Calpain-induced proteolysis of β-spectrins

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Abstract The calcium-activated neutral protease calpain is activated in several pathological conditions. Calpain usually hydrolyses one or only a few peptide bonds in its substrate. One prominent substrate for calpain is spectrin and it has been shown that α-spectrin is the preferred substrate. We now show that the β-chain of spectrin is also a substrate for calpain proteolysis, and that the cleavage site in each β-subunit is located at the very C-terminal part of the molecule. Surprisingly, βIΣ1-spectrin is cleaved at a different site than βIΣ2- and βIIΣ1-spectrins despite their high degree of sequence identity.

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1. Introduction

Injury or lack of oxygen supply in the animal brain leads to a rise in the concentration of the neurotransmitter glutamate [1]. The increased level of glutamate acts on *N*-methyl-D-aspartate receptors, to open an ion channel that allows influx of calcium from the extracellular medium. The elevation of cytoplasmic calcium activates calpain proteases as well as modulating the activities of a wide variety of calcium-binding proteins.

One well-known substrate for the calpain proteases is spectrin [2–4]. The breakdown of spectrin in response to ischaemia has revealed that spectrin proteolysis by calpains closely parallels nerve damage [2,5–8]. Calpain inhibitors administered to animals before induction of ischaemia both inhibit spectrin proteolysis and protect the animals from the spreading of cell death [9,10].

Spectrin is a multifunctional protein, containing several distinct recognition sites for other proteins, such as actin, protein 4.1, ankyrin, adducin, calmodulin and synapsins [11–14]. Moreover, spectrin also contains src [15,16], pleckstrin [17,18] and calponin homology domains [19], indicating a role in signal transduction. Spectrin is a heterodimer, formed by antiparallel association of an ∼280 kDa α-chain and a 245–460 kDa β-chain [12]. Both subunits can be divided into three domains: the N- and C-terminal domains and the repeat domain. The repeat domain consists of several repeats of 106 amino acid residues long, each folded into a triple-helical structure and connected to each other by a short, flexible linker. The α-chain contains 21 repeats whereas the β-chain contains 17 such repeats [12]. The two subunits are the products of several distinct genes; two types of α-spectrin and at least three types of β-spectrin are expressed in humans [12]. Recently, a third β-spectrin gene has been identified, although its expression is unknown. The different isoforms of spectrin show developmentally regulated expression and different subcellular localisation; in neurones and glia αIβIΣ2-spectrin (muscle spectrin) is localised to the cell body and dendrites while αIIβII (brain spectrin) is found in the axon [14,20]. Alternative transcription of the βI-spectrin gene gives rise to αIβIΣ1-spectrin, which is found in the erythrocytes.

The function of spectrin is to link cytoskeletal filament systems, including actin filaments, to transmembrane proteins of the plasma membrane [12]. Spectrin, together with ankyrin, provides a mechanism for restricting the free diffusion of transmembrane proteins in the plane of the membrane. Ion channels, receptors and cell adhesion molecules can thereby be retained at specific points on the cell surface by their interaction with the cytoplasmic structural apparatus. The connection of the plasma membrane to the cytoskeleton also provides a mechanism for generation of cell shape as well as mechanical stability. Therefore, any process that changes the properties of spectrin will also affect the general properties of the cell. This is illustrated by the finding that certain mutations in the spectrin genes are lethal [21,22].

Despite the knowledge of the general basis of membrane-cytoskeleton interaction, the precise connection between disruption of the spectrin-ankyrin system and cell death remains unknown. In part this is due to deficiencies in the knowledge of the biochemistry and molecular biology of this system. Previous work has shown that αII-spectrin but not αI-spectrin is hydrolysed by calpain at a site in repeat 11, giving rise to two nearly equal fragments of ∼150 kDa. In the presence of calmodulin, also βII-spectrin is cleaved by calpain into fragments of 165 and 125 kDa [23]. In this report, we have studied the calpain-induced proteolysis of β-spectrin C-termini, in order to identify the specificity of the proteolytic enzyme. The results show that all three isoforms of β-spectrin are hydrolytically cleaved in the C-terminus by calpain although calpain acts on different peptide bonds.

