Site-Directed Mutagenesis of αII Spectrin at Codon 1175 Modulates Its μ-Calpain Susceptibility†

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ABSTRACT: Intracellular proteolysis by the calpains, a family of Ca^{2+} activated cysteine proteases, is a ubiquitous yet poorly understood process. Their action is implicated in an array of cellular and pathologic processes, including long-term potentiation, synaptic remodeling, protein kinase C and steroid receptor activation, ischemic cellular injury, and apoptosis. Unlike most proteases, the calpains display unusually strict substrate specificity, often cleaving only one or two bonds in proteins with hundreds of potential sites. Studies of synthetic peptides have defined sequences that modulate their specificity, but little data exist in the context of a bona fide protein. A prominent substrate for μ-calpain is αII spectrin (fodrin, brain spectrin), which is cleaved between Tyr_{1176} and Gly_{1177} within spectrin's 11th structural repeat unit. We have cloned and characterized human fetal brain αII spectrin (GenBank no. U26396) and identified a new Thr_{1300} to Ile polymorphism. From this clone, recombinant GST−fusion proteins representing repeat units 8−14 have been prepared and used to systematically explore the *in vitro* determinants of μ-calpain sensitivity. Twenty different amino acids were substituted by site-directed mutagenesis for wild-type Val_{1175}, the penultimate (P2) residue flanking the major calpain cleavage site in αII spectrin. Gly, Pro, and Asp, and to a lesser extent Phe and Glu, substantively inhibited the susceptibility of this site to μ-calpain; other substitutions yielded lesser effects. Dynamic molecular modeling of the 11th structural repeat of human αII spectrin incorporating the various mutations suggests that the calpain cleavage site with its flanking calmodulin binding domain interrupts helix C of αII spectrin's 11th repetitive unit without significantly disrupting the repeat's triple-helical motif. This model predicts that the critical Tyr_{1176}-Gly_{1177} bond occurs in a highly exposed loop juxtaposed between helix C and the calmodulin binding domain and that mutations at the P2 position subtly alter the conformation about this site. We conclude that secondary and tertiary conformational features surrounding the cleavage site, rather than the linear sequence itself, dominate the determinants that define αII spectrin's μ-calpain susceptibility.

The calpains (calcium-dependent neutral proteases, CDNPs,[1] or calcium-dependent proteases, CDPs) are a family of heterodimeric calcium-activated cysteine proteases widely distributed in eukaryotic cells [for reviews, see Murachi (1989) and Saido et al. (1994)]. Their action has been implicated in a variety of cellular and pathologic processes, including long-term potentiation and synaptic remodeling after *N*-methyl-D-aspartate (NMDA) receptor stimulation; glutamate-induced neurotoxicity; ischemic cellular injury; apoptosis; platelet activation; exocrine secretion; neutrophil activation; mitosis; progesterone and estrogen receptor modulation; and the regulation of a variety of kinases such as protein kinase C, phosphorylase kinase, myosin light chain kinase, and calmodulin-dependent kinase and phosphatase. Common targets include cytoskeletal proteins such as αII spectrin[2] (fodrin), adducin, protein 4.1, myelin basic protein, a variety of muscle proteins, neurofilament proteins, vimentin, actin, and myosin [see Glantz and Morrow (1996) and Takahashi (1990) for reviews]. Two forms of calpain are recognized, distinguished by the levels of [Ca^{2+}] required for their activation. One type (μ-calpain, also called calpain I or CDP I) is activated by micromolar levels of Ca^{2+}; the other form (milli-calpain, also called m-calpain, calpain II, or CDP II) is maximally active at millimolar Ca^{2+} concentrations. Besides calcium, both forms are subject to specific regulation by autoproteolysis (Croall & DeMartino, 1990), various phospholipids including polyphosphoinositides (Saido et al., 1992), and by calpastatin, a phosphorylated protein that binds to the large subunit of calpain and inhibits its action (Mellgren & Lane, 1990; Nishimura & Goll, 1991).

Calpain is also regulated *in vivo* by very tight specificity controls on the nature of its substrate. Calpain's cleavage site specificity has only been studied systematically in synthetic peptides, where the affinity of calpain for its substrate is typically 10−100 times weaker than for proteins. These studies fail to reveal a clear consensus cleavage sequence, although general principles seem to have emerged (Takahashi, 1990). However, these principles extend poorly to many proteins for which the cleavage site has been

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[1] Abbreviations: bdp, breakdown product; CaM, calmodulin; CDNPs, calcium-dependent neutral proteases; CDPs, calcium-dependent proteases; DTT, dithiothreitol; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RMS, root mean square; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean.

[2] The nomenclature of spectrin used in this paper follows previous conventions as set forth in recent publications and reviews (Lombardo et al., 1994; Morrow et al., 1996; Winkelmann & Forget, 1993).

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identified, implying that conformational determinants are a significant factor. Typically, calpain will efficiently cleave proteins at only one or two sites, often with values of K_m of 10^{-7} M or less (Nelson & Traub, 1982; Zimmerman & Schaepfer, 1982). In the 286 000 Da protein human αII spectrin (α-brain spectrin, fodrin), μ-calpain cuts predominately only at the single peptide bond joining Tyr_{1176} and Gly_{1177} in spectrin's 11th repetitive unit (Harris et al., 1988, 1989). No other bonds are as readily cleaved, even though this site is within a general region recognized and hypersusceptible to many proteases (Harris & Morrow, 1988).

The action of μ-calpain on αII spectrin is of significant interest because of its putative role in regulation of the spectrin cytoskeleton. *In vivo* and cell culture studies indicate that in the hippocampus and cerebellum spectrin proteolysis may be tightly linked to physiologic NMDA receptor activation (Di Stasi et al., 1991; Siman & Noszek, 1988) and may even be required for the establishment of long-term potentiation (Lynch & Baudry, 1987; Massicotte et al., 1991). *In vitro*, μ-calpain cleavage of the α-subunit of αII,βII spectrin (fodrin) activates a latent sensitivity of spectrin's actin binding and self-association capacities to regulation by Ca^{2+} and calmodulin (Harris & Morrow, 1990). Excessive calpain proteolysis of spectrin has also been implicated as an early event in ischemic injury [for a general review of this area, see Glantz and Morrow (1996)] and in apoptosis (Roberts-Lewis et al., 1993; Squier et al., 1994), in cells ranging from neurons (Arai et al., 1991; Lee et al., 1991; Roberts-Lewis et al., 1994) to kidney (H. Carey, J. S. Morrow, N. Siegel, and M. Kashgarian, in preparation; Doctor et al., 1993).

