Actin-Binding and Dimerization Domains of HeLa Cell Filamin†

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ABSTRACT: HeLa cell filamin is a linear, bivalent, homodimer of high molecular weight subunits (M_r 250000) that may cross-link actin filaments in vivo into supramolecular structures such as networks and bundles. We used millimolar Ca protease from chicken breast muscle to cleave the subunit into smaller fragments that we mapped with respect to the overall structure of the dimer. The enzyme cleaves HeLa filamin into a larger (M_r 192000) and a smaller (M_r 104000) fragment; the smaller fragment is the precursor of a still smaller (M_r 92000) fragment. Only the larger fragment bound to actin in a cosedimentation test, suggesting that it contains the actin-binding region of the subunit. Digestion of HeLa filamin that had been cross-linked with dimethyl adipimidate produced a good yield of the M_r 192000 fragment but a poor yield of the M_r 104000/92000 fragments. Since native filamins are head-to-head dimers, it was expected that cross-linking would proceed most readily at the dimerization site and, therefore, it appears that the M_r 192000 fragment is cleaved from cross-linked filamin because it is distal to the dimerization region, while the M_r 104000/92000 fragments are absent because they lie at the dimerization region and were cross-linked to a form that was not identifiable by sodium dodecyl sulfate electrophoresis.

Filamins are a group of homologous, high molecular weight proteins that can cross-link actin filaments into networks and bundles. Native filamins are believed to be homodimers composed of flexible, linear subunits that incorporate a self-association site at one end and an actin-binding domain at the other end. Self-association is believed to create a bivalent molecule consisting of two actin-binding domains separated by a dimerization domain [reviewed in Weihing (1985)].

This model could be tested, and at the same time, the molecule could be mapped more precisely if controlled proteolytic digestion could be used to cleave the subunit into fragment(s) corresponding to the actin-binding region and other(s) corresponding to the dimerization region. Only one such report is available, and it was found (Davies et al., 1978) that digestion of chicken gizzard filamin with millimolar Ca protease cleaves its subunit (M_r 250000) into a very large fragment (M_r 240000) (heavy merofilamin), which probably retains the actin-binding region, and a very small fragment (M_r 9500), which may be derived from the dimerization region (see Discussion).

Mammalian filamins can also be cleaved into large and small fragments by Ca protease (Wallach et al., 1978; Fox et al., 1985; Weihing, 1986), but the large fragment is somewhat smaller than heavy merofilamin, and the small fragments are about 10 times the molecular weight of light merofilamin. With the exception of an abstract about HeLa filamin (Weihing, 1986), investigations of the function of the Ca protease fragments of mammalian filamins have not been reported. This study reports on the cleavage of HeLa cell filamin by millimolar Ca protease from chicken breast muscle and on experiments relating the fragments to the functional domains of the filamin subunit.

Materials and Methods

Isolation of HeLa Cell Filamin. HeLa cell filamin was isolated by incubating a 100000*g* supernatant fraction of HeLa cells at 25 °C to induce gelation, collecting the gel at 12000*g*, and extracting the precipitated gel with 0.6 M KCl as previously described (Weihing, 1983). For these experiments, the 0.6 M KCl extract was clarified by centrifugation at 14500 rpm in a Sorvall SS-34 rotor for 15 min. The resulting supernatant was dialyzed overnight against 20 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA),[1] 15 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6, and clarified again by centrifugation at 20000 rpm in a Sorvall SS-34 rotor for 15 min. The resulting supernatant fraction was applied to a column of DE-52 anion-exchange cellulose equilibrated with the same buffer and eluted with a linear gradient of NaCl (20–390 mM) in the same buffer [adapted from Mangeat and Burridge (1983)]. PMSF (final concentration 1 mM) was added from a 100-fold-concentrated stock solution in ethanol at the beginning of each step, and all steps were carried out at 0–4 °C unless otherwise indicated. Partially purified filamin was eluted from the column at about 0.1 M NaCl, and its 250-kDa subunit was shown to be identical with the 250-kDa subunit of the filamin previously isolated by gel filtration and designated HMWP (Weihing, 1983) by two tests. First, the 250-kDa subunit of the DE-52-purified filamin reacted in immunoblots (data not shown) with a specific goat antiserum prepared against highly purified HeLa cell filamin (Weihing, 1983). Second, the 250-kDa polypeptide of the DE-52-purified filamin and the filamin purified by gel filtration had the same mobility on SDS–polyacrylamide gels (data not shown).