2. Material and methods

2.1. Isolation of β-spectrin C-terminal gene fragments

The βIΣ1 and βIIΣ1 clones were isolated from a human foetal liver cDNA library (Clontech) by PCR using appropriate primers. The following primers were used: βIIΣ1 forward: GGC GGC CTA **C**CC GGG TGA CAA GG (bp 5923–5945, *Xma*I), βIIΣ1 reverse: GGT AAG AGA AG**C CCG** GGA GGT GAA GG (bp 7414–7439, *Xma*I), βIΣ1 forward: C CAG GAC GTG **GGA TC**C CGT CTG C (bp 5660–5682, *Bam*HI) and βIΣ1 reverse: GC TGC G**GA ATT** CAT CTC GCC TCC (bp 6522–6544, *Eco*RI). (In the primers used, restriction sites for *Xma*I, *Bam*HI or *Eco*RI were introduced. Bold letters indicate introduced mismatches.) Amplified DNA was isolated from agarose gels, digested with *Xma*I, *Bam*HI or *Eco*RI and finally purified by a gene purification system (QIAquick Gel Extraction Kit, Qiagen or GeneClean, Bio 101). Fragments were ligated into the appropriate restriction site of the pGEX-2T vector. These plasmids were then used to transform *Escherichia coli* strain JM109.

Upon purification, using glutathione-Sepharose, it turned out that

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the C-termini of the two β-spectrins were unstable and were degraded fairly quickly. Therefore, the gene fragments were excised by PCR from these plasmids using the following primers: βIIΣ1 forward: GTG CCA TGG CCG ACG ATA TCC (bp 5937–5957, *Nco*I), βIIΣ1 reverse: GGA GAT CTT TTT CTT TTT GCC AAA AAG G (bp 7382–7410, *Bgl*II), βIΣ1 forward: TCT GCA GGG ATC CTA TGC TGG GG (bp 5678–5700, *Bam*HI) and βIΣ1 reverse: TAG GGG TGA GAT CTC CTC TGG CTG C (bp 6482–6505, *Bgl*II). The fragments were purified from agarose gels as before and ligated into pQE-60 expression vector (Qiagen). In this system, a 6×His affinity tag is fused to the C-terminus of the expressed peptide. The 6×His affinity tag allows for simple purification on chelating Sepharose (Amersham Pharmacia Biotech AB) saturated with Ni^{2+}. The produced C-terminal peptides of βIΣ1 and βIIΣ1 include codons 1898–2137 and 1881–2366, respectively.

The plasmid for the C-terminus of βIΣ2-spectrin (GST-βIΣ2) was generously provided by Dr A. Viel and yields a peptide spanning residues 2009–2329 fused to the C-terminus of glutathione *S*-transferase.

2.2. Identification and expression of the recombinant proteins

Positive clones were identified by screening for expressed products with the expected molecular mass. To verify that isolated clones were correct, they were sequenced by the dideoxynucleotide chain termination method using the Cyclist Exo$^-$Pfu DNA sequencing kit (Stratagene) or the ABI Prism Dye Terminator cycle sequencing kit (Perkin-Elmer).

Isolated clones were grown at 23 or 30°C in Luria-Bertani media containing 100 μg/ml ampicillin (or carbenicillin) until the cultures reached mid-log phase ($OD_{600} \approx 0.5$), then protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM. After growth overnight at 23 or 30°C the cell suspensions were harvested by centrifugation. After resuspending the pelleted cells in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8, cells were frozen at −20°C until purification.

2.3. Purification of β-spectrins

His-tagged fusion proteins were isolated from frozen cell suspensions. Thawed and pelleted cells were resuspended in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8, containing 8 M urea, followed by sonication. Triton X-100 was added to a final concentration of 1% and then the lysate was incubated for 1 h on ice before it was centrifuged (48 000×*g* for 10 min). The supernatant was carefully removed and applied to a Ni^{2+}-saturated chelating Sepharose 4B column. After washing the column with 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8, contaminants were eluted with 100 (βIΣ1) or 50 (βIIΣ1) mM imidazole in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8. βIΣ1- and βIIΣ1-spectrin C-termini were eluted with 50 mM EDTA in 25 mM sodium phosphate buffer, 150 mM NaCl, pH 8.