As a first step to exploring the consequences of calpain action specifically on spectrin, human fetal brain αII spectrin cDNA was cloned and sequenced, and the determinants of its μ-calpain sensitivity were explored using recombinant αII spectrin peptides in which the Val_{1175} residue at the P2 position relative to the site of cleavage has been replaced by each of the other 19 amino acids. This site was selected for mutagenesis because of the sensitivity of synthetic peptides to substitution at this location. Our results indicate that spectrin cleavage is nearly unaffected by a broad range of residue substitutions, suggesting that linear sequence determinants *per se*, when presented in the context of the conformation of an intact protein, play only a minor role in modifying calpain susceptibility. Conversely, Gly, Pro, and Asp (and to a lesser extent Glu and Phe) substitutions markedly impair cleavage. Hypothetical models of spectrin's 11th structural repeat unit, derived by energy minimization algorithms, suggest that the calpain cleavage site occurs in an exposed loop juxtaposed between helix C of the spectrin repeat and the calmodulin binding domain. Collectively, these results suggest that secondary and tertiary conformational determinants largely determine the specificity of calpain action, with linear sequence determinants playing a lesser role.

Portions of this work have been previously presented in abstract form (Stabach et al., 1994).

MATERIALS AND METHODS

Cloning of Human Fetal αII Spectrin. Hybridization screening of an oligo(dT) and random hexamer primed human fetal brain lambda ZAP−11 cDNA library (Stratagene) yielded 39 clones from 10^6 plaques. The probe used for screening was a ^{32}P randomly labeled 1.7 kb PCR product constructed from primers derived from the human lung αII spectrin sequence (nucleotides 2455−4211) (Moon & McMahon, 1990). Positive colonies were subcloned *in vivo* into pBluescript (Stratagene) directly from lambda ZAP using helper phage R408, following the protocol accompanying the lambda ZAP product literature. Clone sizes were approximated by restriction digests with *Eco*RI. The ends of each clone were sequenced and overlapping clones were aligned. Clone 18531, encompassing the calpain cleavage site, was completely sequenced in both directions.

cDNA Sequencing. The dideoxy chain-termination method was used to sequence both strands of cDNA using Sequenase (USB) (Sanger et al., 1977). Approximately 3 μg of DNA and 1 pmol of primer were used for each run. Sequencing reactions were loaded on a 40 cm 5% polyacrylamide Long Ranger gel (AT Biochem) and exposed for a minimum of 12 h on autoradiographic film. Approximately 350 bp of sequence was obtained from each run. Each clone was initially sequenced with T3 and T7 primers. New primers were constructed from sequenced regions to fill the gaps. Either the Lasergene computer software package (DNAStar, Inc.) or the Wisconsin genetic analysis software package (Devereux et al., 1984) was used for all sequence analyses.

Prokaryotic Expression Vector Construction and Mutagenesis of the P2 Site. Clone 18531 representing bp 2531−4689 of human αII spectrin was cloned into pBluescript using *Eco*RI adapters and subcloned into the *Eco*RI site of the inducible bacterial expression vector pGEX-3X (Pharmacia) (Kennedy et al., 1991; Smith & Johnson, 1988). Site-directed mutagenesis by PCR was used to replace the valine at codon 1175 in αII spectrin's 11th structural unit with each of 20 possible amino acids. The oligo used for this (10247) contained all four degenerate nucleotides at this codon flanked by 12 matched nucleotides: 5′-CAACAACAG-GAA**NNN**TATGGCATGATG-3′ (Figure 1B). The antisense oligo was a pGEX reverse oligo (pGEX-R) 5′-CGTCATCACCGAAACGCG-3′. The PCR reaction contained a 0.2 mM amount of each dNTP, 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, a 0.5 μM amount of each primer, 2.5 units of *Taq* polymerase (Perkin Elmer), and 15 ng of pBluescript 18531 plasmid as template. The annealing temperature was 42 °C, and reactions were carried out at 72 and 94 °C for 30 cycles. Each cycle was 1 min. The correctly sized 1128 bp band was excised from a 0.75% FMC brand SeaPlaque low-temperature agarose gel and purified by phenol extraction and ethanol precipitation. This material was then used as the reverse primer together with the pGEX forward primer (pGEX-F) 5-′GCAAG-TATATAGCATGGC-3′, in a second PCR reaction to regenerate the full-length clone 18531 sequence incorporating the desired mutations. This PCR product was then digested with *Eco*RI and subcloned back into pGEX-3X and sequenced. In addition to the wild-type valine, 16 other amino acids substitutions were produced using this method. The remaining three amino acid substitutions (His, Met, Ile) were not recovered after screening over 120 colonies. To prepare these, specific oligonucleotides were used: 5′-CAACAA-CAGGAACACTATGGCATGATG-3′ and 5′-CAACAA-CAGGAAAT(GC)TATGGCATGATG-3′. These synthetic oligonucleotides incorporated biotinylated nucleotides to facilitate antisense strand enrichment of the primary PCR

reaction (Mitchell & Merril, 1989), which was needed for the second PCR step. Briefly, the PCR reaction product was ethanol precipitated, rehydrated in 50 μL of TE (10 mM Tris, pH 8.0, 1 mM EDTA), and mixed with an equal volume of a 50% streptavidin−agarose slurry in 0.2 M NaCl, 20 mM Tris, pH 7.5, and 1 mM EDTA. After 10 min at room temperature, the entire slurry was layered atop a G-50 quick spin column (Boehringer Mannhein) and washed twice with 0.5 mL of TE, followed by a 5 min centrifugation at 1000*g*. Single-stranded DNA was harvested from the column with 0.2 N NaOH (for 10 min) and precipitated with equal volumes of 0.3 M NaOAc and 2-propanol. This DNA was washed with 70% ethanol and used as the antisense primer with the pGEX forward primer to regenerate the full-length 18531 sequence in pGEX-3X.

