

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in vitro for two kinases, protein kinase A (PKA)³ and casein kinase 2 (CK2). In living cells, phosphorylation at this site is sensitive to reagents that affect the activity of PKA. Phosphorylation *in vitro* greatly reduces the affinity of interaction with α II, so this unique residue suggests a novel regulatory site in spectrin. In this context, we provide functional evidence that links phosphorylation of β II-spectrin to absolute requirement for spectrin tetramers in the process of neuritogenesis.

EXPERIMENTAL PROCEDURES

Materials—Purified bovine catalytic subunit of PKA was purchased from Sigma (catalogue no. P2645), and purified recombinant human CK2 was purchased from Calbiochem (catalogue no. 218701). Synthetic peptides were prepared by the Protein Science Facility at the University of Kent. Adenosine 5' [γ -³²P] triphosphate triethylammonium salt ([γ -³²P]-ATP: 0.11 TBq·mmol⁻¹, 74 MBq·ml⁻¹) was purchased from Amersham Biosciences. PC12 (rat pheochromocytoma, ATCC catalog no. CRL-1721) and COS-1 (African green monkey kidney cells, ATCC catalog no. CRL-1650) cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). For sequence comparison, protein sequences were retrieved from the International Protein Index (IPI) (15), and genomic sequences were retrieved from Ensembl (16).

Recombinant Proteins—Residue numberings for recombinant proteins or peptides described here are taken from SwissProt:Q01082 short isoform (IPI:IPI00328230). Recombinant GST- β II Σ 2 C-terminal region (CTR; amino acids 2087–2168) was expressed and purified using the plasmid pGEX-2T- β II Σ 2-(2087–2168) as described previously (13). GST- β II Σ 2 CTR contains the C-terminal extension only of the short C-terminal β II variant that stretches beyond the last triple helical repeat.

Recombinant His-tagged β II Σ 2 R16-CTR (amino acids 1900–2168) was prepared using the plasmid pRSET- β II Σ 2-(1900–2168) and was purified by metal chelating chromatography (14). β II Σ 2 R16-CTR encodes the final full triple helix (repeat 16), the partial repeat to which α -spectrin binds (repeat 17) and the short C-terminal extension specific to this variant.

Mutations were incorporated in the plasmid pGEX-2T β II Σ 2-(2087–2168) using site-directed mutagenesis by overlap extension using PCR (18). The primers for the generation of mutated fragments using pGEX-2T β II Σ 2-(2087–2168) as template are listed in Supplemental Table 1S; the mutant cDNA contains a new restriction site not present in the original cDNA. Mutations were incorporated into the recombinant protein encoded by the pRSET- β II Σ 2-(1900–2168) plasmid by subcloning a 195-bp fragment flanked by NcoI and NotI sites. Each construct was verified by restriction digestion and by DNA sequencing (MWG-Biotech, Milton Keynes, UK).

For preparation of EGFP-tagged constructs, cDNAs were subcloned into the vector pEGFP-N1 (Clontech, Cowley, UK). GST- α II-spectrin N-R2 (amino acids 1–145 of the N-terminal region, through the initial α -helix that comprises part of the

α - β self-association site, R1, and the first full triple helix, R2) was expressed and purified as described previously (14).

Phosphorylation of Recombinant Proteins—Purified recombinant proteins were extensively dialysed against 20 mM Tris hydrochloride, pH 7.5, 50 mM KCl, 10 mM MgCl₂ prior to phosphorylation. Phosphorylation reactions were carried out with the indicated concentrations of recombinant protein and 56 μ M [γ -³²P]ATP (35 kBq· μ l⁻¹). The amount of kinase specified in the text below was added to the reaction mixture and incubated at 30 °C. Reactions were terminated at various time points by dilution of the reaction mixture into SDS-PAGE sample buffer (19). Proteins were separated by SDS-polyacrylamide gels, which were briefly stained with fresh Coomassie Blue stain, destained until the background was clear, washed extensively with water, dried, and exposed to Hyperfilm MP (Amersham Biosciences) for autoradiography.

To measure incorporation of ³²P, gel bands of interest were excised and analyzed by liquid scintillation spectrometry. Alternatively, reaction mixtures were captured on phosphocellulose filters after trichloroacetic acid precipitation and analyzed by liquid scintillation spectrometry.

Thrombin Digestion of Purified Recombinant Proteins—Unincorporated radioactive ATP was removed from the reaction mixture using ProbeQuant G50 Microcolumns (Amersham Biosciences). Thrombin was added to a final concentration of 2 units· μ l⁻¹ and incubated for 2 h at 30 °C. Digestions were stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM and the proteins were separated by SDS gel electrophoresis.

Pulldown Assays—Interactions of α II N-R2 and β II Σ 2 R16-C were measured using the pulldown assay described previously (14).

Anti-TP2159 Antibody—Antibody to phosphorylated Thr-2159 in β II Σ 2-spectrin was prepared using a synthetic phosphopeptide. The sequence CFNSRRTPASDQPWS corresponds to residues 2154–2166 plus an N-terminal cysteine for coupling to keyhole limpet hemocyanin (Pierce Inject kit, Perbio, Cramlington, UK). Antibodies were raised in guinea pigs and phosphate-specific IgG isolated chromatographically by standard procedures (20).

Cos-1 Lysate Preparation—Cos-1 cells were cultured by standard techniques using Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (LabTech International) and 1% L-glutamine.

Cells were plated in 6-well plates at a density of approx. 5 × 10⁶ cells/well and incubated overnight at 37 °C with 5% CO₂. For drug treatment, medium in the wells was replaced with fresh medium containing different concentrations of forskolin (Sigma, 0–100 μ M) or pre-treated with H89 (Calbiochem, 0–50 μ M) for 30 min before stimulation. Control reactions with the same amount of Me₂SO were carried out simultaneously. In either case, after the incubation period, cells were washed 3 times with 2 ml of cold phosphate-buffered saline supplemented with phosphatase inhibitors (0.1 mM Na₃VO₄, 1 mM NaF). Cells were scraped into 1 ml of phosphate-buffered saline and collected by centrifugation at 10,000 × g for 1 min. Lysates were prepared by addition of 200 μ l of lysis buffer, 50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, Complete® protease inhibitor mixture (Roche Applied Sci-

³ The abbreviations used are: PKA, protein kinase A; CK2, protein kinase CK2 (casein kinase II); IPI, International Protein Index; CTR, C-terminal region; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase.

