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Mapping the cysteine residues and actin-binding regions of villin by using antisera to the amino and carboxyl termini of the molecule

(cytoskeleton/intestine brush border/peptide mapping/amino acid sequencing/peptide antibody)

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ABSTRACT **Peptide antisera specific for either the amino- or carboxyl-terminal regions of villin were used to locate the position of cysteine residues in immunoblots of villin cleaved with 2-nitro-5-thiocyanobenzoic acid. Maps constructed from the cleavage pattern suggest that villin contains six cysteine residues, two located in its amino-terminal peptide of M_r 44,000, and four located in the carboxyl-terminal peptide of M_r 51,000. Gel overlays of the partial cleavage fragments with ^{125}I-labeled actin identified a calcium-dependent actin-binding region located within the amino-terminal peptide of M_r 32,000 of villin. The peptide antibody method used, called cleavage mapping, should be a convenient technique for mapping residues and ligand binding sites in proteins.**

Villin, a M_r 95,000 actin-binding protein of the intestine microvillus cytoskeleton, is unusual because it possesses two types of actin-binding activities. In calcium concentrations >1 μM villin severs actin filaments into short lengths while in calcium concentrations <1 μM villin crosslinks actin filaments into bundles (1–4). Understanding the mechanism of filament severing and bundling requires identification of the villin-, actin-, and calcium-binding sites.

Villin is cleaved by V8 protease into two fragments, villin core (M_r 87,000) and villin headpiece (M_r 8500). Villin core and headpiece retain the amino and carboxyl termini of villin, respectively. Glenney and co-workers (5, 6) have shown that a calcium-dependent actin-filament-severing activity is located in villin core. The villin headpiece exhibited calcium-independent actin-binding but not -bundling or -severing activity. Villin contains three calcium-binding sites, one of which is nonexchangeable. One of the exchangeable calcium-binding sites is located in villin core, while the other exchangeable site is located in villin headpiece (7, 8). We have extended these studies and shown that trypsin produces a M_r 44,000 amino-terminal fragment of villin (named 44T), which retains the calcium-dependent actin-severing activity (9). The calcium dependence of the binding activity suggests that the exchangeable calcium-binding site in villin core is located within the amino-terminal peptide of M_r 44,000. Although our studies locate an actin- and calcium-binding region within the amino-terminal half of villin, much smaller binding fragments are required to refine further the regulation of actin binding by calcium.

To map such binding regions easily and accurately, we have devised a peptide antibody method for localizing certain amino acids and fragments within the molecule. In our approach we have used polyclonal antisera, which recognize either the NH_2-terminal or COOH-terminal regions of villin, to locate the positions of the cysteine residues in villin. This technique, which we call cleavage mapping, is generally useful for identifying the positions of certain other amino acids, chemical modification sites, ligand binding sites, and chemical or proteolytic fragments. The map of the cysteine residues has enabled us to identify a calcium-regulated actin-binding domain within an amino-terminal villin peptide of M_r 32,000.

MATERIALS AND METHODS

Proteins. Villin was purified from chicken intestine epithelia using the procedure described by Bretscher & Weber (1). The villin proteolytic fragments (villin core, 51T, and 44T) were purified as described (9). Actin was purified from rabbit skeletal muscle by using the procedure of Taylor and Weeds (10). 2-Nitro-5-thiocyanobenzoic acid (NTCB) was obtained from Sigma.

Further treatment of the 44T fragment of villin with trypsin produces a M_r 14,000 peptide, named 44T-14T. Digestion at longer times produces a M_r 13,000 peptide, named 44T-13T. Both peptides were purified by CM-52 and Affigel Blue column chromatography (to be reported elsewhere), and the first 20 residues of each fragment were sequenced by Edman degradation using a modified Beckman 890B automated sequencer (11).

Preparation of Antigen. The peptide Val-Glu-Leu-Ser-Lys-Lys-Val-Thr-Gly-Lys-Thr-Thr-Tyr-Gly was assembled and cleaved as described by Atherton *et al.* (12). The synthetic peptide was coupled to keyhole limpet hemocyanin using bisdiazotized *o*-tolidine, and excess reagents were removed by dialysis against phosphate-buffered saline (13).