To purify GST-βIΣ2, cells suspended in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8, were thawed and sonicated. After incubation with 1% Triton X-100 for 1 h on ice and centrifugation (27 000×*g* for 10 min), GST-βIΣ2 was purified on glutathione-Sepharose (Amersham Pharmacia Biotech AB). Bound material was eluted with 10 mM glutathione in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8, 1 mM EDTA and 150 μM PMSF.

2.4. Electrophoresis and immunoblotting

Isolated recombinant peptides were analysed by SDS-PAGE [24] and Western blotting using anti-6×His monoclonal antibody (Clontech) or INDIA HisProbe-HRP (Pierce) as well as an anti-βIΣ2-spectrin antibody (generous gift from T. Petrucci).

Calpain-induced proteolysis was analysed by electrophoresis under denaturing conditions using either the Laemmli buffer system or a tricine-based buffer system designed for the separation of low molecular weight proteins [25].

2.5. Calpain cleavage

Recombinant peptides were dialysed in 25 mM sodium phosphate buffer, 150 mM NaCl, pH 8, before proteolysis. 3–10 μM β-spectrin peptides were cleaved by adding calpain I (Calbiochem) to 10 μg/ml and $CaCl_2$ to 0.2 mM. Samples, incubated at 25°C, were withdrawn at indicated times and immediately added to SDS-PAGE cocktail and boiled for 3 min to stop the reaction.

2.6. N-terminal sequencing

After separation on tricine SDS-PAGE, proteolytic fragments were transferred to PVDF membranes by semi-dry blotting. Coomassie blue-stained bands were excised and subjected to N-terminal sequencing to identify hydrolytic products and calpain cleavage sites.

2.7. Modelling of spectrin repeat unit

MultiAlin [26] was used to align amino acid sequences of the β-spectrin isoforms with the repeat sequence of α-spectrin. The structure of the incomplete β17 repeat units of βIΣ1 and βIIΣ1 was modelled on the known structure of repeat unit 16 of α-chicken spectrin (PDB ID: 1AJ3) [27] using the SWISS model [28].

