Brain fodrin: Substrate for calpain I, an endogenous calcium-activated protease

(spectrin/cytoskeleton/synaptic membranes/plasticity)

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ABSTRACT The calcium-activated thiol-protease calpain I, which is present in cytosolic and membrane preparations from rat brain, was tested for its capacity to degrade the neuronal spectrin-like protein fodrin. In the presence of micromolar calcium concentrations purified calpain I degraded both purified fodrin and the fodrin present in hippocampal and cerebellar membranes. Fodrin was identified as a high molecular weight protein present in brain membranes by the following criteria: (i) comigration on NaDodSO4/polyacrylamide gels with purified fodrin, (ii) reactivity with antibodies to purified fodrin, and (iii) a proteolytic map following calpain activation comparable to that found after calpain-mediated degradation of purified fodrin. The fodrin breakdown was selective in that calpain I did not affect at least 15 other membrane-associated polypeptides. Fodrin degradation by the protease was rapid and was accompanied by the appearance of a lower molecular weight breakdown product. Calpain I had a high affinity for fodrin, with a $K_a$ for degradation of about 50 nM. Purified calpain I also degraded purified spectrin and the spectrin present in erythrocyte membranes. Calpain I-mediated degradation of spectrin-like proteins could provide a mechanism by which brief increases in intracellular free calcium levels modify the structure of the submembranous cytoskeleton and the distribution of cell surface receptors and alter cell shape.

Fodrin is a rod-shaped protein that lines the cortical cytoplasm of neurons (1). In addition to its submembranous localization and elongated shape, fodrin shares a number of properties with spectrin, the principal component of the erythrocyte cytoskeleton: (i) both proteins are comprised of two subunits (a and b) and likely exist in vivo as tetramers ($\alpha\beta$) (2, 3); (ii) both can bind actin and cross-link F-actin into a viscous gel (3-5); (iii) the higher molecular weight subunits of each (Mr =240,000) bind calmodulin (3, 6-8) and give similar peptide maps (9); (iv) antisera raised against fodrin showed some cross-reactivity with spectrin (9, 10); (v) fodrin adheres to the spectrin-binding proteins of erythrocyte membranes (2, 10). Additionally, fodrin (1, 10) and spectrin (11, 12) antibodies recognize proteins from a variety of tissues, suggesting that fodrin-like proteins are widely distributed among different cell types. It has been suggested that fodrin, in a manner similar to erythrocyte spectrin (reviewed in ref. 13), may regulate cell shape and the position and lateral mobility of cell surface proteins (10, 14). However, the precise functions of fodrin and related proteins and the factors that regulate them are largely unknown.

Synaptosomal plasma membrane (SPM) preparations from rat brain contain calcium-stimulated proteolytic activity that may use fodrin as a substrate. Addition of micromolar calcium concentrations to SPMs decreases the content of a high molecular weight doublet protein, an effect which is blocked by thiol-protease inhibitors (15). Two thiol-proteases that differ in their sensitivities to activation by calcium have been extracted from SPMs and partially purified and characterized (16). These enzymes belong to a family of calcium-activated thiol-proteases, the calpains (EC 3.4.22.17), with the high- and low-calcium-sensitivity forms designated as calpain I and II, respectively (17, 18). Because calpain I is endogenous to SPMs and is activated by those calcium levels that decrease the content of the doublet protein, this enzyme may mediate the breakdown of the doublet. Interestingly, in its polypeptide composition and molecular weight, the doublet protein resembles fodrin. Indeed, a doublet of similar molecular weight present in postsynaptic densities has been identified as fodrin (6).

In the present report we provide evidence that fodrin is a substrate for the endogenous calcium-activated thiol-protease calpain I. Because brain fodrin is part of a family of spectrin-like proteins and erythrocytes contain high levels of calpain I (18, 19), we have also tested the effect of calpain activation on erythrocyte spectrin. Our results indicate that calpain-mediated degradation may be a general mechanism for regulating spectrin-like proteins.