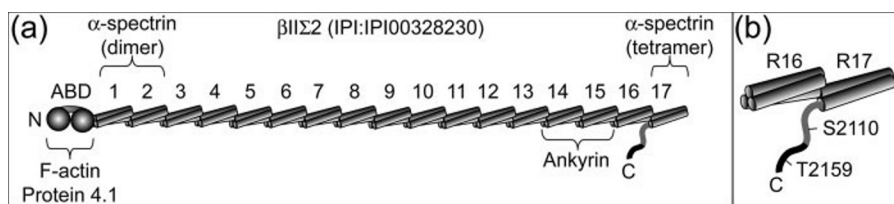


FIGURE 1. Summary of the structure of the short C-terminal isoform of β II Σ 2 spectrin (β II Σ 2). *a*, schematic diagram of the folding structure of this polypeptide. Most of the polypeptide is folded into a series of 16 triple helices; the 17th repeat provides only two helices and interacts with a single helix from α -spectrin in the formation of tetramers. ABD, actin binding domain. Repeats 1 and 2 bind α -spectrin in the formation of spectrin dimers. *b*, repeats 16 and 17 plus the C-terminal sequence are shown in more detail. At the C terminus is a short sequence that contains no recognized folding structures but has a number of potential phosphorylation sites (Ser-2110 and Thr-2159 are indicated). The sequence shown in *dark shading* represents the differentially spliced sequence characteristic of the short C-terminal isoform.

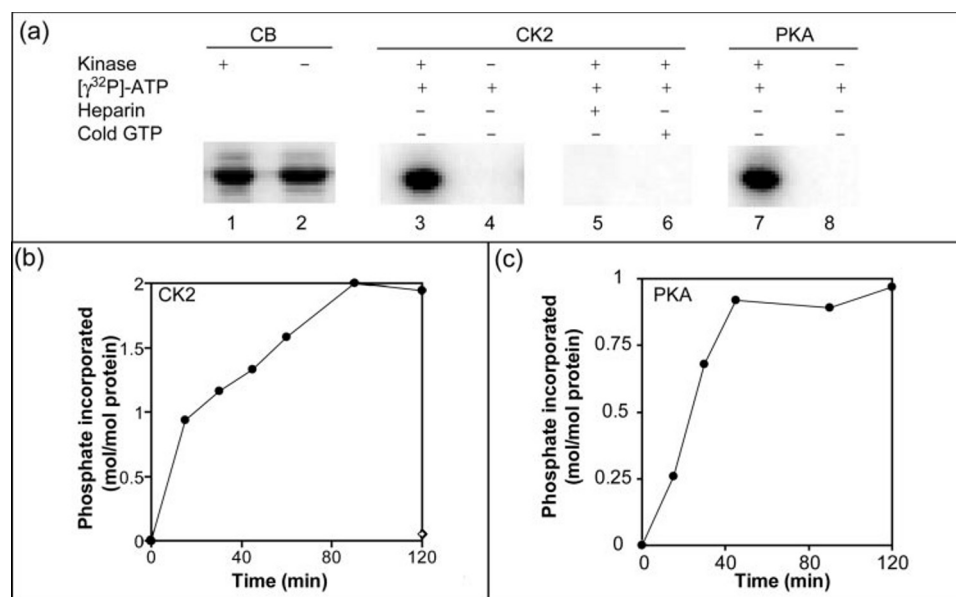


FIGURE 2. *In vitro* phosphorylation of β II Σ 2 spectrin C-terminal region and identification of phosphorylation sites. *a*, a GST-fusion protein containing the C-terminal region of human β II Σ 2 spectrin (residues 2185–2168) was treated with protein kinases, 20 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and other reagents as indicated in the figure. After *in vitro* phosphorylation, the protein was analyzed by SDS gel electrophoresis and staining by Coomassie Blue (CB, lanes 1 and 2) or by autoradiography (lanes 3–8). CK2 (lane 3) and PKA (lane 7) in the presence of ATP phosphorylated this construct. Phosphorylation was dependent on the presence of kinase (lanes 4 and 8). Heparin is a CK2 inhibitor; 5 nM heparin blocked phosphorylation of the construct (lane 5). CK2 is distinctive in that it is one of few kinases to use GTP as a substrate; addition of an excess of unlabeled (25 μM “cold”) GTP blocked radiolabeling of the construct by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (lane 6). *b*, quantification of phosphorylation of the β II Σ 2 CTR by CK2. *c*, quantification of phosphorylation of the β II Σ 2 CTR by PKA.

ence), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4 , 1 mM NaF. After 10 min of incubation, the lysate was cleared by centrifugation at $10,000 \times g$ for 10 min at 4°C . Gel samples of cleared lysates were prepared as before (19).

PC12 Culture and Transfection—PC12 cells were cultured on collagen-coated coverslips in RPMI 1640 medium with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin for 24 h before differentiation in the presence of 5 ng/ml nerve growth factor for 1–3 days. Cells were cultured for 24 h in antibiotic-free medium before transfection. For transfection, cells were incubated with 2 μl of Lipofectamine reagent (Invitrogen) and 0.8 μg of each β II-spectrin fragment cDNAs in pEGFP-N1 diluted in 100 μl of Optimem (Invitrogen) followed immediately by differentiation with nerve growth factor. At the required time after transfection, cells were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde in

phosphate-buffered saline. Phase and fluorescence images were recorded digitally on a Leica DMRB microscope. Cells that showed green fluorescence were scored for neurite production.

RESULTS

Phosphorylation of β II Σ 2-Spectrin C-terminal by PKA and CK2— β II Σ 2-Spectrin is abundant in brain and heart (13, 21). A schematic diagram indicating the folding structure of the polypeptide is shown in Fig. 1*a*. Note that the C-terminal region lacks the pleckstrin homology domain and has only a short region with no known domains (Fig. 1*b*).