Digestion of HeLa Cell Filamin by Millimolar Ca Protease. The DE-52-purified filamin or cross-linked DE-52-purified filamin prepared as described below was treated with millimolar Ca protease from chicken breast muscle (generous gift of Dr. D. E. Goll, University of Arizona) by mixing the filamin with an amount of millimolar Ca protease equal to $^1/_{25}$th or $^1/_{50}$th the weight of filamin and incubating for various lengths

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DE-52, microgranular (diethylaminoethyl)cellulose, manufactured by Whatman; SDS, sodium dodecyl sulfate; DMA, dimethyl adipimidate; HMWP, high molecular weight protein; kDa, kilodalton(s); PMSF, phenylmethanesulfonyl fluoride.

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of time at 29.5 °C in the presence of 1.6 mM $CaCl_2$ and 2.5 mM 2-mercaptoethanol. The reaction was stopped by adding 5-fold-concentrated sample buffer for SDS gel electrophoresis or by adding 100 mM EGTA to a final concentration equal to 5–10 times the concentration of $CaCl_2$.

Cosedimentation Assay for Actin Binding. Undigested filamin or filamin treated first with millimolar Ca protease and then with EGTA to stop the digestion was mixed with polymerized rabbit muscle actin (in 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 2 mM Tris-HCl, 100 mM KCl, and 2 mM $MgCl_2$, pH 8) to a final concentration of 0.1–0.2 mg/mL actin. After incubation at 29.5 °C for 10–30 min, the samples were centrifuged at 35000 rpm in a Beckman type 40 rotor at room temperature. The resulting pellets and the lyophilized supernatant fractions were boiled in electrophoresis sample buffer, and equivalent samples were analyzed by SDS gel electrophoresis.

Cross-Linking HeLa Filamin with Dimethyl Adipimidate. The general procedure was that previously used by Wang (1977) for chicken gizzard filamin. HeLa filamin was first dialyzed against two changes of 0.5 M KCl in 0.1 M triethanolamine hydrochloride, pH 8.5. Filamin at 0.2–0.3 mg/mL was mixed with solid dimethyl adipimidate to give a final concentration of 1–2 mg/mL and incubated at 25 °C for 20 min. Longer incubations did not change the proportion of cross-linked to un-cross-linked filamin but did decrease the overall yield of filamin. The reaction was quenched by adding 1 M NH_4Cl to a final concentration of 0.1 M, and one portion was dialyzed against 0.054 M Tris–sulfate, pH 6.03, to prepare it for gel electrophoresis while the rest was dialyzed against 100 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, and 20 mM Tris–acetate, pH 7.6, to prepare it for digestion.

Immunoblotting. Electrophoretic transfer of proteins from 5% or 6% polyacrylamide gels to nitrocellulose paper was performed according to Towbin et al. (1979) using the following modifications. The transfer apparatus was assembled from a glass chromatography chamber, a plastic grid, and platinum wire (Bittner et al., 1980), and the power supply was a dual battery charger (6/2 A, 6/12 V, Model SE-82-6, Schumacher Electric Corp., Chicago, IL) (Fischer, 1986). Blotting was for 5 h with the battery charger set at 12 V and 6 A. Under these conditions, enough protein was transferred for localization by the peroxidase reaction (see below), but Coomassie blue staining of the gel after blotting revealed incomplete transfer of the 250-kDa subunit and the 192-kDa, Ca protease fragment of filamin (not shown).

Washing and treatment of the blot with a goat antiserum against HeLa filamin were performed as described (Weihing, 1983). After this treatment, the papers were treated with peroxidase-conjugated rabbit anti-goat IgG diluted 1/500, and antigen–antibody complexes were located by incubating the blot in 4-chloro-1-naphthol and H_2O_2 (Hawkes et al., 1982).