Preparation and Purification of Recombinant Proteins. The pGEX constructs containing the #18531 clone incorporating 20 different codons at the P2 position were expressed in either the DH5α or CAG-456 strains of *Escherichia coli*. As before (Kennedy et al., 1991), overnight bacterial cultures were diluted 1:10 in fresh media, grown for 1 h, and then induced for 1−3 h with IPTG before harvesting by centrifugation. Lysis was achieved by gently agitating the bacterial pellet for 30 min at 4 °C in 100 μL of 40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM benzamidine, 10 μg/mL aprotinin, 0.5 mM Pefabloc, 1 mM DTT, and 1 mg/mL lysozyme (Sigma), after which the samples were frozen at −70 °C and rapidly thawed at 30 °C. Triton X-100 was added to a final concentration of 1% (v/v), and the sample was incubated at 4 °C for an additional 30 min and then sonicated three times for 10 s each. Under these conditions, approximately 10−20% of the total recombinant peptide was released in the soluble pool. The 15 000*g* supernatant of the lysate was affinity absorbed on 200 μL of glutathione−agarose beads (Sigma) at 4 °C and washed five times with 4 mL of PBS with 1 mM DTT, followed by a 4 mL wash with 50 mM Tris and 1 mM DTT, pH 8. The bound peptide was eluted in 100 μL of the same buffer containing 10 mM glutathione. Proteins were stored at 4 °C in this solution for up to 2 weeks before use. Alternatively, to enable more rapid screening assays of calpain activity, the pellet from 1 mL of IPTG-induced bacterial culture was treated as above and directly resuspended in digestion buffer (see below). This generated a preparation that was highly enriched in the recombinant αII spectrin peptide and readily analyzed by SDS−PAGE for its sensitivity to μ-calpain.

μ-Calpain Digestion. Purified μ-calpain (calpain I) prepared from porcine erythrocytes was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Its advertised purity was >99%, with less than (1×10^{-7})% contamination with other protease enzymes. Its specified activity was 140 units/mg, with half-maximal activity at ≈2 μM free calcium. It was used without further purification. Control digestions with purified bovine brain αII spectrin were carried out to confirm that its activity against spectrin was as before (Harris et al., 1988). For the initial activity screens, the Triton-washed pellets from 1 mL of induced cultures were suspended with brief sonication in 100 μL of PBS (phosphate-buffered saline) on ice; 15 μL of this solution was mixed with an equal volume of 2× digestion buffer (0.3 mM $CaCl_2$, 10 mM DTT) and 1 μL of 50 mM Tris-HCl, pH 7.5, and 1 mM DTT, containing various amounts of μ-calpain (as specified in the figure legends). Reactions were carried out at 30 °C for 20 min and stopped by the addition of 4× SDS sample buffer [5 M urea, 250 mM Tris-HCl, pH 7.5, 12% (w/v) SDS]. For digestions employing affinity-purified recombinant peptides, purified peptides were dialyzed into PBS before use and then reacted as above. Alternatively, purified peptides in elution buffer were diluted with a stock buffer to achieve final reaction conditions of 75 mM NaCl, 150 μM $CaCl_2$, 5 mM DTT, and 1 mM Tris, pH 7.5, containing various amounts of calpain. All samples were analyzed by SDS−PAGE.

Calmodulin Binding. The ability of the recombinant peptides to bind calmodulin in a Ca^{2+}-dependent manner was assessed by their ability to be retained on calmodulin−Sepharose (Pharmacia) affinity columns (Harris et al., 1988). The bacterial lysates containing various recombinant αII spectrin peptides were passed through the calmodulin−Sepharose column in 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$ and washed exhaustively. The calmodulin-bound peptide was eluted in the same buffer containing 4 mM NaEDTA and analyzed by SDS−PAGE. Its identity as the recombinant peptide was verified by its affinity for a reduced glutathione column.

Data Analysis. Protein in the band representing intact peptide as well as the large breakdown product (bdp) at 69 kDa was evaluated by densitometric scanning of Coomassie blue stained SDS−polyacrylamide gels using a HP Scanjet II® in conjunction with the Scan Analysis (Biosoft) program. Different dilutions were scanned and compared with standard samples to assure that all measurements were in the linear range of instrumental responsiveness.

Dynamic Protein Modeling. The coordinates of the crystal structure of the *Drosophila* α-spectrin 14th repeat unit were used as the starting point for all modeling studies (Yan et al., 1993). The sequence of the 11th unit of αII spectrin was aligned with the 14th repeat unit of *Drosophila* α-spectrin using the program BestFit (Devereux et al., 1984). The corresponding residues of human αII spectrin were then graphically incorporated into the published crystallographic coordinates of fly spectrin, using the program Insight (Biosym Technologies, 1993), creating a *starting structure* for the dynamic modeling computations. These computations, combined with energy minimization algorithms, are fully described under Results. Minimized structures were displayed using Insight (Biosym Technologies, Inc.) molecular display software. All computations were carried out using an Indigo^2 Extreme workstation (Silicone Graphics, Inc.).

Other Procedures. Protein concentrations were measured by the Bradford assay (Bio-Rad), using bovine γ-globulin as a standard. All molecular biological techniques followed standard procedures unless otherwise noted (Sambrook et al., 1989). All synthetic oligonucleotides were purchased from the synthesis facility of Yale's Critical Technologies Laboratory. SDS−PAGE followed the method of Laemmli (1970); proteins were visualized by Coomassie blue staining. Digitally recorded images were processed for publication using Adobe Photoshop version 2.5.1 operating on a Macintosh computer and printed using a Fuji or Kodak color printer.

RESULTS

Cloning of Human Fetal αII Spectrin Identifies a THR_{1300} to Ile Polymorphism. PCR-derived oligonucleotide probes