In brain, spectrin has been previously shown to be a physiological substrate for casein kinases and PKA (22). The amino acid sequence of the C-terminal region of β II Σ 2-spectrin contains consensus sequences for phosphorylation by (among others) PKA and CK2. To determine whether spectrin is a substrate for these kinases *in vitro*, recombinant GST- β II Σ 2 CTR (as shown in Fig. 1*b*) (13) was labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of either CK2 or PKA. The recombinant protein became phosphorylated in the presence of the enzymes (Fig. 2*a*). Phosphorylation of GST- β II Σ 2 CTR by CK2 was blocked by cold GTP or heparin, two characteristics of phosphorylation by CK2 (23, 24). The protein (0.51 mg/ml) was maximally phosphorylated by CK2 (25 units/100 μl) after ~ 80 min incubation at 30°C , whereas with PKA phosphorylation levels plateaued after ~ 40 min (identical substrate concentration and units of enzyme activity). Wild-type protein incorporated ~ 2 mol phosphate/mol protein when phosphorylated by CK2 and 1 mol/mol PKA (Figs. 2, *b* and *c*, respectively).

Identification of Phosphorylation Sites in β II Σ 2-Spectrin C-terminal Region—To map the sites phosphorylated by these kinases, first we took advantage of an internal thrombin site within β II Σ 2 CTR. Thrombin cleaves the Arg-2140-Val-2141 bond in this sequence (Fig. 3*a*, mapped by electrospray mass spectrometry (and data not shown)). Thus, cleavage of GST- β II Σ 2 CTR using thrombin releases fragments representing residues 2087–2140 and 2141–2168 from the GST fusion protein. Thrombin digestion of GST- β II Σ 2 CTR previously phosphorylated by purified recombinant CK2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ revealed the incorporation of labeled phosphate into both spec-

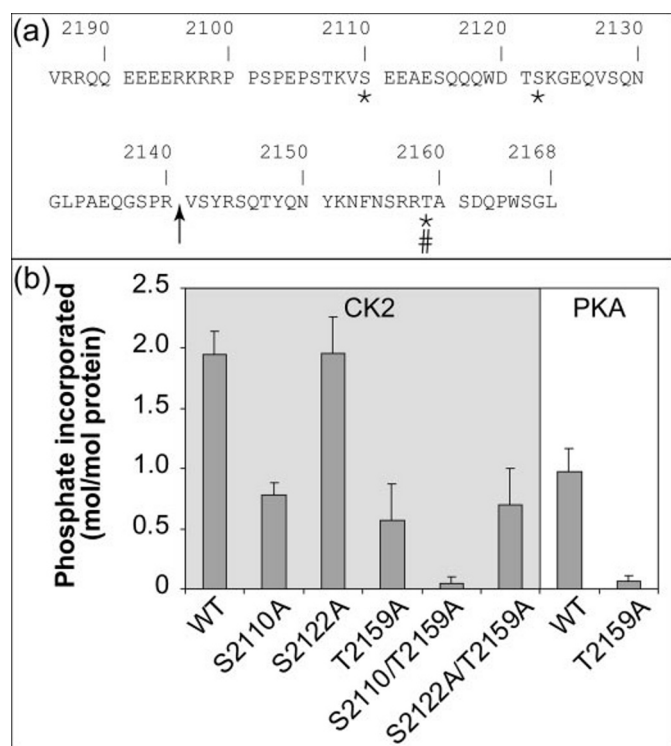


FIGURE 3. Identification of substrate sites for CK2 and PKA. *a*, candidate residues for substrate sites in the β II Σ 2 C-terminal region. The sequence is numbered as in the SwissProt:Q01082 short isoform (IPI:PI00328230). * indicates residues that fall into consensus substrate sequences for CK2 (2110, 2122, and 2159), and # indicates PKA consensus substrate (2159). The site of splice variation between long and short C-terminal variants is between Arg-2140-Val-2141 (arrow), which is also cleaved *in vitro* by thrombin. *b*, identification of *in vitro* substrate sites for purified CK2. Ser/Thr residues 2110, 2122, and 2159 were mutated to Ala in the GST- β II Σ 2 CTR construct or the double mutants S2110A/T2159A and T2122A/T2159A. Quantification of phosphate incorporation after 120 min under conditions shown in Fig. 2 indicate that Thr-2110 is a substrate for CK2 and that Thr-2159 is a substrate for both PKA and CK2.

trin fragments (data not shown). By contrast, PKA phosphorylation only phosphorylated one of the spectrin fragments, residues 2141–2168 (data not shown).

To identify sites of phosphorylation, alanine mutants were prepared of three candidate phosphorylation sites (Fig. 3*a*). The mutations were S2110A, S2122A, and T2159A. These residues were chosen as candidates for analysis because they are within the CK2 consensus based on S*/T*-[D/E/S(P)_{1–3}X_{2–0}] (23, 24). Additionally, Thr-2159 is in a PKA consensus R-X-S*/T* (25). This residue is located in the 2141–2168 thrombin fragment.

Phosphorylation reactions were performed on the purified wild-type and mutated recombinant proteins, and the level of incorporation of ³²P was quantified after 120 min (Fig. 3*b*). Purified mutant proteins were more labile to proteolysis as compared with the wild-type protein. The mutation S2122A made no appreciable difference to maximal phosphorylation by CK2. However, S2110A and T2159A incorporated ≤ 1 mol/mol, identifying each as a substrate for CK2. The double mutant S2110A/T2159A incorporated little phosphate, consistent with the results with the single mutants. The double mutant S2122A/T2159A incorporated approximately the same amount of phosphate as the single T2159A mutant. T2159A incorporated little or no phosphate above background in the presence of PKA (Fig. 3*b*). Therefore, T2159 is a substrate for both kinases.

Residues Equivalent to Human Thr-2159 Are Conserved Throughout Land-based Vertebrates—The availability of multiple genomes makes possible an evolutionary analysis of the prevalence of the phosphorylation sites equivalent to human Thr-2159. Analysis of genomic sequences of three bony fish species (*Tetraodon nigroviridis*, *Danio rerio*, and *Fugu rubripes*) reveals that β II Σ 2 isotype is not encoded in their β II-spectrin genes. However, the β II Σ 2 isoform is encoded in all available amphibian, bird, and mammal β II Σ 2 C termini (representative sequences are aligned in Fig. 4*a*). In each case, threonine residues at positions equivalent to human Thr-2159 are present within consensus sequences for PKA phosphorylation (RRT) and CK2 phosphorylation (TXXD). These data indicate a conserved role for threonine phosphorylation in regulating spectrin network function throughout the land-based vertebrates.