MATERIALS AND METHODS

Purification of Fodrin, Calpain I, and Calpastatin. Fodrin was purified from rat brain membranes by the method of Levine and Willard (1). The purified material, as determined by NaDodSO4/polyacrylamide gel electrophoresis, consisted predominantly of fodrin but contained some minor polypeptides. No calpain or other protease activity could be detected in this preparation. Calpain I was purified from rat erythrocyte cytosol according to Murachi et al. (18). Erythrocyte lysate was fractionated on DEAE-cellulose (Whatman) and Ultragel AcA 34 (LKB) as described (16). Active fractions from the latter column were designated as "purified calpain I" and contained no other apparent proteolytic activity and no fodrin-like proteins. Calpastatin, the endogenous inhibitor protein of the calpains, was purified from erythrocytes obtained from outdated human bank blood as described by Takano and Murachi (20). The inhibitor (Mr =70,000) was >90% pure as analyzed by NaDodSO4/polyacrylamide gel electrophoresis.

Fodrin Proteolysis. SPMs were prepared from rat brain as described (15) and were suspended in 50 mM Tris-HCl, pH 7.5/5 mM 2-mercaptoethanol/0.1 mM EGTA at a concentration of 6 mg of protein per ml. To determine the activity of exogenous calpain I against fodrin or SPM polypeptides, 10 $\mu$ of purified fodrin (10 $\mu$g of protein) or SPMs and 35 $\mu$ of purified calpain I (5 $\mu$g of protein) were incubated at 30°C for 10 min. The reaction was started by the addition of 5 $\mu$ of either water or CaCl2 (200 $\mu$M free concentration). After 20 min (Fig. 1) or various times (Fig. 2) the reactions were stopped by dilution into 0.5% sodium dodecyl sulfate and heating. The $\alpha$-fodrin polypeptide was quantitated by NaDodSO4/polyacrylamide gel electrophoresis. The autoradiograms were then scanned with a densitometer and the areas under the peaks were calculated. A similar approach was used to determine the effect of calpain I on a SPM doublet. The autoradiograms were scanned as above and peak areas were calculated. The work presented here is part of a series to be published subsequently. A preliminary account of these results has been presented in abstract form (15).
stopped by the addition of electrophoresis sample buffer (15). Samples were heated to 90°C for 5 min, and aliquots were on 6% slab gels, after which the gels were stained and then destained and the polypeptides were quantified by densitometry (15).

\[^{14}\text{C}]\text{Fodrin was prepared by reductive methylation using}^{14}\text{C}\text{[formaldehyde (50 mCi/ml; 1 Ci = 37 GBq; ICN) (21). To measure}^{14}\text{C}\text{[fodrin degradation, 15 }\mu\text{l of}^{14}\text{C}\text{fodrin (3000–30,000 cpm) and 30 }\mu\text{l of purified calpain 1 were incubated in the presence or absence of 100 }\mu\text{M }\text{CaCl}_2\text{ for 5 min at 30°C. The reaction was stopped by the addition of 50 }\mu\text{l of 50% trichloroacetic acid and 250 }\mu\text{l of bovine serum albumin (2 mg/ml). Samples were kept at 4°C for 1 hr and then centrifuged; radioactivity in the trichloroacetic acid supernatants was counted by liquid scintillation spectrometry. Trichloroacetic acid-soluble radioactivity increased linearly with time over the incubation period. Degradation of}^{14}\text{C}\text{fodrin by calpain 1 was taken as the difference in trichloroacetic acid-soluble radioactivity between reactions run in the presence or absence of calcium, each performed in triplicate.}

Preparation of Erythrocyte Membranes and Spectrin. Erythrocytes were prepared from heparinized rat blood by centrifugation at 1000 × g for 10 min, and the supernatant and buffy coat were discarded. Cells were washed three times with 10 vol of 0.15 M NaCl/2.5 mM 2-mercaptoethanol/0.5 mM EGTA/2 mM Tris-HCl, pH 7.4. To prepare membranes, erythrocytes were lysed for 2 min in 2 vol of 7.5 mM Hepes, pH 7.4/5 mM 2-mercaptoethanol/0.1 mM EGTA and the lysate was centrifuged at 27,000 × g for 20 min. Membranes were washed twice in 20 vol of lysis buffer.

Spectrin was purified from erythrocyte membranes according to Marchesi (22). To determine spectrin degradation by exogenous calpain, erythrocyte membranes (100 }\mu\text{g of protein) or purified spectrin (3 }\mu\text{g of protein) were incubated at 30°C for 20 min with or without calcium and were processed as described above for brain fodrin.