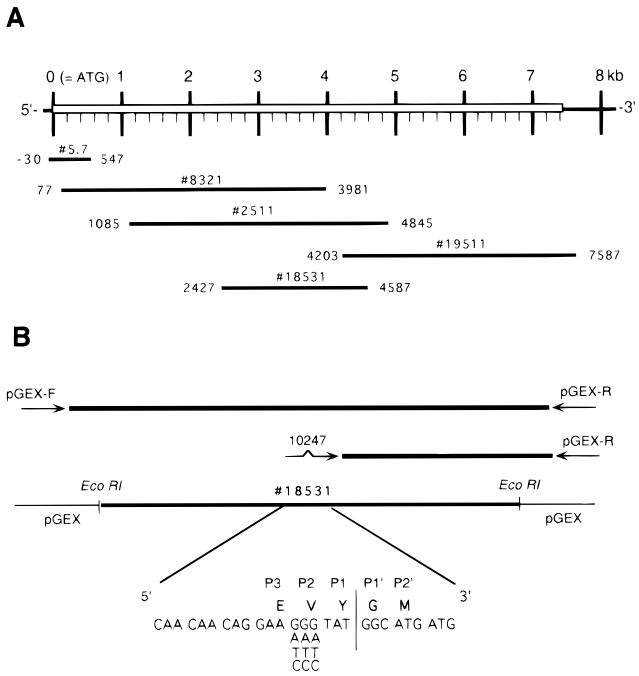

FIGURE 1: Cloning and mutagenesis of human fetal brain αII spectrin. (A) Screening of a human fetal brain library with a PCR-derived probe based on the published human lung fibroblast sequence (Moon & McMahon, 1990) identified 39 clones. Representative clones are depicted. Sequencing confirmed their origin as human αII spectrin and identified the relationships shown. The clone used in these experiments is labeled #18531 and represents codons 809−1529 of human αII spectrin (Moon & McMahon, 1990). The sequence of this clone is accessioned as GenBank no. U26396. The open boxed area represents the open reading frame. Two nucleotide polymorphisms, yielding one predicted amino acid substitution relative to the published lung fibroblast sequence, were identified in the #18531 clone (see text). (B) The #18531 clone was subcloned into pGEX-3X at the *Eco*RI site. Mutations were introduced at the P2 position (equivalent to codon 1175 of full-length αII spectrin) by PCR using a primer degenerate at this position (primer 10247). For residues His, Met, and Ile, specific oligonucleotides were used since codons representing these residues were not identified after screening over 120 colonies generated from the degenerate primer.

were used to screen approximately 10^6 plaques of a human fetal brain cDNA library. Thirty-nine overlapping clones were identified and sequenced (Figure 1A). Collectively these clones encompassed all but the very 5′-portion of the open reading frame for αII spectrin. Clone #18531 encompassed codons 809−1529 of αII spectrin and represented structural repeat units 8−14 of αII spectrin. The sequence of this clone was confirmed by repetitive bidirectional sequencing, as well as by sequencing the overlapping clones #8321 and #2511. Compared to the human lung fibroblast sequence, the sequence of #18531 (GenBank no. U26396) displayed two nucleotide substitutions, at bp 2631 and 3899 (numbered with the nucleotide #1 = A of the ATG at codon 1). Of these, the substitution at bp 3899 alters the residue at position 1300 from Thr to Ile. Given the otherwise near identity of the fetal brain and lung sequences, we conclude that this variation at the amino acid level as well as the translationally silent variations at the nucleotide level represents polymorphisms in this protein. Their functional significance (if any) is unknown.

Twenty Different Amino Acids Were Introduced into the P2 Position of αII Spectrin. Mutations were introduced into codon 1175 (based on αII spectrin) using a PCR-derived strategy (Figure 1B). Initially, a degenerate oligonucleotide sense primer was used (10247) that incorporated all possible nucleotides at codon 1175. This codon is (−2) relative to the site of μ-calpain cleavage in human αII spectrin (Figure 2a) (Harris et al., 1988). By convention, this site is termed P2 (Takahashi, 1990). After screening over 120 colonies, clones expressing the wild-type valine as well as 16 additional residues at this position were identified. For most of these, multiple clones with degenerate codon usage were also identified. However, no clones expressing His, Met, or Ile were identified. To obtain these substitutions, two specific oligonucleotides were used in place of oligonucleotide 10247. These oligonucleotides also incorporated biotinylated nucleotides, which enabled the enrichment of the antisense strand of the PCR product and subsequent amplifications with the pGEX-F sense primer (Figure 1B).

The final constructs were cloned into pGEX and expressed in *E. coli* to produce a GST−fusion protein encompassing repeats 8−14 of αII spectrin, with all mutations at the P2 position (Figure 2a). DNA sequencing of these constructs confirmed their fidelity. Clones with nucleotide substitutions due to *Taq* polymerase errors were discarded. The sequence of codon 1175 in each construct used in these experiments is shown in Figure 2b.

Gly, Asp, and Pro Most Strongly Limit the Susceptibility of αII Spectrin to μ-Calpain. Each of the constructs was expressed as recombinant proteins in *E. coli*. For the initial determinations of calpain sensitivity, all constructs were screened by digestion with two different concentrations of μ-calpain in buffer containing 150 μM (total) Ca^{2+}. For these screening studies, it was found that the cleavage of the recombinant peptide could be readily and simply monitored by Coomassie blue staining (Figure 3a). The intact recombinant peptide (wild type) had a calculated M_r of 109 060. Cleavage by μ-calpain at the same site as in native αII spectrin would be expected to generate fragments of M_r 68 724 and 40 336 Mr (Figures 2a and 4b). Separate studies employing cleavage-specific antibodies to the αII spectrin breakdown product (bdp) (del Lacoste et al., 1992; Glantz & Morrow, 1996) confirmed that, at least for the valine construct, cleavage was occurring at precisely the same site as in native αII spectrin. In general, both of the predicted breakdown fragments could be detected with most constructs (see Figure 4b), although the larger fragment was the more easily demonstrated, and is the bdp shown in the gels in Figure 3a.

The extent of cleavage varied as a function of the substituted residue. Substitutions of Phe, Trp, and Arg destabilized the initial cleavage product, such that further degradation of the initial breakdown product rapidly occurred, as evidenced by a net loss of spectrin peptide from the gel. Conversely, substitution of Gly, Pro, or Asp at the P2 position, and to a lesser extent Glu and Phe, greatly protected the intact peptide from the action of μ-calpain. These results are summarized quantitatively in Figure 3b. Note that the differences between the various constructs were most apparent at low (6 nM) enzyme concentrations (hatched bars) and with the exception of the five substitutions cited above were diminished at higher enzyme concentrations (30 nM, solid bars). To exclude the possibility that the protective effect of these residues arose from interactions with contaminating bacterial proteins or from other features of the bacterial fraction used for these