General Methods and Reagents. Growth and harvesting of HeLa cells, protein analysis, gel electrophoresis, and preparation of myosin were done as described previously (Weihing, 1977). Muscle actin was purified from an acetone powder of rabbit skeletal muscle by using the method of Spudich and Watt (1971) as previously described (Weihing, 1983). When necessary, actin was purified further by gel filtration on Bio-Gel A15-m eluted with 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl, pH 8, followed by batch treatment with DE-52 anion-exchange cellulose as described by Pardee and Spudich (1982). Actin was stored at 0–4 °C after polymerization with 0.1 M KCl and 2 mM $MgCl_2$ and addition of 0.02% sodium azide to prevent bacterial growth and could be used for several months. α-Actinin from pig muscle (for electrophoretic standards) was kindly provided by Dr. D. E. Goll. Chemical reagents were generally from Sigma Chemical Co., St. Louis, MO, or Mallinkrodt Co., St. Louis, MO; dimethyl adipimidate was from Pierce Chemical Co., Rockford, IL; 2-mercaptoethanol and triethanolamine were from Eastman Kodak Co., Rochester, NY; Bio-Gel A15-m, acrylamide, and bis(acrylamide) were from Bio-Rad Laboratories, Richmond, CA; DE-52 anion-exchange cellulose was from Whatman International Ltd., Maidenstone, Kent, England; nitrocellulose paper was from Schleicher & Schuell, Inc., Keene, NH; rabbit anti-goat IgG was from Cappel Laboratories, Cochranville, PA. Ca protease from chicken breast muscle was kindly provided by Dr. D. E. Goll, University of Arizona, as a powder that had been lyophilized in the presence of sucrose. The enzyme was reconstituted by dissolving the powder in 5 mM EDTA, pH 7.4, with 0.02% sodium azide and dialyzing it at 0–4 °C against two changes of the same buffer. It retained activity during storage for over 1 year at 0–4 °C.

RESULTS

Digestion of HeLa Filamin with Millimolar Ca Protease. The ability of millimolar Ca protease from chicken breast muscle to digest HeLa cell filamin was tested by determining the time course of production of fragments of filamin. Filamin in the buffer in which it was eluted from DE-52 was first mixed with 2-mercaptoethanol and a suitable aliquot of millimolar Ca protease and incubated at 29.5 °C for 2 min. $CaCl_2$ was then added to start the reaction, and samples were removed and mixed with sample buffer for SDS gel electrophoresis at various times after the reaction was started. Analysis by SDS gel electrophoresis (Figure 1) showed that initially the enzyme cleaves the 250-kDa subunit into a large, 192-kDa fragment and a smaller 104-kDa fragment. Subsequently, the 104-kDa fragment disappears and is replaced by a 92-kDa fragment (or possibly a pair of fragments). This pattern of digestion suggests that initially the enzyme cleaves the 250-kDa subunit into a 192- and a 104-kDa fragment and that subsequently the 104-kDa fragment is cleaved to a 92-kDa fragment.

To test the possibility that the 92-kDa fragment is produced by digestion of the 104-kDa fragment, the latter was separated from the 250-kDa subunit and the 192-kDa fragment and then subjected to digestion with millimolar Ca protease. To prepare partially purified 104-kDa fragment, the DE-52-purified filamin was digested with Ca protease for 1 min, a time which was sufficient to produce a usable amount of 104-kDa fragment but very little 92-kDa fragment. Digestion was stopped by adding EGTA, and the mixture was fractionated by size on Bio-Gel A15-m. This produced fractions which were highly enriched in the 104-kDa fragment relative to the 250-kDa subunit and the 192-kDa fragment. The 104-kDa enriched fraction was then digested for 5, 15, and 40 min and subjected to SDS gel electrophoresis. Immunoblot analysis of the separated peptides (Figure 1, lanes i–l) showed that as the 104-kDa fragment disappeared, the 92-kDa fragment appeared concomitantly, indicating that the 92-kDa fragment is derived at least in part from the 104-kDa fragment.

Actin Binding by Ca Protease Fragments. The actin-binding ability of the Ca protease fragments of HeLa filamin was tested by determining which of the fragments could be consedimented with actin. Filamin was first digested for 5 min to produce the large fragment and both smaller fragments, the reaction was quenched with EGTA, and the peptides were mixed with actin and centrifuged as described under Materials and Methods.