Immunochemical Methods. Antibodies to electrophoretically homogeneous fodrin were raised in rabbits by modification of the method of Levine and Willard (1). Briefly, purified fodrin was run on 6% slab gels, and the fodrin polypeptides were excised, homogenized, mixed with adjuvant, and injected at multiple subdermal sites. Booster injections were given 2 and 4 wk after the initial injection, and serum was obtained 10 days after the second booster injection. The presence of antibodies to fodrin was verified by immunoprecipitation of fodrin–antibody complexes, followed by analytical NaDodSO₄/ polyacrylamide gel electrophoresis. Antifodrin was purified from immune serum by affinity chromatography using a column of fodrin coupled to Sepharose 4B (1).

Affinity-purified antifodrin was radioiodinated by the lactoperoxidase method (23), employing 0.5 mg of antifodrin and 1 mCi of Na[125]I (New England Nuclear). Labeled antifodrin was separated from free Na[125]I on a Sephadex G-25 (Pharmacia) column, followed by dialysis against 0.15 M sodium chloride/10 mM sodium phosphate, pH 7.4/0.1% sodium azide and was stored at −70°C prior to use.

The binding of [125]I-labeled antifodrin (125I-antifodrin) to electrophoretically separable polypeptides was determined by the gel overlay method described by Burridge (24). Antifodrin-binding polypeptides were located by staining gels with Coomassie brilliant blue R, followed by slicing each lane into 4-mm-thick pieces (30 pieces per lane) and assaying each piece in a gamma counter (Beckman).

RESULTS

The first experiment tested the possibility that the doublet protein (Mr ~240,000) present in SPM preparations is fodrin.

**Fig. 1.** Degradation of purified and membrane-bound fodrin by purified calpain 1. (A) Calpain 1, purified from rat erythrocyte cytosol, was incubated with purified brain fodrin (lanes 2 and 3) or SPMs prepared from hippocampus (lanes 4–8) or cerebellum (lanes 9–12). Reaction mixtures were run on 6% NaDodSO₄/polyacrylamide slab gels, and the polypeptides were visualized and quantified. Lane 1, purified fodrin; lane 2, purified fodrin with calpain; lane 3, same as lane 2, with 200 }\mu\text{M }\text{CaCl}_2\text{; lanes 4 and 9, SPMs; lanes 5 and 10, SPMs with 200 }\mu\text{M }\text{CaCl}_2\text{; lane 6, SPMs with calpain; lanes 7 and 11, SPMs with calpain and 200 }\mu\text{M }\text{CaCl}_2\text{; lanes 8 and 12, same as lanes 7 and 11, with 100 }\mu\text{M leupeptin. The upper arrow indicates the position of fodrin, whereas the lower arrow points to the fodrin breakdown product (BDP). Molecular weights of standard proteins are indicated as }\text{Mr} \times 10^{-3}.\text{ (B) Identification of }125\text{I-antifodrin-binding polypeptides. Purified fodrin (top) or hippocampal SPMs (bottom) were incubated with purified calpain 1 in the absence (o) or presence (c) of 200 }\mu\text{M calcium and were run on a 6% slab gel. The gel was overlaid with }125\text{I-antifodrin, washed, stained, sliced, and assayed for radioactivity. Molecular weights of standard proteins are indicated as }\text{Mr} \times 10^{-3}\text{ at the arrows. Note the recognition of the }\text{Mr} \sim 240,000\text{ doublet in SPMs by }125\text{I-antifodrin and the similarities between the calpain-mediated degradation of purified and SPM-associated fodrin.}

As shown in Fig. 1A, two closely spaced polypeptides in hippocampal and cerebellar SPMs comigrated on NaDodSO₄/ polyacrylamide gels with purified fodrin. *The }\text{Mr} \sim 240,000\text{ doublet has been shown to be a substrate for a calcium-activated protease endogenous to hippocampal SPMs (15). To further study the identity of the doublet protein, purified fo-