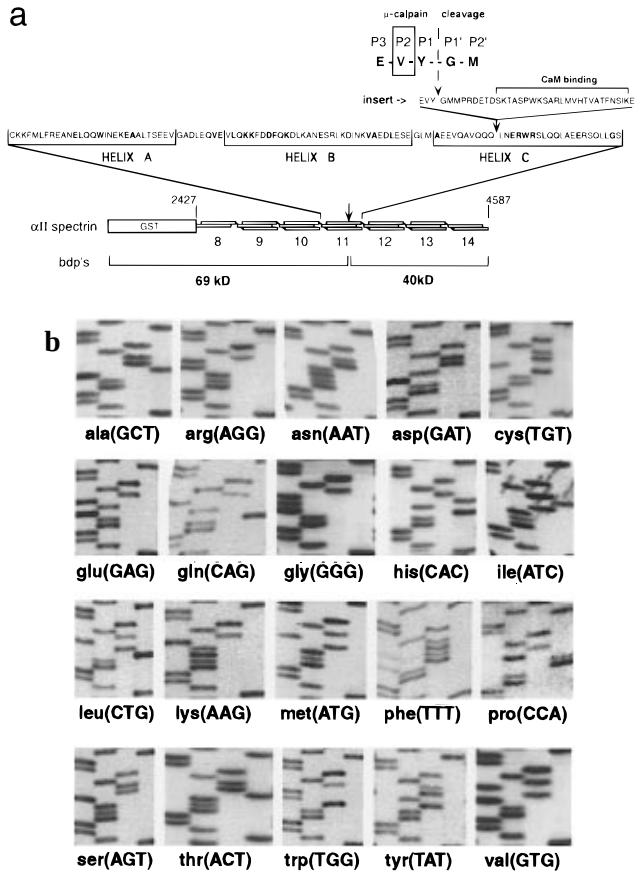

FIGURE 2: DNA sequencing confirms that mutations encoding each of 20 different amino acids were introduced at the −2 position (P2) relative to the μ-calpain cleavage site in human αII spectrin. (a) The μ-calpain cleavage of human αII spectrin occurs within spectrin's 11th repetitive unit in the protease hypersensitive region between Y_{1176} and G_{1177} and flanks the calmodulin binding domain (Harris et al., 1988, Harris & Morrow, 1988). The recombinant proteins used in this study represented repeat units 8−14 (residues 809−1529) of αII spectrin and were prepared as fusion proteins with GST. The sequence of the residues flanking the cleavage site is shown in single-letter code. Substitutions were made at P2; the wild-type protein contains valine at this position. An alignment with the *Drosophila* spectrin 14th repeat unit, for which the crystal structure is known, suggests that the calpain cleavage site and the calmodulin binding domain interrupt helix 3 (insert) and identifies 29 identical residues (bold) between the two spectrins within the predicted three helices. Based on this alignment, the predicted location of the three helices in the spectrin repeat unit is as indicated (also see Figure 5). (b) After mutagenesis and construction of the expression plasmid, each construct was sequenced to verify the sequence of the insert. The use of degenerate oligonucleotides encoding all possible codons at P2 allowed the identification of mutations encoding for 17 different amino acids (including wt valine). Specific oligonucleotides were used to prepare constructs encoding His, Met, and Ile at P2. In many cases (not shown), clones were identified encoding the same residue but with alternate codon usage. Depicted are the relevant regions of representative sequencing gels identifying the nucleotides at codon P2 in the constructs used for this study. The fidelity of the remaining sequence in these constructs was also verified by sequencing and was identical to wt in all constructs (data not shown). The order of loading on these gels from the left is GATC.

screening experiments, the sensitivity of affinity-purified peptides was examined. Compared to the purified wild-type valine peptide, which was nearly half-digested by as little as 1 nM enzyme in 20 min under the conditions of these experiments (Figure 4a), constructs containing Gly or Pro were largely resistant. These differences were also apparent in separate experiments with prolonged calpain digestion,

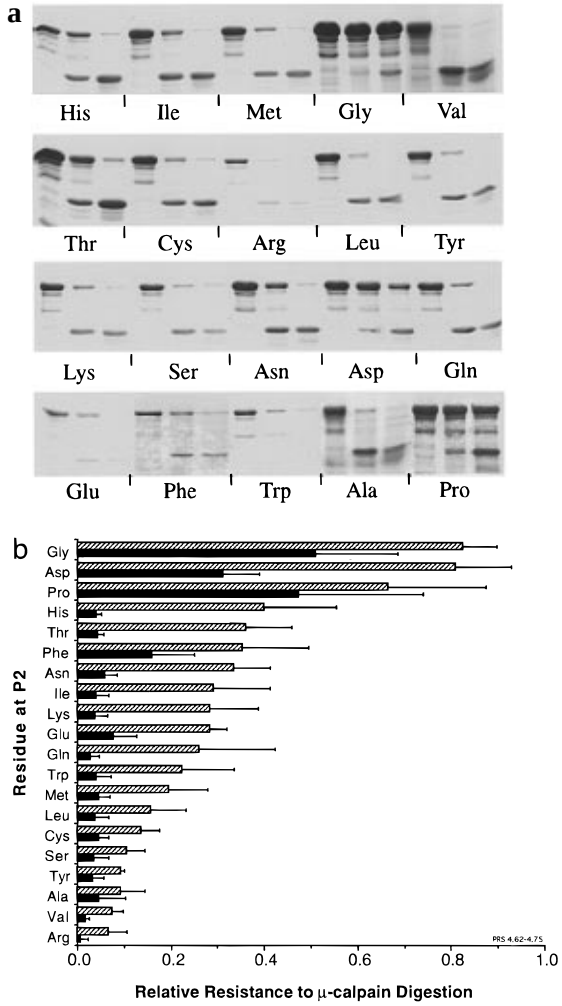

FIGURE 3: Gly, Asp, and Pro, and to a lesser degree Glu and Phe, at the P2 position limit the susceptibility of αII spectrin to μ-calpain. (a) Recombinant αII spectrin−GST fusion peptides harboring mutations at the P2 position of the calpain cleavage site were compared for their susceptibility to μ-calpain at 30 °C. The residue at the P2 position is given below each set of three lanes. For each triplet, the left lane is without enzyme; the center lane is with 30 nM enzyme; and the right lane is with 6 nM enzyme. The stated activity of the calpain used was 140 units/mg. All gels are Coomassie blue stained. (b) The relative sensitivity of the αII spectrin peptides to μ-calpain was determined by densitometric evaluation of the intact peptide under the conditions of digestion shown (a). Results are expressed relative to the integrated OD of each starting peptide and represent the fraction of peptide remaining unproteolyzed. Hatched bars represent 6 nM enzyme; solid bars are for 30 nM enzyme. Note the marked resistance of peptides carrying Gly, Asp, or Pro at the P2 position. All determinations represent three to five separate experimental determinations. Error bars = ±SEM.

in which the half-life of the undigested valine peptide was less than half that of peptides containing Gly, Pro, or Asp at the P2 position. To exclude the possibility that the relative resistance of the Gly, Pro, Asp, and Glu peptides was an artifact of varying enzyme/substrate ratios (since these peptides were among those produced in the best yields), the experiments were repeated in triplicate with the purified peptides all in solution at an identical concentration (5 μM), in identical buffers, with a constant amount of the same preparation of enzyme (Figure 4b). Again, these studies demonstrated the reduced calpain susceptibility compared to the valine peptide of peptides containing Gly, Pro, Asp, or Glu.