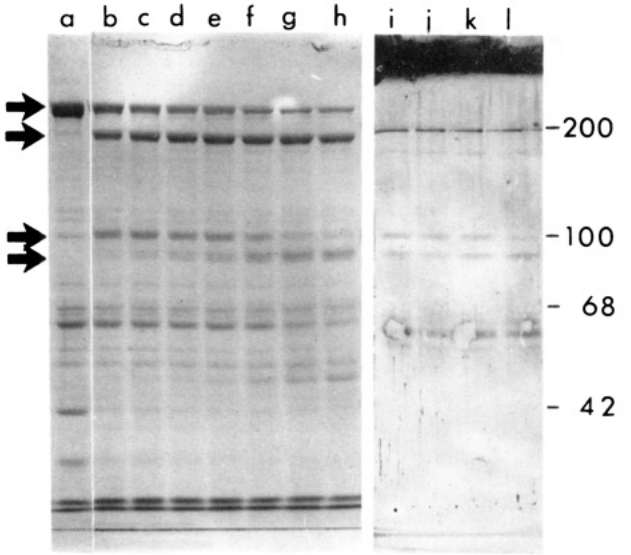

FIGURE 1: (Lanes a–h) Time course of digestion of HeLa cell filamin with millimolar Ca protease from chicken breast muscle. HeLa cell filamin was digested for various lengths of time and analyzed by SDS gel electrophoresis as described in the text: (a) 0 min; (b) 2 min; (c) 3 min; (d) 4 min; (e) 5 min; (f) 10 min; (g) 20 min; (h) 30 min. (Lanes i–l) Time course of digestion of the 104-kDa fragment of HeLa filamin. The 104-kDa fragment was partly purified by gel filtration and then digested with Ca protease. Peptides separated by SDS gel electrophoresis were identified by immunoblotting using an antiserum against filamin: (i) 0 min of digestion; (j) 5 min; (k) 15 min; (l) 40 min. The bands at 200 and 60 kDa are artifacts which for unknown reasons were also present in lanes that contained only sample buffer. Numbers to the right of lane l indicate the position of standard proteins; from top to bottom, these are heavy chain of rabbit skeletal muscle myosin (200 kDa), pig skeletal muscle α-actinin (100 kDa), bovine serum albumin (68 kDa), and rabbit skeletal muscle actin (42 kDa). Arrows on the left side of lane a indicate the following polypeptides (from top to bottom): the 250-kDa subunit of filamin and the 192-, 104-, and 92-kDa fragments of filamin.

Sedimentation in the presence of actin results in a striking increase in the staining intensity of the 192-kDa fragment in the actin-containing pellet fraction as compared to the pellet formed in the absence of actin (Figure 2, lanes f and g). There was also a concomitant decrease in the staining intensity of the 192-kDa fragment in the corresponding supernatant fraction (Figure 2, lanes c and d). This pattern, which was consistently observed in several such experiments, means that the 192-kDa fragment retains actin-binding activity. Similar but much less striking changes in staining intensity were sometimes observed for the smaller fragments, so it is not clear if they retain actin-binding activity. Proof that the 192-kDa fragment accounts for the actin-binding activity of the subunit would require quantitative binding studies that are not yet possible because it has not been possible to prepare 192-kDa fragment free of 250-kDa subunit.

Cross-Linking and Ca Protease Digestion. The relation of the fragments to the dimerization region was studied by analyzing the products released from filamin that had first been cross-linked with DMA. It was expected that cross-linking would proceed most readily at the dimerization site, and therefore that the fragment(s) containing this region would not be present in a digest of cross-linked filamin.

Filamin was cross-linked with DMA and prepared for digestion as described under Materials and Methods. Because the filamin was not completely cross-linked, it was important to rule out the possibility that any fragments produced were actually derived primarily from the un-cross-linked dimer that remained after treatment with DMA. Filamin cross-linked with DMA was therefore subjected to gel filtration on Bio-Gel