Peptide Antisera Production. Antisera were raised in rabbits by injecting 1 mg of the peptide-hemocyanin conjugate in complete Freund's adjuvant at multiple sites on the back. This was followed by injections at 2-week intervals in incomplete Freund's adjuvant. The rabbits were exsanguinated at week 10. The titers of the antisera were quantified by ELISA using either the synthetic peptide or undenatured villin adsorbed to microtiter plates. Swine anti-rabbit IgG coupled to peroxidase (Dakopatts) was used as the secondary antibody. The plates were developed with diaminobenzidine, and absorbance was monitored at 490 nm. The specificity of the antisera (R211.3) was determined from immunoblots (14) against villin proteolytic fragments. Antisera (R200.2) against the villin headpiece peptide were described (9).

Quantitation of Cysteine Residues. Villin or its proteolytic fragments was reduced and the cysteine residues were carboxymethylated with iodo[2-^{14}C]acetic acid or iodo[1-^{14}C]acetamide (Amersham) (15). Incorporation of radioactivity was quantitated by liquid scintillation counting. The number of cysteine residues was calculated from the specific activity of the radiolabeled villin.

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Abbreviations: NTCB, 2-nitro-5-thiocyanobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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Cysteine content was also measured by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) titration (16) in either $NaDodSO_4$ or 6 M guanidine·HCl.

NTCB Cleavage. Villin was reduced and denatured by dialysis into 6 M guanidine·HCl/10 mM dithiothreitol/0.1 mM EDTA at 37°C. After 6–12 hr, the sample was rapidly desalted against 6 M guanidine·HCl/0.2 M Tris·HCl (pH 8.0)/0.1 mM dithiothreitol/0.1 mM EDTA. The protein concentration was determined by absorbance at 280 nm using an absorptivity of 1.28 liters/g·cm (7). The reduced and denatured villin was cleaved with NTCB (17, 18). NTCB was dissolved in 50 mM sodium phosphate, pH 7.6, mixed with villin at a 5-fold excess over total thiols, and incubated in the dark at 37°C. The reaction was quenched after different times by adding acetic acid to 50% (vol/vol) and the cleaved products were dialyzed against 10% acetic acid (vol/vol) in M_r 3500 cutoff dialysis tubing. The cleavage products were lyophilized and stored at −20°C. As control experiments, villin was either incubated in the absence of NTCB under reduced and denaturing conditions or reduced, denatured, carboxymethylated, and then incubated with NTCB. The cleavage fragments of villin were electrophoresed in $NaDodSO_4$ on 10% or 10–22.5% polyacrylamide microslab gels (19).

Cleavage Mapping. The NTCB cleavage fragments of villin were electrophoresed on 10% $NaDodSO_4$/polyacrylamide microslab gels and were immunoblotted with rabbit polyclonal antisera specific for the amino terminus (R211.3) or the headpiece region (R200.2) at a dilution of 1:200 or 1:2000, respectively. The filters were incubated at a 1:400 dilution with swine anti-rabbit IgG coupled to peroxidase and then were developed with 4-chloro-1-naphthol.

The molecular weights of the peptides were calculated from their electrophoretic mobility on $NaDodSO_4$/polyacrylamide gels using markers with M_r 95,000, 77,000, 66,300, 45,000, 25,700, and 17,200.

^{125}I-Labeled Actin Gel Overlay. NTCB fragments which bound actin were identified using ^{125}I-labeled actin (20). The fragments and villin proteolytic markers were electrophoresed on 10% microslab gels. The gels were incubated with ^{125}I-labeled actin in buffer containing either 1 mM EGTA or 0.2 mM $CaCl_2$ and then processed for autoradiography.

RESULTS

The rationale we followed for mapping cleavage sites is illustrated in Fig. 1 for a hypothetical protein M_r 100,000 that contains two cleavage sites (Fig. 1*a*). Limited or partial cleavage at these sites (Fig. 1*b*) should produce five fragments in addition to the intact protein. Two fragments should retain the same amino terminus as the intact protein, while two other fragments should retain the original carboxyl terminus. The polypeptides can be separated on $NaDodSO_4$/polyacrylamide gels (Fig. 1*c*, lane 1). Immunoblots with antisera specific to the amino (lane 2) or carboxyl terminus (lane 3) should reveal the fragments that retain either terminus of the protein. The molecular weight of each detected fragment then maps the position of the cleavage site from the end of the protein identified by the antisera. Mapping from both ends should produce the same "cleavage map" of the protein.