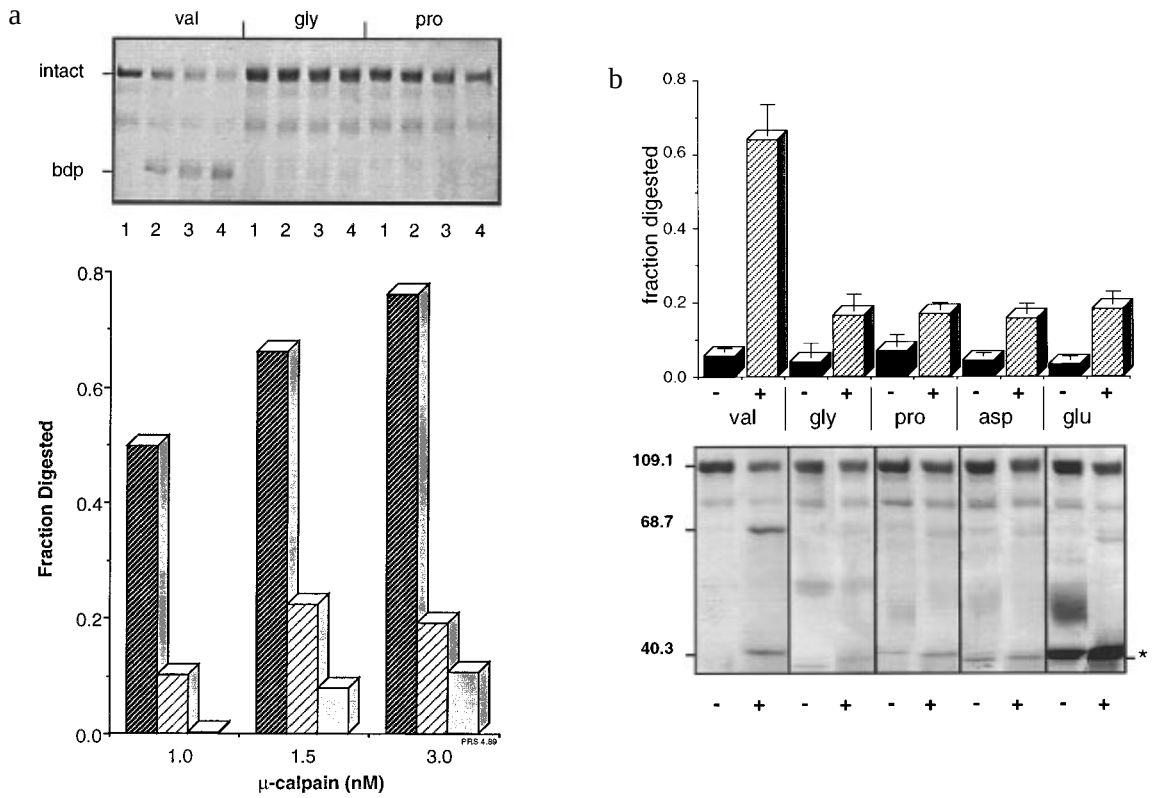

FIGURE 4: Purified αII spectrin peptides with Gly, Pro, or Asp for Val at P2 resist prolonged μ-calpain exposure. To exclude the possibility that other inhibitors or the state of the recombinant peptide in the high-salt-extracted bacterial fractions used in the screening experiments caused an inhibition of μ-calpain activity, the effect of calpain on purified peptides was examined. (a) Recombinant αII spectrin peptides containing the wild-type valine, glycine, or proline at P2 were subjected to 20 min of digestion with increasing concentrations of enzyme. (Top) SDS−PAGE analysis: lane 1, no enzyme; lane 2, 1.0 nM enzyme; lane 3, 1.5 nM enzyme; lane 4, 3.0 nM enzyme. The fraction of intact peptide remaining was evaluated and is presented in the bottom panel. Note the appearance of the spectrin breakdown product (bdp) at M_r ≈69 000 in the wt but not the peptides containing Gly or Pro at P2. (b) To further confirm the selective resistance of certain mutations at the P2 position and to exclude contributions from alterations in the enzyme/substrate ratio, purified peptides carefully matched to an identical concentration (5 μM) were compared under identical conditions with the same (6 nM) enzyme preparation. This experiment was carried out at pH 7.5 in 1 mM Tris, 75 mM NaCl, and 150 μM $CaCl_2$ at 30 °C for 20 min. Note the marked resistance of peptides containing Gly, Pro, Asp, Glu, and Phe compared to the peptide containing Val. The lanes marked (−) are without enzyme; (+) designates with enzyme. The error bars represent ±1 SD of three separate determinations. The positions of the intact peptides (M_r ≈109 060) and their cleavage products at M_r ≈68 724 and ≈40 336 (calculated values based on valine peptide) are indicated. The (*) marks a gel artifact in which the tracking dye and the band at M_r 40 000 merged. The fraction of peptide cleaved in each lane is depicted in the bar graph.

Another measure of the conformational integrity and activity of these peptides containing substitutions at P2 is their ability to bind calmodulin in a Ca^{2+} sensitive manner, since the calmodulin binding domain of αII spectrin is immediately adjacent to the P2 site (Figure 2a; Harris et al., 1988). All of the cleavage-resistant peptides that were examined in this assay (P2 = Gly, Pro, and Asp) were retained on calmodulin affinity columns in a Ca^{2+} sensitive manner, as was the wild-type peptide. These results indicate that this activity is clearly preserved in the recombinant peptides.

Molecular Modeling Suggests That Calpain Recognizes Complex Conformational Determinants. Since the substitutions in αII spectrin that altered its μ-calpain sensitivity did not correlate with the usual parameters of amino acid hydrophobicity, charge, or bulk, it seemed likely that features of spectrin's secondary and tertiary structure were the most important parameters. Detailed information on the structural unit of spectrin exists only for the 14th repeat of *Drosophila* α-spectrin (Yan et al., 1993) and for the 16th repeat unit of chicken α-spectrin (Pascual et al., 1996). Sequence alignment of the 11th structural repeat unit of human αII spectrin with the *Drosophila* 14th unit suggested that the calpain cleavage site and its adjacent calmodulin binding site represented 37 residues of nonhomologous sequence interspersed within a typical α-spectrin structural repeat unit. On the basis of this alignment, the positions of helices "A", "B", and "C" in αII spectrin's 11th repetitive unit and their relationship to the intervening nonhomologous sequence containing the calpain cleavage site and the CaM binding site were estimated (Figure 2a). Of note is the relatively low level of identity between the fly and human sequence, with only 29 of 106 residues perfectly conserved.