Fig. 4*b* shows comparison of the C termini of the human β I- and β II-spectrin short C-terminal variants. The phosphorylation sites identified by Tang and Speicher (26) are annotated on the β I sequence, and the two sites identified here are annotated on β II. Note that none are conserved between the two proteins and that the C termini are very different.

Phosphopeptide Antibody Anti-TP2159—Because Thr-2159 is most unusual in being a substrate *in vitro* for both PKA and CK2, we wanted to establish whether it is phosphorylated in living cells. To investigate phosphorylation of Thr-2159 *in vivo*, we raised an anti-phosphopeptide antibody that would recognize β II Σ 2 phosphorylated at this residue (see “Experimental Procedures”). This antibody is designated anti-TP2159. We also took advantage of an antibody to the C-terminal region of β II Σ 2 described previously (13). The antigen used to raise this antibody was a synthetic non-phosphorylated peptide representing the C-terminal 14 residues of β II Σ 2.

Phosphorylated and control proteins were subjected to analysis by SDS gels and immunoblotting. Note that the phosphorylated protein migrated more slowly in the SDS gel than unphosphorylated (Fig. 5, lanes 1 and 2, Coomassie Blue stain). The same is seen in lanes 2–4, the equivalent immunoblot processed with the phosphate-insensitive anti- β II Σ 2 C-terminal antibody. Lanes 5 and 6 are an immunoblot with anti-TP2159; note the absence of reactivity in the control lane (lane 5) and strong reactivity in the phosphorylated sample (lane 6). Immunoreactivity with anti-TP2159 toward the PKA-phosphorylated protein was competed out in the presence of the phosphorylated peptide (Fig. 5, lanes 9 and 10) but not when unphosphorylated peptide was used for competition (lanes 7 and 8). No reactivity of the antibody was detected when the mutant T2159A was incubated with or without PKA and ATP (lanes 11 and 12). The anti-TP2159 antibody also reacted with the wild-type construct after incubation with CK2 and ATP (lane 14) but not with the unphosphorylated control (lane 13). These data clearly identify anti-TP2159 as a specific antibody to β II Σ 2 phosphorylated at Thr-2159 and confirm that this residue is an *in vitro* substrate for both PKA and CK2. Competition with phosphopeptides representing the equivalent sequences of mouse and rat β II Σ 2 showed that the antibody does not recognize the rodent proteins (data not shown), despite these sequences being very similar to human (only 2 residues differ

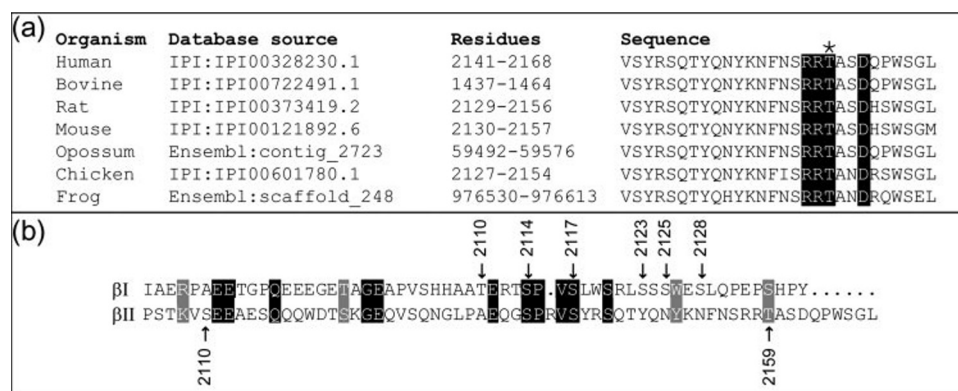


FIGURE 4. **Conservation of the phosphorylatable threonine through the land-based vertebrates.** *a*, the figure shows alignment of C-terminal sequences of the short β II-variant from a variety of land-based vertebrates. Sequences are taken from the IPI data base, where available, or from genomic sequence in Ensembl. The short C-terminal variant of β II-spectrin probably arose with the tetrapods; no similar sequence is present in the accessible bony fish genomes or invertebrate spectrin genes. Note the absolute conservation of Thr at the position equivalent to Thr-2159 of human (*). The motifs specifying PKA phosphorylation (RRR) or CK2 phosphorylation (TXDD) are also perfectly conserved. Note also that this whole region is very highly conserved from frog to human. *b*, comparison of the C-terminal regions of β I-spectrin (IPI: IPI00216513.3) and β II-spectrin (IPI: IPI00328230.1) short C-terminal variants. Phosphorylation sites in β I-spectrin are taken from Ref. 26. Note the lack of conservation of sequence and phosphorylation sites between β I and β II.