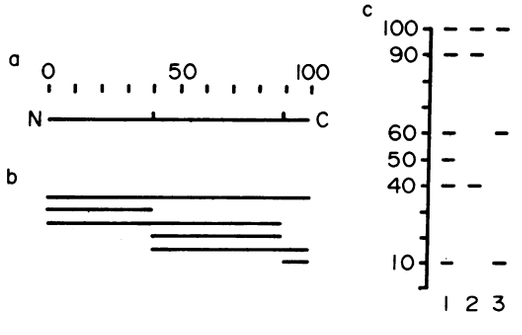

FIG. 1. (*a*) Mapping the positions of two cleavage sites in a hypothetical M_r 100,000 protein requires the generation of partial cleavage fragments (*b*) and methods for sizing (lane 1) and detecting peptides which retain the amino (lane 2) or carboxyl terminus (lane 3) of the native protein (*c*). The molecular weight of the detected peptide identifies the position of the cleavage site relative to that of the intact end.

Characterization of Amino-Terminal Specific Antiserum. We synthesized the peptide Val-Glu-Leu-Ser-Lys-Lys-Val-Thr-Gly-Lys-Thr-Thr-Tyr-Gly. This peptide represents the amino-terminal 12 residues of villin (9) followed by Tyr-Gly. The synthetic peptide was crosslinked through its tyrosine residue to hemocyanin and the mixture was injected into rabbits. The antiserum recognized undenatured villin and the synthetic peptide (Fig. 2*a*).

The specificity of the antiserum to the amino terminus was tested by immunoblots containing villin proteolytic fragments whose locations in the protein have been characterized by protein sequencing (Fig. 2*b*, lane 1). The antiserum (lane 2) recognized villin, villin core, and 44T. The amino termini of these fragments have been confirmed by sequence analysis, and all contain the villin amino terminus. As expected, this antiserum did not recognize the 51T fragment, which comes from the carboxyl-terminal end of the protein. Our characterized villin headpiece antiserum (9) recognized villin and 51T but not villin core or 44T (lane 3).

We isolated two small tryptic fragments of 44T (M_r 14,000 and 13,000) and sequenced their amino termini. The larger fragment, 44T-14T, retained the original amino terminus of villin while the smaller fragment, 44T-13T, started at the lysine residue at position 6. Using these fragments the specificity of the amino-terminal antisera was checked on immunoblots (Fig. 2*c*, lanes 4–7), and it was found that the antiserum recognized only the fragment with the intact amino terminus. Loss of the amino-terminal five residues eliminated all detectable antibody binding.

Quantitation of Cysteine Residues. Because villin has not been completely sequenced, we did not know the number of cysteine residues in the protein. Furthermore, cysteine content cannot be reliably quantified from amino acid analysis. Therefore, we measured cysteine content with two methods, carboxymethylation and the DTNB reaction. Our measurements (Table 1) indicate one or two cysteine residues in the amino-terminal peptide of M_r 44,000, two or three in the carboxyl-terminal peptide of M_r 51,000, and three or four residues in the intact protein. However, since cysteine residues are easily oxidized, our measurements of cysteine content represent only a minimum estimate.

Partial NTCB Cleavage. Villin was cleaved by NTCB (Fig. 3) under denaturing conditions. A time course of cleavage shows the major cleavage fragments are produced by 12 hr (lane 3). At longer times (lanes 4–6) smaller fragments appear. The number of fragments (15–20) resolved on gels is consistent with random cleavage at five or six cysteine residues.