*In cerebellar membranes fodrin migrated as a triplet. The fastest migrating of the three bands likely is similar to β-spectrin and has been observed in cerebellar membranes (25).
radioiodinated, binding method were for the other molecule. Both the AN and AM were produced 15% decreasing by (26). BDP produced 15% of fodrin. The breakdown of was prevented without sulfonyl dogenous purified. We next examined whether fodrin could serve as a substrate for the calcium-activated thiol-protease calpain I. Rat erythrocytes were selected as a source of calpain I because of their availability, high cytosolic enzyme content, and lack of other calcium-activated proteases (18, 19). Incubation of purified fodrin in the presence of purified calpain I and 200 μM CaCl₂ caused an 81% decrease in fodrin content (Fig. 1A, lanes 2 and 3). Both the α and β subunits of the protein were degraded. In the absence of either calpain I or CaCl₂, fodrin levels were not affected. In addition to causing a loss of the fodrin polypeptides, calcium induced the appearance of a lower molecular weight (Mₐ = 150,000) breakdown product (BDP, lane 3). The BDP cross-reacted with antibodies to fodrin (Fig. 1B, top, ○), indicating that it is derived from the fodrin molecule. Both the fodrin degradation and the appearance of the BDP were completely prevented by 100 μM leupeptin, a thiol-protease inhibitor (not shown). Similar results were obtained by using calpain I purified from rat brain cytosol (not shown).

In addition to its capacity to degrade fodrin in solution, calpain I also acts on endogenous fodrin in situ (Fig. 1). Incubation of hippocampal (lane 7) and cerebellar (lane 11) SPMs with erythrocyte calpain I and 200 μM CaCl₂ induced 80% and 66% decreases in fodrin content, respectively. In the absence of added calpain, calcium produced smaller effects on fodrin in hippocampal SPMs (lanes 4 and 5; 78% of control; four experiments) and caused no detectable degradation of fodrin from cerebellar SPMs (lanes 9 and 10; 98% of control; three experiments). Activation of purified calpain I also reduced 125I-antifodrin binding to the fodrin present in hippocampal SPMs (Fig. 1B, bottom, ○). Furthermore, the decrease was accompanied by the appearance of a lower molecular weight 125I-antifodrin-binding polypeptide (bottom, ○). In this BDP of SPM-associated fodrin conjugated with the BDP produced by calpain-mediated proteolysis of purified fodrin (top, ○).

Added calpain I degraded SPM polypeptides other than fodrin. In particular, the polypeptides migrating like the high molecular weight microtubule-associated proteins (more slowly migrating than fodrin; barely visible in Fig. 1) were decreased following calpain I activation. However, at least 15 other SPM-associated polypeptides were largely unaffected by calpain I activation.

The calcium-induced fodrin breakdown was a specific consequence of calpain I activation. The thiol-protease inhibitors (26) leupeptin (Fig. 1A, lanes 8 and 12) and antipain prevented the degradation of SPM-bound fodrin by purified calpain I, whereas the serine protease blocker phenylmethylsulfonyl fluoride and the pepsin inhibitor pepstatin A were without effect (Table 1). Furthermore, calpastatin, the endogenous inhibitor protein of the calpains (20, 27, 28), completely prevented fodrin degradation (Table 1). Calpastatin has previously been found to be a highly selective calpain blocker, as it has no influence on any of several other thiol or serine proteases (28). Thus, the inhibition profile of calcium-induced fodrin degradation is identical with that of the inhibition of purified calpain I (27).

The time course of the breakdown of purified fodrin by purified calpain I is shown in Fig. 2. Fodrin degradation by calpain I was rapid, with a 47% loss of the α and β subunits (higher and lower molecular weights, respectively) occurring in 1 min. Breakdown of β-fodrin was complete by 5 min, whereas α-fodrin levels declined at a slow rate for >30 min.