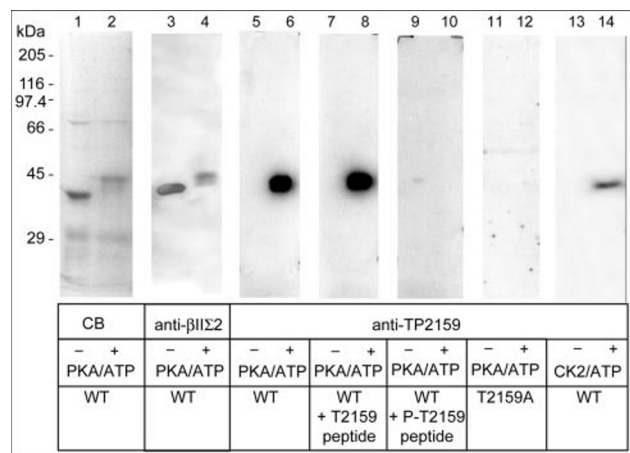


FIGURE 5. Characterization of anti-TP2159 antibody. Antibody to a phosphopeptide containing phospho-Thr-2159 was prepared as described under "Experimental Procedures." The figure shows SDS gels either stained with Coomassie Blue (CB) or transferred to nitrocellulose for probing with anti- β II Σ 2 antibody (2 ng·ml⁻¹ IgG) or anti-TP2159 (5 ng·ml⁻¹ IgG). In each panel, *odd numbered lanes* show the His-tagged β II Σ 2 R16-CTR construct; *even numbered lanes* show the same construct treated with the indicated kinase and ATP *in vitro*. Note that in *lane 2*, the phosphorylated fragment migrates more slowly (apparent M_r ~ 45,000) than the unphosphorylated form in *lane 1* (apparent M_r ~ 38,000). *Lanes 3 and 4* show the reactivity of the construct with anti- β II Σ 2; note that both phosphorylated and unphosphorylated proteins are recognized. *Lanes 5 and 6*, anti-TP2159 reactivity is only found in *lane 6* but not *lane 5*, indicating that the antibody reacts specifically with the phosphorylated protein. In *lanes 7 and 8*, 5 μ g·ml⁻¹ of the unphosphorylated antigen peptide was preincubated for 2 h with the antibody solution. In *lanes 9 and 10*, 5 μ g·ml⁻¹ of the phosphorylated antigen peptide was added to the antibody solution. Note that only the phosphorylated peptide is capable of displacing antibody reactivity. In *lanes 11 and 12* the antibody was reacted with the nonphosphorylatable mutant T2159A. No reactivity was found with this whether or not it had been treated with kinase and ATP. *Lanes 13 and 14* show that anti-TP2159 recognizes the wild-type (WT) construct phosphorylated with CK2.

out of 14 amino acids in the peptide) and the motif RRTXXD being conserved.

Phosphorylation of β II Σ 2-Spectrin at Thr-2159 in Vivo in Intact Cells—Anti-TP2159 was used to investigate phosphorylation at residue 2159 of endogenous β II Σ 2-spectrin of cultured

mammalian Cos-1 cells *in vivo*. These are cells of primate origin, and the sequence is expected to be close enough to the human antigen that reactivity would be detected.

Cos-1 cells contain endogenous β IIIS2-spectrin. A single band at 250 kDa is detected by immunoblotting with phosphate-independent anti- β IIIS2 antibody on Cos-1 cell lysate (Fig. 6a, lanes 1 and 2). Immunoblotting of Cos-1 cell lysate with anti-TP2159 revealed a band that migrated identically (Fig. 6a, lanes 2 and 3). This band was competed out with soluble phosphopeptide (data not shown); therefore a proportion of β IIIS2-spectrin in growing Cos-1 cells is phosphorylated at Thr-2159.

To investigate *in vivo* require-

cell β IIIS2-spectrin, we took two approaches. First, adenylate cyclase was stimulated using forskolin (27). This is a standard method to increase the cytoplasmic content of cAMP, which would activate PKA. Fig. 6*a*, lane 4 reveals that 20 μ M forskolin dissolved in Me₂SO generates an increase in the intensity of the band detected with anti-TP2159 compared with Me₂SO alone (Fig. 6*a*, lane 3), consistent with increased phosphorylation at Thr-2159. Fig. 6*a*, lanes 5 and 6 show corresponding samples probed with anti-actin to control for protein loading.

A dose-response analysis of Cos-1 cells (Fig. 6*b*, *main graph*) revealed an increase in the immunoreactivity of anti-TP2159 when treated with forskolin up over the range 0.1–100 μ M, greatly in excess of a small increase observed with Me₂SO alone. Thus an activator of adenylate cyclase promotes phosphorylation of Thr-2159.

A second approach was to combine forskolin treatment with a protein kinase inhibitor. H89 is one of the most specific inhibitors of PKA available, although it also inhibits a limited range of other kinases (28). Fig. 6*b* (*inset*) shows that increasing concentrations of H89 progressively decreased anti-TP2159 reactivity. Inhibition of up to 40–45% of the forskolin stimulation was obtained with 30–50 μM H89.

These data, taken together with the observation that T2159 is phosphorylated by PKA *in vitro*, are consistent with Thr-2159 being a natural substrate for PKA but do not exclude the possibility that other kinases (such as S6K1 or MSK1, which are also inhibited by H89 (28)) also phosphorylate this residue. The lack of complete inhibition of forskolin-stimulation by H89 might suggest that in addition to PKA other kinases are activated by forskolin.

Phosphorylation of Spectrin C Terminal Region, Modulation of the Tetramerization Affinity—Although the phosphorylation site Thr-2159 is located outside the region of β -spectrin, which interacts with α -spectrin (Fig. 1), we tested for the possibility that phosphorylation at this site influences binding to α .

Purified wild-type β IIIS2 R16-CTR became phosphorylated when incubated with labeled ATP and the catalytic domain of