To ensure cleavage is specific at cysteine and not at other residues, two types of controls were performed. First, reduced and denatured villin was incubated in the absence of NTCB (lane 1). This control tests whether the buffer cleaves villin. Second, villin was carboxymethylated at its cysteine residues before incubation with NTCB (lane 7). Under appropriate conditions only cysteine residues are carboxymethylated by iodoacetic acid or iodoacetamide, and the modified cysteine cannot be cleaved with NTCB. Thus, if NTCB cleaves carboxymethylated villin, then cleavage must

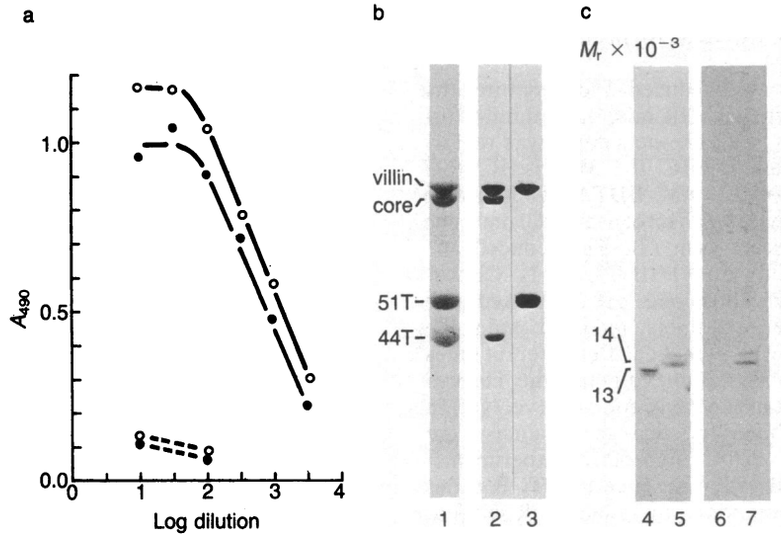

FIG. 2. (*a*) Amino acid sequence (single-letter code) of the peptide that was synthesized and coupled to hemocyanin has the first 12 residues of the amino-terminal sequence of villin plus Tyr and Gly. (*b*) The relative amount of the preimmune (dashed line) or immune (solid line) antisera bound to either native villin (open circles) or synthetic peptide (closed circles) was detected by ELISA. (*c*) Immunoblots of villin and villin proteolytic products (villin core, 51T and 44T). Nitrocellulose was stained with amido black (lane 1), amino-terminal antisera (lane 2), and carboxyl-terminal antisera (lane 3). (*d*) Immunoblots of the 44T proteolytic fragments of M_r 14,000 and 13,000 transferred to nitrocellulose and stained with amido black (lanes 4 and 5) or amino-terminal antisera (lanes 6 and 7).

occur at residues other than cysteine. The control experiments showed little cleavage occurred under conditions which had produced the NTCB cleaved fragments.

Cysteine Cleavage Map. The NTCB cleavage fragments of villin (Fig. 4, lane 1) were immunoblotted with antisera specific for the amino terminus (lane 2) or carboxyl terminus (lane 3) of villin. Five peptides including villin were detected with the amino-terminal antisera while seven peptides were detected with the carboxyl-terminal antisera. When the cleavage sites were plotted on a linear scale (Fig. 4), three sites detected by the amino-terminal antiserum coincided with the positions of the sites detected by the carboxyl-terminal antiserum. One site, identified by an amino-terminal M_r 66,400 peptide, was resolved into two sites by carboxyl-terminal peptides of M_r 28,500 and 29,000. Regions where the cleavage sites were difficult to map lie at the ends of the protein. In one case, the site nearest the amino terminus was identified by immunoblots from 10% gels with the carboxyl-terminal- but not the amino-terminal-specific antiserum. However, this cleavage site was detected by the amino-terminal-specific antiserum on immunoblots from higher percentage polyacrylamide gels (unpublished data) of NTCB-cleaved villin. The cleavage sites mapped nearest to the carboxyl terminus by the antisera differ in position by a peptide of M_r 3000. The difference may represent anomalous mobility of the smaller peptide.

Table 1. Measurement of cysteine content by DTNB titration and carboxymethylation

Method	Polypeptide	SH/polypeptide, mol/mol
DTNB	Villin	3.9
	44T	0.63, 1.13
	51T	2.5
$I^{14}CH_2COOH$	Villin	3.1
	44T	1.1, 0.84
	51T	2.84
$ICH_2{}^{14}CONH_2$	Villin	2.6
	44T	1.9

Denatured villin, 44T, or 51T was reduced and treated with DTNB, iodoacetic acid, or iodoacetamide.