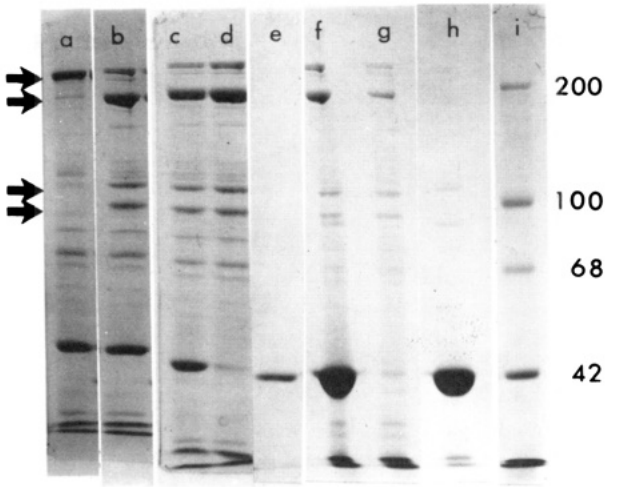

FIGURE 2: Cosedimentation of Ca protease fragments of HeLa cell filamin with rabbit skeletal muscle actin. HeLa filamin was digested with Ca protease and then mixed with polymerized actin and centrifuged, and equivalent fractions of the resulting supernatant and pellet fractions were analyzed by SDS gel electrophoresis as described in the text. (a) Undigested filamin; (b) digested filamin; (c, d, and e) supernatant fractions; (f, g, and h) pellet fractions; (c and f) mixture of digested filamin and actin; (d and g) mixture of filamin and actin buffer; (e and h) mixture of filamin buffer and actin; (i) standard proteins described in the legend to Figure 1. Arrows on the left side of lane a indicate the polypeptides mentioned in Figure 1.

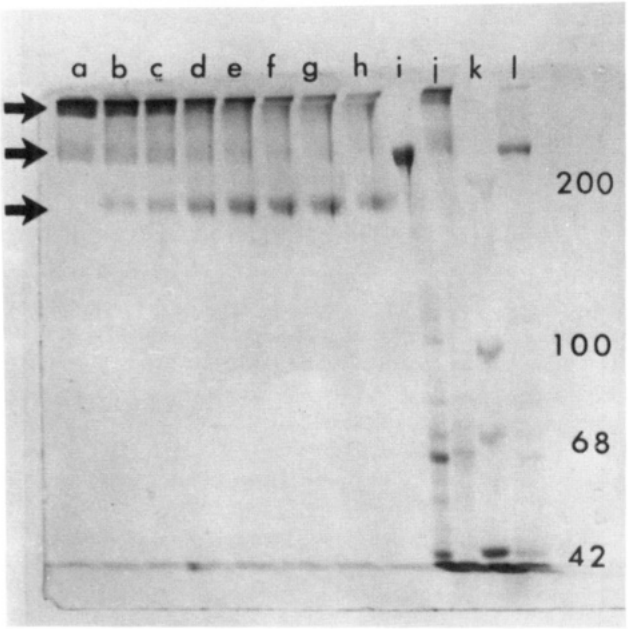

FIGURE 3: Time course of digestion of DMA-cross-linked filamin. HeLa filamin was cross-linked with DMA and subjected to gel filtration on Bio-Gel A15-m as described in the text. A Bio-Gel fraction selected because of its very high ratio of cross-linked to un-cross-linked subunit (compare lane a to lane j) was mixed with millimolar Ca protease, and samples removed after 0.5, 1, 3, 5, 10, 20, and 30 min of digestion were analyzed by SDS gel electrophoresis as described in the text. Lane a, undigested Bio-Gel fraction of cross-linked filamin; lanes b–h, digestion products after the following times of digestion: (b) 0.5 min; (c) 1 min; (d) 3 min; (e) 5 min; (f) 10 min; (g) 20 min; (h) 30 min; (i) un-cross-linked filamin; (j) cross-linked filamin before gel filtration on Bio-Gel; (k) standard proteins described in Figure 1. Arrows on the left side of lane a indicate the position of the cross-linked dimer, the 250-kDa subunit, and the 192-kDa fragment of filamin.

A15-m which produced fractions that were enriched in the cross-linked species relative to the 250-kDa subunit (compare lanes a and j of Figure 3). Ca protease digestion of such an enriched fraction for up to 30 min produced a good yield of