To better estimate the conformational environment about the calpain cleavage site, dynamic molecular modeling of the human αII spectrin 11th repeat unit was undertaken, based on the crystal structure of the *Drosophila* spectrin repeat. To test the reliability of the computations, the 14th structural unit of *Drosophila* α-spectrin was also evaluated. The starting structure for modeling this spectrin was based on its crystal structure (Yan et al., 1993). The molecular dynamics at 300 K of this structure were initially modeled in a vacuum, using the Verlet method (Verlet, 1967). It was found that this approach yielded helices at the NH_2 and COOH termini that unwound too much, and the orientation of the amino acid side chains was also changed compared to the crystal structure. This same result was obtained when a simulated annealing method starting at high temperature

was used. These results suggested that simulation in a vacuum was inappropriate for this superhelix of the three helices and suggested that interactions between spectrin and the aqueous environment were critical. To solve this problem, the effects of modeling these structures in a 6 Å water shell using both molecular dynamics at 300 K and simulated annealing from high temperatures were evaluated; this approach yielded better agreement with the known crystal structure (see below).

Given practical limitations on the computational complexity that could be managed, a 6 Å water shell was chosen for the simulation of the spectrin structural repeat. Both the *Drosophila* α-spectrin 14th repeat and the 11th structural unit of human αII spectrin were modeled. The *starting structures* (see Materials and Methods) were "placed" in a 6 Å thick water shell that contained about 1100 water molecules. Five hundred steps of Powell conjugate gradient energy minimization (Powell, 1977) were first performed to minimize the system energy. Then Verlet molecular dynamics was used to accomplish the simulated annealing. The simulated temperature was raised to 1000 K and then cooled to 110 K in steps of 10 K. At each temperature, 400 time steps of dynamic calculation were performed. Time-step increments (TS_k) were determined by the formula:

$$TS_k = \left\{\frac{18}{100 - k}\right\} \text{fs} \qquad k = 0, 1, ..., 89$$

The total simulation time was 16.5 ps. The initial velocity was randomly set according the Maxwell velocity distribution. The frictional coefficient was set to 100 ps^{-1} for all atoms. A structure was sampled after each temperature step. The 90 sampled structures were energy minimized by the Powell conjugate gradient energy minimization with 2000 steps, and the 10 structures with lowest energy minima were averaged. Energy minimization was then again performed on the averaged structure with 2000 steps. The tolerance for the norm of the gradient of E_{total} was 0.01 during all of the energy minimization procedures.

The simulated structure of the 14th structural unit of *Drosophila* α-spectrin derived by this method was superimposed onto its crystal structure. The orientations of the side chains matched well with the crystal structure. The root mean square deviation between the crystal structure and that estimated by the above modeling procedure was 1.99 Å for the backbone atoms and 2.42 Å for all heavy atoms. In separate studies, this modeling procedure also has successfully predicted the consequences of all known mutations in spectrin's α,β self-association domains (Zhushan, Weed, and Morrow, in preparation). These results collectively give some confidence that this modeling procedure, as applied to different spectrin repeats, has validity.

This procedure was then applied to the 11th structural repeat of human αII spectrin and to this repeat harboring the various mutations at the P2 position. A model of the wild-type αII spectrin 11th repeat unit was constructed (Figure 5). This model is significant in that, even though helix C is interrupted by the presence of the calpain cleavage site and the adjacent calmodulin binding domain, the modeling algorithms predict that helix C is essentially preserved, as is the fundamental triple-helical spectrin repeat motif. Within helix C, the nonhomologous sequence containing the calpain cleavage site (* in Figure 5) and the CaM binding domain are predicted to loop out of the helix and to thereby form a distinct structural domain, with the critical bond between Tyr_{1176} and Gly_{1177} highly exposed along a portion of the peptide that joins helix C to the CaM binding sequence. Several mutations at P2, including Gly and Pro, are predicted to subtly alter the conformation of this loop, although the modeling algorithms were not precise enough to identify any consistent pattern of conformational change that correlated well with sensitivity to calpain.

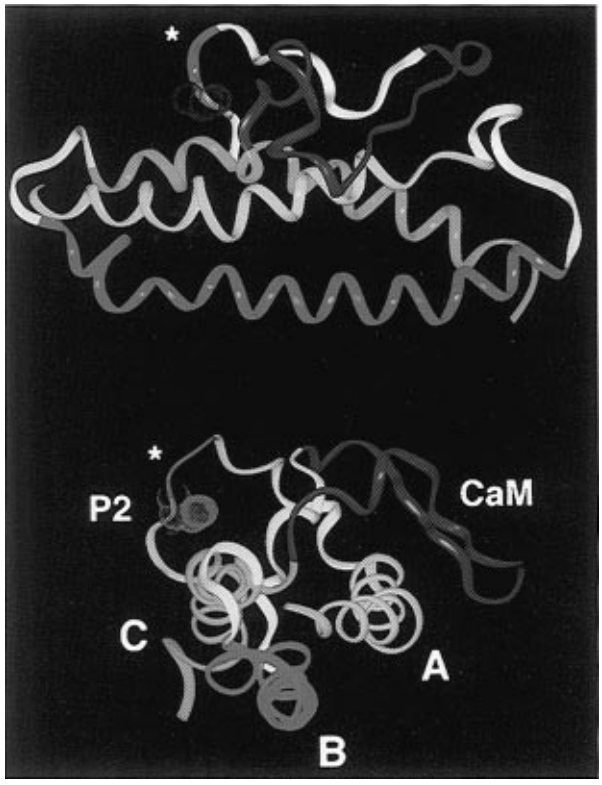

FIGURE 5: Molecular modeling suggests that the calpain cleavage site appears on an exposed loop of peptide joining the CaM binding domain to helix C of the spectrin repeat. After molecular dynamic simulation, energy minimization was carried out to estimate the relationship of the nonhomologous calpain cleavage site and calmodulin binding domain to the 11th structural unit of αII spectrin. The crystal structure of the *Drosophila* 14th repeat of α-spectrin was used as a starting point for all calculations. The predicted structure of the wild-type 11th repeat of human αII spectrin preserves the triple-helical motif of the classic spectrin repeat, with the calpain cleavage and CaM binding sequences forming an independent structural domain that interrupts helix C. Calpain cleavage occurs on the short stretch of exposed peptide (*) joining helix C to the calmodulin binding domain (blue). The P2 residue that was mutated in these studies is as marked. It is anticipated that, in the presence of CaM and Ca^{2+}, the relatively unstructured CaM binding domain predicted here will assume a more helical conformation, as happens with other CaM binding peptides (Vogel & Zhang, 1995).