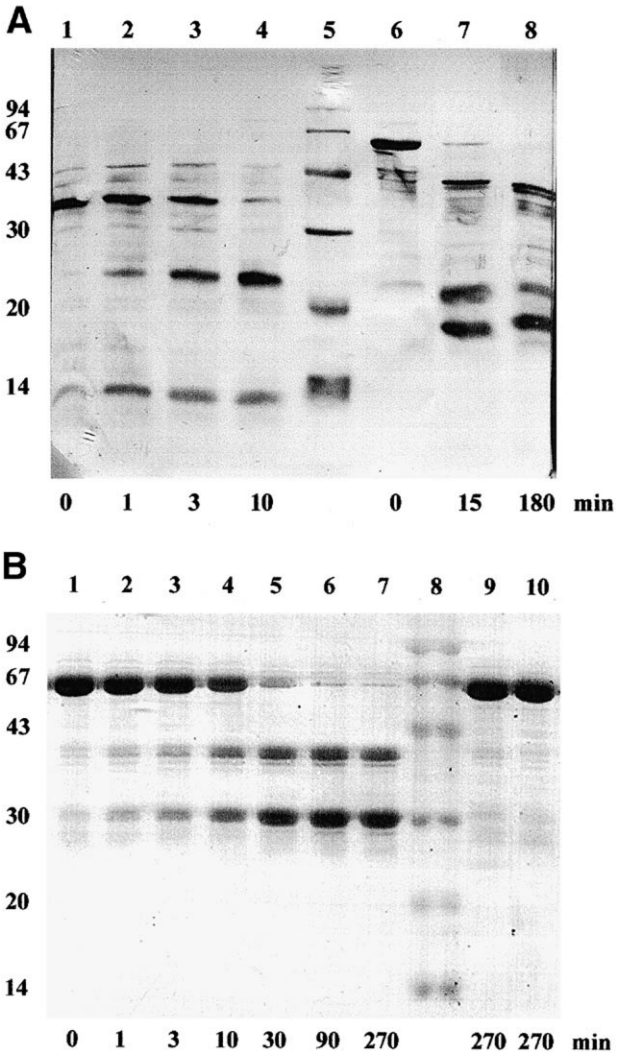

Fig. 1. Calpain-induced degradation of spectrin. A: βIΣ1- (lanes 1–4) and βIIΣ1-spectrin (lanes 6–8) were degraded by calpain (10 μg/ml) at 25°C. Samples were withdrawn after the indicated time, mixed with SDS cocktail, boiled for 3 min and analysed by 16% SDS-PAGE. Lane 1: 0 min; lane 2: 1 min; lane 3: 3 min; lane 4: 10 min; lane 5: molecular weight standards, from top to bottom: 94, 67, 43, 30, 20 and 14 kDa; lane 6: 0 min; lane 7: 10 min; and lane 8: 180 min. B: βIΣ2-spectrin was degraded similarly, and cleavage products were analysed by 13.5% SDS-PAGE. Lane 1: 0 min; lane 2: 1 min; lane 3: 3 min; lane 4: 10 min; lane 5: 30 min; lane 6: 90 min; lane 7: 270 min; lane 8: molecular weight standards (as above); lane 9: 270 min in the absence of calcium; and lane 10: 270 min in the absence of calpain.

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Repeat 15                                                                                            ______________helix C_____________
βIΣ1                                                                                     RL.QTAYAGEKAEAIQNKE..QEVSAAWQALLDACAGRRTQLVD
βIΣ2
βIIΣ1                                                                                                ADDIQKRE..NEVLEAWKSLLDACESRRVRLVD

Repeat 16__________helix A_____________        _____________helix B_____________        ______________helix C_____________
βIΣ1      TADKFRFFSMARDLLSWMESIIRQIETQ.ERPRDVSSVELLMKYHQGINAEIETRSKNFSACLELGESLLQRQHQA..SEEI.R.EKLQQVMSRRKEMNEKWEARWERLRM
βIΣ2
βIIΣ1     TGDKFRFFSMVRDLMLWMEDVIRQIEAQ.EKPRDVSSVELLMNNHQGIKAEIDARNDSFTTCIELGKSLLARKHYA..SEEI.K.EKLLQLTEKRKEMIDKWEDRWEWLRL

Repeat 17__________helix A_____________        _____________helix B_____________
βIΣ1      LLEVCQFSRDASVAEAWLIAQEPYL.ASGDFGHTVDSVEKLIKRHEAFEKSTASWAERFAALEKPTTLELKE
βIΣ2       LEVCQFSRDASVAEAWLIAQEPYL.ASGDFGHTVDSVEKLIKRHEAFEKSTASWAERFAALEKPTTLELKE
βIIΣ1     TLEVHQFSRDASVAEAWLLGQEPYL.SSREIGQSVDEVEKLIKRHEAFEKSAATWDERFSALERLTTLELLE