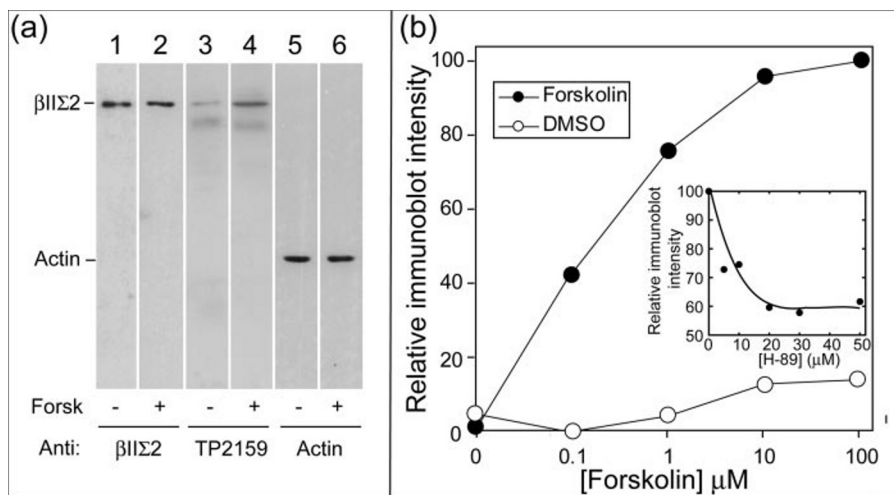


FIGURE 6. β II Σ 2 Thr-2159 is a physiological substrate for cAMP-activated kinase in COS-1 cells. *a*, immunoblot analysis of endogenous β II Σ 2 in COS-1 cells. COS-1 cell lysates were analyzed by immunoblotting with anti- β II Σ 2 (phosphate insensitive) antibody (lanes 1 and 2), anti-TP2159 antibody (lanes 3 and 4), or anti-actin (as a control for total protein loading) (lanes 5 and 6). Even numbered lanes (noted + below the lanes) were samples prepared after treatment with 10 μ M forskolin (Forsk) dissolved in Me₂SO. Odd numbered lanes were samples prepared after treatment with Me₂SO alone. Note the increased reactivity of antibody in lane 4 relative to lane 3, but unchanged total β II Σ 2 or actin. *b*, quantification of the effect of forskolin and its inhibition by H89. COS-1 cells were treated with 0–100 μ M forskolin dissolved in Me₂SO or with corresponding amounts of Me₂SO alone. Cell lysates were analyzed by immunoblotting with anti-TP2159 antibody. Note that reactivity increased with forskolin treatment. Me₂SO alone induced little change in reactivity. *Inset*, compound H89 is an inhibitor of cAMP-activated kinases. Cells were incubated with H89 at the indicated concentrations for 1 h before addition of 10 μ M forskolin. H89 reduces the reactivity of the antibody to ~60% of maximum.

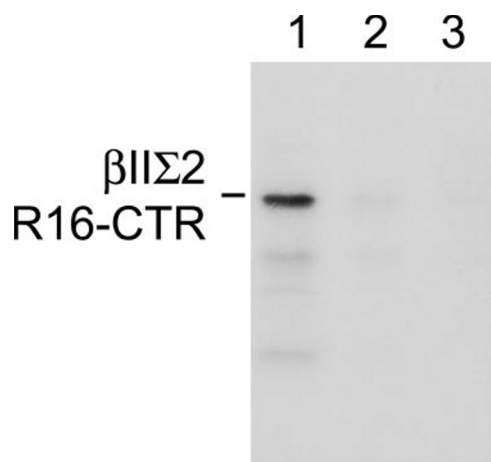


FIGURE 7. Interaction with α II-spectrin inhibits phosphorylation of β II Σ 2 Thr-2159. Wild-type β II Σ 2 R16-CTR (lanes 1 and 3) or T2159A mutant (lane 2, 0.3 mg/ml) were incubated with an equal concentration of α II N-R2 overnight at 4 °C to allow an α - β complex to form (lane 3) or with buffer alone (lanes 1 and 2). PKA and [γ -³²P]ATP were added to the mixtures, which were then incubated for a further 3 h at 30 °C before the reaction was terminated and the mixtures analyzed by SDS gel electrophoresis and autoradiography.

PKA (Fig. 7, lane 1). When reactions were performed in similar conditions with β II Σ 2 R16-CTR T2159A, no reactivity was detected (Fig. 7, lane 2); the lack of labeling indicates the site specificity of the reaction. When the phosphorylation reaction was performed after pre-incubation of the wild-type R16-CTR with α II N-R2, no phosphorylation above background was detected (Fig. 7, lane 3).

These data suggest that binding to α -spectrin occludes Thr-2159. This observation led us to wonder if phosphorylation at Thr-2159 would influence interaction of β II Σ 2 with α II. To test this possibility, purified recombinant protein β II Σ 2 R16-CTR

was incubated with ATP in the presence or absence of the catalytic domain of PKA for the phosphorylation and control reactions. The interaction with the N-terminal region of α II-spectrin was studied using purified recombinant protein GST- α II N-R2 in a pull-down assay as described previously (14). Fig. 8 shows the results of this assay. The wild-type unphosphorylated β II Σ 2 R16-CTR bound α II with an affinity calculated as 29.9 ± 4.1 nM, a value close to that reported previously using this assay (14). A much reduced binding of phosphorylated β II Σ 2 R16-CTR was found (Fig. 8*a*). The interaction was so weak that a dissociation constant for the interaction of phosphorylated β II Σ 2 R16-CTR could not be calculated with certainty because the plateau in the binding curve was not reached; however, half-maximal binding was >0.3 μ M, and from this we are able to infer that the affinity

of the interaction is reduced at least 10-fold. β II Σ 2 R16-CTR aggregates at high concentrations so we were unable to obtain the concentrations required to attain saturation.

The mutant T2159E was also prepared. This mutant replaces the neutral Thr-2159 with an acidic glutamate and partially replicates the effect on residue charge of phosphorylation. A moderate reduction in affinity was noted with this mutant (Fig. 8*a*).

Fig. 8*b* shows a similar binding assay performed with the mutant β II Σ 2 R16-CTR T2159A in which the threonine residue is replaced with the uncharged and nonphosphorylatable residue, alanine. Note that incubation of the protein with PKA and ATP makes no substantial difference to affinity.

We conclude that Thr-2159 is a regulatory site unique to the short C-terminal variant of β II-spectrin. Phosphorylation of this threonine reduces the affinity of the short β II-spectrin variant for the major α -spectrin of solid tissues, α II.

Phosphorylation of Thr-2159 and Neurite Outgrowth—It is clear from analyses of both worms and mammalian systems that spectrin is required for the production of the nervous system. Nishimura *et al.* (29) have suggested that spectrin is part of a “clutch module” required to engage the motor of neurite extension in the process of neurite initiation. Therefore, we tested whether there is a role for Thr-2159 in neuritogenesis.

PC12 cells are one of the standard models for neurite formation (30, 31). They are a rat pheochromocytoma cell line that differentiates in the presence of nerve growth factor into a sympathetic neuron phenotype. They have been used extensively to dissect the signaling processes in neurite outgrowth.