Table 1. Effects of protease inhibitors on calcium-induced fodrin degradation

<table>
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<th>Additions</th>
<th>Inhibition of calpain I activity</th>
<th>% inhibition of fodrin degradation</th>
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<tr>
<td>Leupeptin 3 μM</td>
<td>Yes</td>
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<tr>
<td>30 μM</td>
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<td>Antipain, 50 μM</td>
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<td>PhMeSO₄F, 1 mM</td>
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<td>Pepstatin A, 0.5 mM</td>
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Rat hippocampal SPMs were incubated at 30°C for 10 min with the indicated agents and then were treated with purified calpain I (3 μg of protein) and either 1 mM EGTA or 200 μM calcium. After 20 min, reactions were stopped and fodrin levels were analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. The percent to which each agent inhibited fodrin degradation was determined by comparing degradation with the agent present with the degradation in the absence of agent (71% loss of fodrin). The data are the means from two separate experiments, which differed by <15%. PhMeSO₄F, phenylmethylsulfonyl fluoride.

*From ref. 27.

The time course of the appearance of the BDP was somewhat different. Whereas nearly 50% of the fodrin was lost by 1 min, at this time there was little elevation in the content of the BDP polypeptide. The BDP content was 240% of control level at 5 min and rose to 600% of control level at 30 min. The delayed appearance of the BDP relative to fodrin disappearance is consistent with the BDP being a fodrin BDP and suggests that fodrin degradation by calpain I is a multistep process.

Varying amounts of fodrin were incubated with a fixed amount of calpain I to determine the affinity of the protease for fodrin (Fig. 3). In the first experiment we used [14C]fodrin and measured trichloroacetic acid-soluble radioactivity following calpain activation. [14C]Fodrin was incubated with calpain I for 5 min, a duration over which trichloroacetic acid-soluble radioactivity increased linearly with time. [14C]Fodrin degradation saturated at fodrin concentrations of 100 nM or higher. As determined by a Lineweaver–Burk

![Fig. 2. Time course of degradation of purified fodrin by purified calpain I. Purified fodrin was incubated at 30°C with purified calpain I for the indicated times. Reactions were stopped and polypeptides were separated and quantified by NaDodSO₄/polyacrylamide gel electrophoresis. Fodrin and BDP levels after treatment with calpain I and 200 μM CaCl₂ were taken as percentages of their respective levels following treatment with calpain I in the absence of CaCl₂. Each point is the mean of three determinations. This experiment has been repeated with essentially the same result. Fodrin and BDP levels were not altered by incubations with calpain I in the absence of calcium.](image-url)
plot, the $K_m$ of calpain I for fodrin was about 45 nM. In the second experiment fodrin degradation was determined at different fodrin concentrations by using NaDodSO4/polyacrylamide gel electrophoresis. The $K_m$ of calpain I for fodrin obtained by this method was about 53 nM (not shown), in good agreement with the value determined by using $[^{14}C]$fodrin.

Because brain fodrin is part of a family of spectrin-like proteins, we tested whether another member of the family, erythrocyte spectrin, could also serve as a substrate for calpain I. As shown in Fig. 4, calpain I degraded both purified spectrin and the spectrin present in erythrocyte ghost membranes. The levels of the $\alpha$ and $\beta$ subunits of spectrin declined 45% and 61%, respectively, following incubation in calpain I and 100 $\mu$M calcium (lanes 1 and 3). The breakdown was blocked by 100 $\mu$M leupeptin (lane 4) and was not observed when calpain I was omitted (lane 2). Similarly, calpain I activation caused a 49% loss of both spectrin subunits from erythrocyte ghost membranes (lanes 5 and 7), an effect that was also leupeptin sensitive (lane 8) and calpain I dependent (lane 6). This loss represents spectrin degradation rather than solubilization since spectrin levels were measured in the membranes with incubation medium. In addition to its action on spectrin, calpain I degraded band 3, the predominant integral membrane protein (13), whereas actin (band 5) was relatively resistant to the protease.