C-term
βIΣ1      ..RQIAERPAEETGPQEEEGETAGEAPVSHH....AATERTSPVSLWSRLSSSWESLQPEPSHPY
βIΣ2      ..RQIAERPAEETGPQEEEGETAGEAPVSHH....AATERTSP....GEEEGTWPQNLQQPPPPGQHKDGQKSTGDER.PTTEPLFKVLDTPLSEGDEPATLPAPRDHGQ
βIIΣ1     VRRQQEEEERKRRPPSPEPSTKVSEEAESQQQWDTSKGEQVSQNGLPAEQGSPRMAETVDTSEMVNGATEQRTSSKESSPIPSPTSDRKAKTALPAQSAATLPARTQETP
C-term
βIΣ1
βIΣ2      SVQMEGYLGRKHDLEGPNKKASNRSWNNLYCVLRNSELTFYKDAKNLALGMPYHGEEPLALRHAICEIAANYKKKKHVFKLRLSNGSEWLFHGKDEEEMLSWLQGVSTAI
βIIΣ1     SAQMEGFLNRKHEWEAHNKKASSRSWHNVYCVINNQEMGFYKDAKTAASGIPYHSEVPVSLKEAVCEVALDYKKKKHVFKLRLNDGNEYLFQAKDDEEMNTWIQAISSAI
C-term
βIΣ1
βIΣ2      N.........ESQSIRVKAQSLPLPSLS.GPDASLGK......KDKEKRFSFFPKKK
βIIΣ1     SSDKHEVSASTQSTPASSRAQTLPTSVVTITSESSPGKREKDKEKDKEKRFSLFGKKK
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Fig. 2. Alignment of βIΣ1-, βIΣ2- and βIIΣ1-spectrin. The C-termini of the three isoforms of β-spectrin were aligned using MultiAlin according to the suggested phasing of the repeat units of α-spectrin. The three α-helices (A, B and C) are indicated above the sequence. Bold letters indicate calpain cleavage sites.

3. Results and discussion

When the isolated recombinant β-spectrin C-termini were incubated in the presence of calpain and calcium, all three isoforms were cleaved proteolytically, as shown in Fig. 1. The initial 35 kDa βIΣ1-spectrin gave rise to two major fragments, as expected, with molecular sizes of about 12 and 23 kDa. The 60 kDa βIIΣ1-peptide gave rise to a 40 kDa fragment as well as two smaller fragments of 18 and 22 kDa. The reason for the occurrence of two and not a single smaller peptide is unknown. It is possible that there are other proteolytic sites becoming accessible upon the initial calpain cleavage. The muscle β-spectrin was also degraded by calcium-activated calpain, though in this case the initial 65 kDa GST-βIΣ2 was cleaved into two peptides of around 30 and 37 kDa. Thus, in all cases, the sizes of the produced fragments add up to the initial size of the peptide.

The C-terminal products of βIΣ1, βIΣ2 and βIIΣ1 after proteolysis were separated by SDS-PAGE, transferred to PVDF membranes, excised and subjected to N-terminal sequencing. The results indicated that these isoforms were not cleaved at one single site in the C-termini (Fig. 2). Alignment of the sequences, both with the three β-spectrin isoforms and with the putative repeat structure, implies not only that there is a difference in the calpain-dependent proteolytic sites but also that these sites are located to a suggested helical region and not, as expected, to a turn or linker region.

Although the recognition sites of calpain substrates probably involve higher orders of structure some features appear to be common: there is usually a large aliphatic or aromatic side chain at position 1, 2, or 3 at the N-terminal side and a basic or large aliphatic residue at position 1 at the C-terminal side of the cleavage site [29]. The cleavage of βIΣ1 and βIΣ2 seemed to follow this as they were cleaved after tryptophan and threonine, respectively, contrary to βIIΣ1 which was hydrolysed between two alanines.

The difference in cleavage site may depend on slight differences in structure of the isoforms. We have applied molecular