Fragments of spectrin that contain the tetramerization site are known to compete with native spectrin for tetramer formation in whole red cell membrane preparations (32, 33). We reasoned, therefore, that a fragment of β II-spectrin from repeat 16

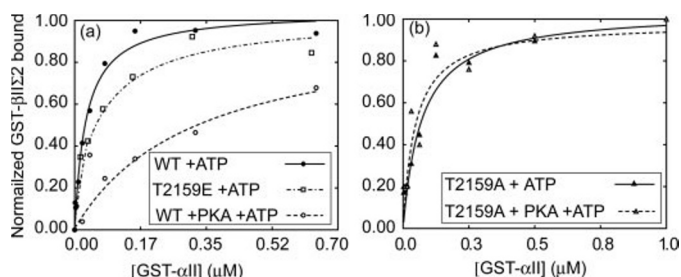


FIGURE 8. Phosphorylation of β II Σ 2 C-terminal fragment inhibits its interaction with α II N-terminal fragment. A pull-down assay with recombinant GST- α II N-R2 spectrin and β II Σ 2 R16-CTR (wild type (WT) and mutants) was used to quantify the effect of phosphorylation on interaction with α II β II Σ 2 construct (0.75 μ M) was incubated for 3 h at 22 °C, either alone or in the presence of differing amounts of GST- α II, in a final reaction volume of 50 μ l. Free and bound species were separated with glutathione-Sepharose beads. Complexed His-tagged proteins were quantified from Coomassie Blue stained gels or from immunoblots with anti-pentaHis antibodies by densitometry. *a*, wild-type β II Σ 2 interacts with $K_D \sim 30$ nM in this assay as described elsewhere. T2159E mutant interacts with an affinity approximately half as strong as wild type. Phosphorylation by PKA and ATP *in vitro* inhibits the interaction greatly; half-maximal binding occurs at ~ 0.35 μ M. *b*, the T2159A mutant is insensitive to phosphorylation. T2159A mutant was incubated with ATP alone or with ATP and PKA. No effect on binding was observed, and the affinity of the interaction was indistinguishable from wild type.

to the C terminus should compete with β -spectrin endogenous to PC12 cells for interaction with α chains. We prepared EGFP-fusions with R16-CTR of β II Σ 2-spectrin, either wild-type, T2159A, or T2159E. These were transfected into PC12 cells at the same time as application of nerve growth factor. Cells were then retained for various periods of time, up to 3 days, to allow neurite outgrowth. All three constructs were expressed to approximately equivalent levels in PC12 cells (and COS-7 cells, data not shown) as judged by integration of fluorescence signals from the transfected cells. The cells were examined by fluorescence microscopy, and EGFP-positive cells were scored for neurite production. The data are summarized in Fig. 9.

In our hands, 70–85% of untransfected cells, or cells transfected with GFP alone, produce neurites in many different experiments (data not shown). Cells transfected with the β II Σ 2 R16-CTR constructs remained viable over the 3 days of the experiment. However, cells transfected with the T2159A mutant became extremely misshapen. Many of the wild-type or T2159E transfectants were flatter than their untransfected counterparts and in many cases produced neurites that were wider than those of untransfected cells. Nevertheless, cells expressing the wild-type construct produced recognizable neurites ($80 \pm 11\%$ of the transfected cells). T2159E allowed neurite production in $74 \pm 11\%$ of cells. Thus, cells transfected with a phosphorylation mimic (T2159E) or a construct that can be phosphorylated allow neurite production indistinguishable from non-transfected or EGFP-only transfected cells. By contrast the mutant, T2159A, gave cells that showed reduced neurite outgrowth ($14 \pm 13\%$ of fluorescent cells produced neurites).

T2159A mutants, which cannot be phosphorylated, bind α II-spectrin with high affinity (Fig. 8*b*). Our data are consistent with this mutant acting as a high affinity competitor of tetramer formation and argue that spectrin tetramers are required in processes leading to neurite outgrowth. Furthermore, because the wild-type β II C-terminal region can be phosphorylated and

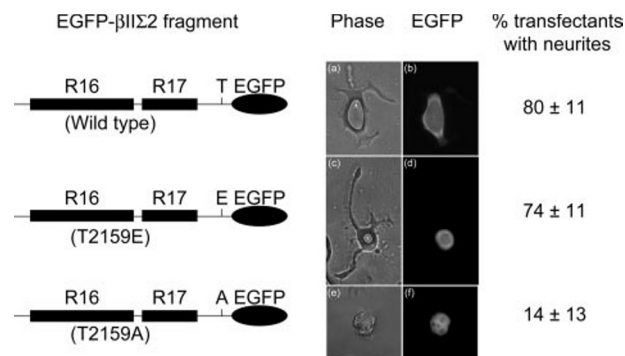


FIGURE 9. Transfection of fragments of β II Σ 2-spectrin into PC12 cells. PC12 cells were transfected with the indicated EGFP-conjugated constructs of β II Σ 2 spectrin R16-CTR. Cells were cultured further for 3 days and were then examined by phase and fluorescence microscopy (representative cells are shown in *central panels*). The proportion of EGFP-positive cells producing neurites was scored. Note that both wild type (*a* and *b*) and T2159E mutants (*c* and *d*) allow neurite outgrowth, but this is severely reduced in the T2159A mutant (*e* and *f*). For quantification of neurite outgrowth, data were collected from 10 separate fields of cells, and these are expressed as the means plus or minus the standard deviation.

still allows outgrowth, our data suggest that Thr-2159 becomes extensively phosphorylated in PC12 cells during neurite formation and, therefore, that formation of spectrin tetramers is regulated as a dynamic process during neuritogenesis.

DISCUSSION

In this article, we provide evidence that a threonine residue unique to the β II Σ 2-isoform of β -spectrin is phosphorylated both *in vivo* and *in vitro*, that phosphorylation modulates the affinity of α II- and β II Σ 2-spectrin subunits at the tetramerization site, and that phosphorylation at this site occurs during the process of neurite production by PC12 cells. Our data further indicate a requirement for spectrin tetramers in this process.

The C-terminal region of β II Σ 2-spectrin is phosphorylated at Thr-2159 and Ser-2110 *in vitro*; CK2 recognizes both sites, but only Thr-2159 is phosphorylated by PKA (Figs. 3 and 7). In our assay, PKA phosphorylates the C-terminal region of β II Σ 2-spectrin more rapidly than CK2, even though equivalent units of enzyme activity were present in each reaction. Ser-2110 and Thr-2159 are located within recognized consensus sequences for their kinases. Notably, Ser-2110 is common to both the long and short C-terminal isoforms of β II-spectrin, but Thr-2159 is unique to β II Σ 2, the short C-terminal form. Thus, phosphorylation of Thr-2159 presents a novel isoform-specific mechanism for regulation of spectrin function. This residue is unusual in being a substrate for both PKA and CK2 enzymes. It is also conserved in all available amphibian, bird, and mammal sequences (Fig. 4) indicating a fundamental role for this residue in spectrin regulation in most (possibly all) land-based vertebrates.