Mapping Actin-Binding Sites. The NTCB cleavage fragments were overlaid with ^{125}I-labeled actin (Fig. 5) in the presence of EGTA (lanes 1–3) or calcium (lanes 4–6). The radiolabeled actin recognized a set of NTCB-cleaved fragments, as well as previously characterized proteolytic fragments, only when incubated in buffers containing calcium (lanes D–F). The smallest fragment that bound actin was M_r 32,000 (lane F). The number and sizes of the actin-detected fragments was similar to the pattern obtained with immunoblots using the amino-terminal-specific antisera and suggests that a calcium-dependent actin-binding region is located within the amino-terminal peptide of M_r 32,000 of villin. However, within this region lies a cysteine residue located in an amino-terminal peptide of M_r 10,000. We were

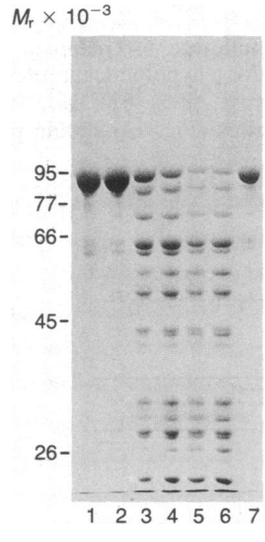

FIG. 3. $NaDodSO_4$/polyacrylamide gels of NTCB-cleaved villin. Reduced and denatured villin was incubated with NTCB and quenched with acetic acid after 0, 25, 15, 24, 36, and 48 hr (lanes 2–6). In control experiments either villin was reduced, denatured, and incubated without NTCB (lane 1) or carboxymethylated villin was incubated with NTCB for 24 hr (lane 7).

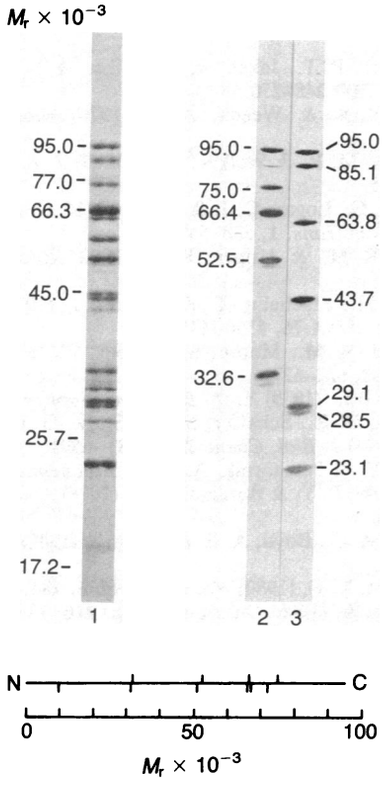

FIG. 4. The partial cleavage fragments of villin were separated on 10% NaDodSO$_4$/polyacrylamide gels (lane 1) and immunoblotted with antisera specific for the amino (lane 2) or carboxyl (lane 3) termini of villin. The positions of the cleavage sites are plotted on a linear scale for the amino-terminal (tick marks above line) and carboxyl-terminal (tick marks below line) antisera. Since NTCB cleaves specifically at cysteine residues, the cleavage map identifies the positions of these residues in the protein.