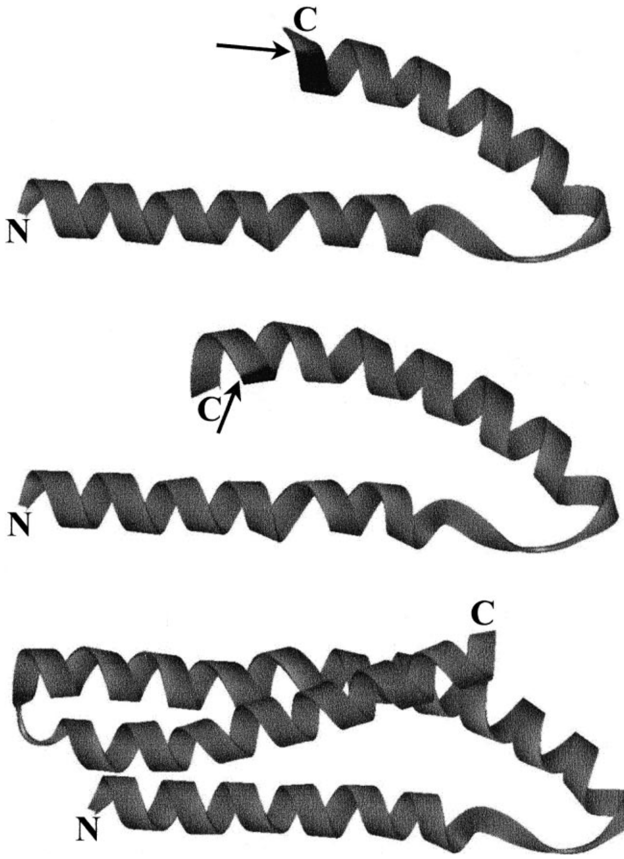

Fig. 3. Models of the incomplete repeat unit 17 of βIΣ1- and βIIΣ1-spectrin. The structures of the incomplete β17 repeat units of βIΣ1 (middle) and βIIΣ1 (upper) were calculated by the SWISS model using the known structure of repeat 16 of chicken brain α-spectrin (lower). Arrows indicate the new C-terminal amino acid residues after cleavage.

modelling [28] on repeat 17 of the β-spectrins to investigate this further, using the known structure of repeat 16 of chicken α-spectrin [27] as a template. Circular dichroism measurements indicated a high helical content [30], implying that the N-termini, containing repeats 16 and 17, are α-helical whereas the C-termini are much less ordered. Secondary structure prediction analysis of the peptides also supported this suggestion. Although repeat 17 probably is mostly α-helical, there is no evidence as to whether this repeat folds independently into an incomplete triple-helical structure or not. However, the modelling indicated, as shown in Fig. 3, that not all residues of the suggested helix B of repeat β17 may fold into a helix, when modelled on α16; in both βIΣ1- and βIIΣ1-spectrins only the N-terminal half of the suggested helix B appeared to fold into a helical structure. Helix B seemed to be incomplete in both cases compared to α16 and it was apparent that helix B of repeat 17 in βIΣ1 was longer than that of βIIΣ1. The models of β17 also indicated that the determined cleavage sites would be accessible to calpain in all three isoforms (Fig. 3) independent of the folding. However, it must be noted that the alignment of the repeat units of β-spectrin is based on the repeat structure of α-spectrin. Therefore it cannot be excluded at this stage that the suggested phasing of the β-repeat unit is incorrect.

Previously, we identified a calpain-like activity strongly associated with erythrocyte spectrin [31] that cleaved βIΣ1-spectrin at a single site. It was not possible to isolate the released peptide, but the size of the remaining β-chain decreased by 10–20 kDa. Since one of the peptides released from the βIΣ1-peptide was around 12 kDa, similar to the size calculated from the determined cleavage site, this might indicate that these proteolytic events are catalysed by the same activity. This peptide is quickly degraded further, which may explain why it was not possible to isolate it in the previous studies [31].

It has been shown that in αIIβII-spectrin, only the αII-chain is cleaved by calpain and that proteolysis of the βII-chain also requires the presence of calmodulin [23]. In contrast, in erythrocyte spectrin only the βI-chain but not the αI-chain is degraded by calpain [31]. It has been suggested [23] that calmodulin and calpain co-ordinately regulate the interaction of αIIβII-spectrin with actin and that as long as the β-chain is intact spectrin is still able to cross-link actin filaments. Since the spectrin isoforms show different subcellular localisation [14,20] and since the calpain site in the α-chain is missing in αIβI-spectrin [32], it is possible that in neurones the effect of calpain-induced proteolysis is different in the axon compared to the cell body and dendrites. Further studies of the different spectrin isoforms and their susceptibility to calpain with the objective of understanding the molecular details of the calpain-induced degradation of spectrin are in progress.

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