DISCUSSION

Human fetal brain αII spectrin has been cloned, and novel polymorphisms in this gene have been identified. Using this gene, recombinant peptides have been used to explore the determinants of μ-calpain sensitivity in αII spectrin. Three mutations at the P2 position most strongly limit the sensitivity of the recombinant peptides to μ-calpain digestion *in vitro*. These results suggest that the primary determinants of calpain action in proteins are secondary and tertiary structural features about the cleavage site. These conclusions are

supported by three lines of evidence: (i) Of 20 recombinant peptides representing repeats 8−14 of human αII spectrin and differing only in the P2 position, those with Gly, Pro, or Asp, and to a lesser degree Glu and Phe, most strongly resisted digestion; many other substitutions were without effect. (ii) The sensitivity of different substitutions at P2 to digestion did not correlate with any (obvious) characteristics of the P2 residue. (iii) Molecular models of spectrin's 11th repeat unit suggest that the calpain cleavage site occurs on an exposed loop of the peptide joining helix C to a calmodulin binding domain and that the P2 residue is positioned at a junction of this loop with the spectrin backbone. Collectively, these results indicate that the action of calpain against a specific protein substrate is controlled by features well beyond the sequences immediately flanking the cleavage site and raise the possibility that calpain's action might be subject to regulatory control at the substrate level as well as at the level of the enzyme.

The P2 position was chosen for these experiments on the basis of the sensitivity of small peptides to substitutions at this locus [see Takahashi (1990) for review]. The cleavage sites of a variety of synthetic peptides, neuropeptides, and other naturally occurring peptides, and many proteins, have been reported. In general, the activity of calpain against proteins or large peptides is greater than it is against small peptides, an observation consonant with the conclusion that conformational determinants resident in large peptides and proteins markedly enhance the affinity of the substrate for the enzyme. On the basis of peptide studies, it appears that, at the P1 site, the preferred residues are those with bulky or basic side chains, such as Tyr, Lys, or Arg. At the P2 position, Leu and Val are most commonly found, suggesting a preference for residues with bulky aliphatic side chains. At the P3 position, residues with bulky or aromatic side chains are often found. At sites beyond P3 (or P1′), no specificity has been identified (Takahashi, 1990). The P3 to P1′ sequence in native αII spectrin fits these substrate specificities well (Figure 2a). However, many of the P2 mutations (exclusive of Gly, Pro, and Asp) do not and yet are still cleaved readily. Conversely, Pro at the P2 position in small peptides does not limit their calpain sensitivity (Takahashi, 1990) but does in αII spectrin.

Another factor in interpreting these studies may be the choice of calpain used. Each calpain isozyme may display somewhat different substrate specificity (Takahashi, 1990). In our previous work, bovine heart muscle calpain was used, which cleaved αII spectrin between Tyr_{1176} and Gly_{1177} (Harris et al., 1988). Using antibodies generated to the novel epitope created by the cleavage after Tyr_{1176}, we have confirmed that *in vivo* endogenous calpains cleave at an identical site in human, bovine, and rat brains (del Lacoste et al., 1992; also Glantz and Morrow, unpublished observations). In the present experiments, we have used porcine erythrocyte calpain. On the basis of the exclusive reactivity of the cleaved but not the intact peptides with the above epitope specific antibody, we conclude that porcine erythrocyte, bovine cardiac muscle, and endogenous human, bovine, or rat brain calpain all cleave spectrin at the identical site. Thus, while these three enzymes may differ quantitatively in their K_{cat} and K_m's, it is unlikely that differences between these enzymes would change the qualitative conclusions of this study.

The modeling of the structure of the 11th repeat of human αII spectrin relies on the fidelity of the energy minimization algorithms to identify the true minimal energy conformation from an unknown number of false minima. It is for this reason, and to manage an otherwise impractical computational complexity, that the modeling began with the proposed structure of the *Drosophila* spectrin repeat as derived from crystallographic studies (Yan et al., 1993). While there can be no assurance that the models deduced here represent the true minimal energy state, several features of the model and the modeling process lend credibility. When the same procedure was applied to the *Drosophila* sequence, the deduced model fit the published crystal structure well, with a root mean square (RMS) deviation of less than 2.4 Å for all of the 1728 heavy atoms. In addition, the model of the wild-type αII spectrin 11th repeat depicted in Figure 5 is compatible with all expectations of a legitimate protein. All atoms are accounted for without steric interference; hydrophobic side chains are buried; salt bridges are satisfied; and the invariant Trp (at position 16 in the structural repeat) is buried between and presumably stabilizes helices A and B. The model also preserves the overall tertiary and secondary structure of the fly repeat, despite the presence of an inserted nonhomologous sequence within helix C, in accord with other studies of protein superfamilies that indicate a strong conservation of secondary and tertiary structure despite variations in amino acid sequence (Chen & Vierling, 1991; Peterson & Piatigorsky, 1986).

To the extent that the predicted structure is valid, it provides a potentially important first look into the secondary and tertiary changes in spectrin that may relate to its calpain sensitivity. What stands out in this model is the placement of the calpain cleavage site on an exposed loop of peptide adjacent to a CaM binding domain, all without disrupting helix C or the packing of the spectrin repeat motif. This model lends a mechanistic interpretation to previous observations that CaM binding enhances the sensitivity of spectrin to calpain (Harris et al., 1989), as well as to the observation that calpain cleavage *per se* does not lead to the dissociation of the cleavage products (Harris & Morrow, 1990), a result that fits well with the model shown in Figure 5. In future studies, it will be of interest to directly determine the detailed structure of this repeat. We also anticipate that spectrin mutants can now be prepared that retain CaM binding but resist the action of μ-calpain *in vivo*. Such molecules should facilitate studies on the role of the calpains in biology and pathology.

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