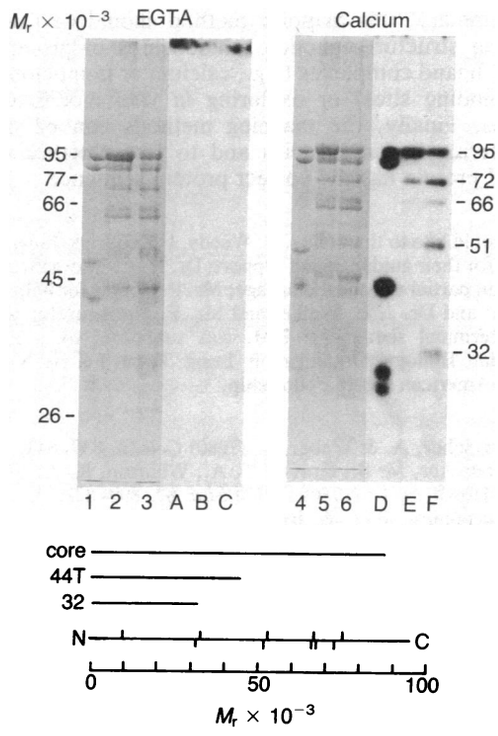

FIG. 5. ^{125}I-labeled actin gel overlay of NTCB-cleaved villin. Villin, villin core, 51T, 44T (lanes 1 and 4), 6-hr NTCB-cleaved villin (lanes 2 and 5), 12-hr NTCB-cleaved villin (lanes 3 and 6) were electrophoresed on 10% NaDodSO$_4$/polyacrylamide gels and overlaid with ^{125}I-labeled actin in EGTA (lanes 1–3)- or calcium (lanes 4–6)-containing buffer, and stained with Coomassie brilliant blue. Their corresponding autoradiograms (lanes A–C and D–F) reveal the fragments which retain calcium-dependent binding activity. The diagram compares the size and location of the smallest actin-binding fragment generated with NTCB with the previously characterized villin core and 44T proteolytic fragments.

unable to detect actin binding to either the M_r 10,000 or M_r 22,000 fragments that would be produced by cleavage at this site.

DISCUSSION

Previously we purified a M_r 44,000 amino-terminal fragment of villin and showed this fragment retained a calcium-regulated actin-severing activity (9). In this report we use a different approach to locate a calcium-regulated actin-binding region within the amino-terminal M_r 32,000 peptide of villin. Furthermore, our studies show that cleavage at the cysteine residue located in an amino-terminal M_r 10,000 peptide inhibits actin binding and, thus, suggests that this region is important for villin function. This suggestion is consistent with our preliminary biochemical studies on the 44T-14T fragment. This fragment retains the amino terminus of villin and displays a calcium-regulated actin-depolymerizing activity similar to villin and 44T as measured by pelleting assays (unpublished data).

With antibodies specific for either terminus of villin, we have identified the number and location of the NTCB cleavage sites. Our assignment of cysteine residues will only be confirmed when the amino acid sequence of villin is completed. However, the number of cysteine residues mapped in the 44T and 51T domains of villin is similar to the number of cysteine residues calculated for each domain by carboxymethylation and DTNB titration. Because cysteine residues are easily oxidized, quantitation of these residues by conventional biochemical methods can lead to an underestimate of their number. The peptide antibody method is an integral counting method with the same advantages as the chemical modification methods described by others (21–23).

Other methods for mapping cleavage sites have been described that involve either metabolic labeling of the carboxyl terminus (24) or Edman degradation and labeling of the amino terminus (25). These methods are direct and thus offer advantages such as speed and simplicity. Although our method requires the lengthy preparation of amino- and carboxyl-terminal peptides and production of antisera, there are particular advantages of this approach. One advantage of the peptide antibody approach is that cleavage maps can be produced from either or both ends of the protein even from proteins with blocked amino termini. Mapping from both ends increases the accuracy of the cleavage map. Since the peptide antibody method is very sensitive because of amplification by the second antibody, the number and positions of the cleavage sites can be calculated even if the efficiency of cleavage is low and few fragments are produced. A third advantage of this method is that specific antisera should allow mapping studies in the presence of other proteins. This feature might allow binding regions to be mapped directly in protein complexes or cellular structures *in vitro* and *in vivo* by using an approach involving protection from chemical modification. In addition, the peptide antibody approach allows us to map protease sensitive sites on undenatured proteins. This advantage could be used in renaturation experiments to identify whether regions of a protein are correctly refolded by comparing the cleavage maps from native and denatured samples.

In summary, this mapping method should be useful for exploring structure–function relationships of proteins and protein–ligand complexes (e.g., calcium or monoclonal antibody binding sites) or exploring *in vivo* modification of residues. Finally, the mapping methods can be used to characterize fusion proteins and to test whether the expressed protein has the correct protein sequence.

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