**DISCUSSION**

In this report we show that brain fodrin, both in purified form and in its native state in SPMs, is a substrate for the calcium-activated thiol-protease calpain I. The $M_i \approx 240,000$ doublet present in SPMs has been identified as fodrin. This doublet comigrates with purified fodrin on polyacrylamide gels, binds antibodies raised against purified fodrin, and has a calpain-produced peptide map identical with that of purified fodrin. The conclusion that fodrin is a substrate for calpain I is based on the following evidence: (i) the levels of both subunits of purified fodrin are decreased by incubation with purified calpain I and CaCl$_2$; (ii) no decrease occurs when either calpain I or CaCl$_2$ is omitted; (iii) fodrin content of hippocampal and cerebellar SPMs decreases following incubation with calpain I and CaCl$_2$; (iv) no decrease occurs in the presence of either leupeptin or antipain, specific thiol-protease inhibitors (26), or in the presence of the endogenous calpain inhibitor calpastatin (20, 27, 28); (v) calpain-mediated fodrin degradation is saturable and of high affinity, with a $K_m$ of about 50 nM. This mechanism is not limited to brain fodrin, for calpain activation also leads to degradation of erythrocyte spectrin (Fig. 4). In view of the widespread distributions of both spectrin-like proteins (11, 12) and calpain I (18) in various cell types, proteolytic degradation could provide a general mechanism for the regulation of the functions of spectrin-like proteins.

Calpain I-induced degradation of fodrin occurs in isolated synaptic membranes in the presence of micromolar concentrations of calcium. We have previously reported that a calcium-activated protease present in SPM preparations degrades a high molecular weight doublet protein (15). Studies of calcium-dependent proteases endogenous to SPMs indicate that these membranes contain a thiol-protease, calpain I, that is activated by those calcium levels that decrease the doublet protein (16). This substrate protein has been identified as fodrin (Fig. 1). Thus, in SPMs fodrin is an endogenous substrate for the endogenous protease calpain I. Importantly, proteolytic degradation of fodrin occurs rapidly (Fig. 2), indicating that intraneuronal free calcium levels, which at steady state are below the threshold for calpain I activation (30), need rise for only a brief period to activate fodrin degradation.

In neurons both fodrin (1) and calpain I (16) are part of the submembranous cytoskeleton. Because the cytoskeleton is considered to control cell shape and the disposition of cell surface proteins, calpain-mediated degradation of fodrin could play a role in these phenomena. Indeed, the related protein spectrin serves such a function in erythrocytes. Extraction of spectrin from erythrocyte ghosts leads to an enhanced lateral mobility of cell surface proteins (31) and dissolution of the ghosts into smaller vesicles (13). Calpain activation could provide a means by which physiological stimuli produce similar shape or cell surface changes in neurons. Additionally, fodrin is a major actin- (3, 5) and calmodulin- (3, 6–8) binding protein, and all three proteins are present in postsynaptic density preparations (6, 32, 33). Proteolytic regulation of fodrin levels could alter actin and calmodulin interactions with postsynaptic membranes. Actin has been suggested to be a key element in controlling the shape of dendritic spines (34). In this regard, erythrocytes, a relatively homogeneous population of cells with a characteristic shape, could be a useful system with which to investigate the roles of calpain, fodrin-like proteins, actin, and calmodulin in regulating cell shape.

When considering potential physiological roles for the cal-
pain-induced degradation of fodrin it is important to note that low levels of calcium have been shown to increase the binding of L-glutamate to neuronal membranes prepared from hippocampus and cerebral cortex (35). This effect, which appears to be due to an increase in the number rather than a change in the affinity of glutamate-binding sites, occurs rapidly and cannot be reversed by removal of the added calcium (35). Moreover, the effect of calcium is virtually abolished by leupeptin in concentrations that block calpain activity (15). These findings indicate that transient activation of endogenous calpain uncovers previously masked glutamate-binding sites. Since there is considerable evidence indicating that these binding sites form part of a postsynaptic neurotransmitter receptor (36), it is possible that the calpain-mediated fodrin breakdown produces a lasting modification of synaptic transmission in certain regions of the brain.

The capacity of added calpain I to degrade cerebellar and hippocampal fodrin (Fig. 1) indicates that fodrin can serve as a calpain substrate in these brain regions. However, we have found here (Fig. 1) and elsewhere (15) that calcium additions lower fodrin levels in hippocampal but not cerebellar SPMs. This raises the possibility that in some brain regions, such as cerebellum, fodrin levels are deficient in calpain I. It cannot be excluded, however, that the enzyme is present in cerebellar membranes but lacks access to the fodrin.

In sum, the cytoskeletal protein fodrin is a substrate for calpain I, a calcium-activated thiol-protease endogenous to SPMs. This mechanism might provide a means by which brief fluctuations in intracellular calcium levels result in long-lasting modification of cell shape or cell-surface receptor distribution.

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