We identify Thr-2159 as a likely substrate for PKA *in vivo*. Our anti-TP2159 antibody identifies that the same residue is phosphorylated both *in vitro* by purified PKA (Fig. 5) and *in vivo* by reagents that modulate the activity of PKA (Fig. 6). Phosphorylation of Thr-2159 reduces the affinity of the C-terminal region of β II Σ 2 for the N-terminal region of α II (Fig. 8). Furthermore, mutation of this residue alters the activity of β II-spectrin fragments transfected into PC12 cells (Fig. 9). These

data strongly indicate that Thr-2159 is a physiological regulatory site.

Bearing in mind that many investigators have sought a role for C-terminal phosphorylation of erythrocyte β -spectrin in controlling the interactions of α I- β I-spectrin (e.g. Refs. 34 and 35), it was surprising to find that phosphorylation controls the interaction of our β II Σ 2 fragment with α II fragment. We were prompted to perform this experiment by the observation that incubating β II Σ 2 fragments with α II fragments reduced the phosphorylation of β II Σ 2 (Fig. 7). This suggests that the very C-terminal region is intimately connected with the tetramerization site and that, once bound in an α - β complex, Thr-2159 becomes inaccessible to kinase. Physiologically, it seems likely that when bound to α II-spectrin in a tetramer, this phosphorylation is inhibited. There have been a number of reports of β -spectrins not associated with α -spectrin (36–38), so this phosphorylation may occur on free β -chains. Alternatively or in addition, phosphorylation of nascent β -chains might limit their incorporation into tetramers until signals that elevate cAMP are attenuated, or until phosphatase(s) is/are activated. It is also interesting that although the T2159E mutation has only a moderate effect on affinity for α -spectrin, it allows neurite outgrowth, in contrast to T2159A. The spectrin system in PC12 cells gives the impression of being poised on a fine balance such that the dynamics of the spectrin cytoskeleton are so closely controlled that only the highest affinity interactions allow incorporation of nascent spectrin monomers/dimers into the cytoskeleton. Furthermore, because the locations of PKA and spectrin are both tightly limited by their binding partners, it is possible only the small fraction of β II Σ 2-spectrin closest to the site(s) of neurite outgrowth, need be phosphorylated during (or just prior to) outgrowth.

We also noted that each of the β II Σ 2-spectrin fragments (wild-type and the two mutants) were retained to a very large extent in the PC12 cell bodies (Fig. 9). This mirrors our observation (13) that β II Σ 2-spectrin is largely retained in the cell bodies of cerebellar neurons both in brain and in primary culture. β II Σ 2-Spectrin is targeted differently from β II Σ 1-Spectrin, which is present throughout axons (13). Our data are consistent with some targeting information being encoded in the C-terminal region of the β II Σ 2 variant. At present we do not know the nature of the targeting information, but β II Σ 2 is probably targeted by factors additional to any interaction with α -spectrin.

The functions of β II-spectrin and its invertebrate β -spectrin homologs have been probed extensively in genetic experiments. There is a clear requirement for β -spectrin in development of both invertebrate and vertebrate nervous systems. In *Caenorhabditis elegans*, β -spectrin is encoded by the *UNC70* gene. Animals null for this gene make fewer axons, and those that do form fasciculate less and also have guidance defects (2, 3). In mice, animals null for the gene encoding β II-spectrin (*SPTBN1*) do not form the primary brain vesicles properly (5). A relationship between spectrin and axon outgrowth was first suggested by Sobue and Kanda (39); they found spectrin concentrated in PC12 cell growth cones. Such a relationship is further suggested by the presence of β II-spectrin in axonal sprouts in developing neuroblasts (4). Nishimura *et al.* (29) have sug-

gested a model that includes a spectrin activity in the initiation of neurite outgrowth. The cell adhesion molecule L1-CAM binds ankyrin, which in turn binds spectrin. In the Nishimura model, engagement of L1-CAM with a stationary extracellular ligand promotes binding of ankyrinB to the cytoplasmic domain of L1-CAM. This in turn recruits other components of a clutch complex that links to cytoplasmic actin. Such linkage of actin to a stationary extracellular ligand enables transmission of traction force from F-actin flow to the extracellular substrate. Because a mutation in ankyrinB that abolishes spectrin-binding blocks association of ankyrinB with F-actin flow, Nishimura *et al.* (29) suggest that spectrin is part of the clutch complex. Further evidence for a requirement for spectrin in neuritogenesis comes from experiments in which antibodies to the actin-binding domain of β -spectrin were microinjected into neuroblastoma cells; this treatment blocks neurite extension in response to dibutyl-cAMP (40).

Our data support a role for spectrin in neurite formation. A fragment of the short C-terminal β II-spectrin with the mutation T2159A binds α II-spectrin with high affinity. Fragments of spectrin containing the tetramerization site alone make effective inhibitors of tetramer formation (32, 33) so such fragments can be used to test the requirement for spectrin tetramers in physiological processes. The T2159A fragment is an efficient inhibitor of neurite outgrowth (Fig. 9). The mutant, T2159E, displays a reduction in affinity for α II-spectrin *in vitro* (Fig. 8), and it does not block neurite outgrowth. Furthermore, even at the early stages of neurite outgrowth, *i.e.* after 1 day in culture when the cells elongate somewhat before neurites actually appear, the wild type and T2159E do not block the remodeling of cell shape; in contrast cells transfected with T2159A do not elongate significantly (data not shown). These data argue that spectrin tetramers are required in neurite outgrowth. Most interestingly, the wild-type short C-terminal β II-spectrin fragment does not block neurite formation. A likely mechanism for this is that the fragment becomes phosphorylated at Thr-2159 during neurite formation. PKA has a central role in axon outgrowth (e.g. Refs. 41–43) so the identification of Thr-2159 as a PKA substrate indicates a link between cAMP signaling and the dynamics of spectrin tetramers.

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