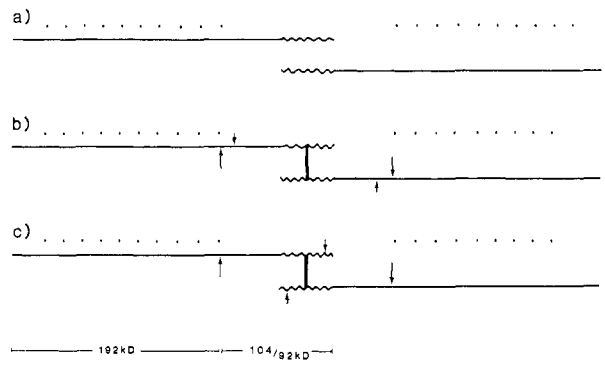

FIGURE 4: Possible maps of the intramolecular location of the Ca protease fragments of HeLa filamin. The un-cross-linked native dimer (diagram a) is shown as two linear subunits [reviewed in Weihing (1985)] that may interact by overlap (Castellani et al., 1981) of a dimerization site (wavy lines). The cross-linked dimer (diagrams b and c) is diagrammed as linear subunits joined by a single bar at the dimerization site. Only one cross-link is shown because this is sufficient to account for the presence of the 192-kDa fragment and the absence of the 104- and 92-kDa fragments in digests of DMA-cross-linked HeLa filamin. The number of cross-links has not been measured, however. The long arrow indicates the approximate location of the primary cleavage of the 250-kDa subunit into the larger 192-kDa fragment and the smaller 104-kDa fragment. The different positions of the short arrows in diagrams b and c indicate two of the possible ways in which cleavage of the 104-kDa fragment could produce the 92-kDa fragment. The dotted lines indicate only that the 192-kDa fragment retains actin-binding activity, but the location of the activity within the fragment has not been mapped.

the 192-kDa fragment but virtually none of the 104- or 92-kDa fragments (Figure 3, lanes a–h). Most of the 192-kDa fragment produced in this experiment must have been derived from the cross-linked species because the initial staining intensity of the 250-kDa subunit that remained after cross-linking but before digestion was much lower than the staining intensity of the 192-kDa fragment that was produced by 20–30 min of digestion (Figure 3).

Discussion

These results indicate that millimolar Ca protease from chicken breast muscle initially cleaves the HeLa filamin subunit into polypeptides with distinct characteristics. One, the 192-kDa fragment, is released from DMA-cross-linked filamin and therefore appears not to participate in the dimerization function of the subunit. However, this fragment consistently binds to actin and therefore must be derived from the actin-binding domain(s) of the subunit of HeLa filamin. Quantitative studies of the binding of the purified fragment to actin will be required to decide if it accounts for all the actin-binding activity of the subunit. The others, the 104- and 92-kDa fragments, are not observed after Ca protease digestion of DMA-cross-linked filamin, and therefore, they appear to participate in the dimerization function of the subunit. Digestion of the 104-kDa fragment freed of 250-kDa subunit and 192-kDa fragment produced 92-kDa fragment, indicating that the 104-kDa fragment is a precursor of the 92-kDa fragment. Quantitative studies will be required to determine if the 92-kDa fragment is derived only from the 104-kDa fragment.

How do these fragments fit into the known structure of filamin? So far, it is known that native filamin is a flexible, linear homodimer (Weihing, 1985) and that self-association may occur by overlap of the dimerization region (Castellani et al., 1981). The actin-binding domains of the dimer appear to be separated by the dimerization domain, judging from rotary shadowing of actin filaments cross-linked by macrophage actin-binding protein (Hartwig & Stossel, 1981), a homologue of filamin (Weihing, 1985). The present data extend this model in the following way. The 192-kDa actin-binding fragment lies at the free ends of the dimer, while the 104- and 92-kDa fragments presumably overlap in the dimerization region of the dimer. The precise relationship of the 104- and 92-kDa fragments to the dimerization end of the monomer cannot yet be determined because one cannot deduce from the present data whether the material removed during conversion of the 104-kDa fragment to the 92-kDa fragment comes from the distal end, the proximal end, or both ends (relative to the dimerization domain) of the 104-kDa fragment. Two possible relationships (which are not the only ones possible) are diagrammed in Figure 4.

Two other mammalian filamins, guinea pig vas deferens filamin (Wallach et al., 1978) and platelet actin-binding protein (Fox et al., 1985), are cleaved by Ca-dependent proteases in the same general pattern of cleavage to fragments of roughly 192 and 104 kDa, followed by further cleavage of the 104-kDa fragment to a 92-kDa fragment and other, unidentified products. This similarity suggests that these fragments will be mappable to the respective native dimers in the general way that has just been discussed for HeLa filamin, but this has not yet been tested experimentally.

All the filamins that have been tested contain sites that are sensitive to Ca protease, but the physiological significance of this observation is obscure. In platelets, it is known that cleavage of filamin by Ca protease appears to occur shortly after activation of the platelets. This may result in detachment of an integral glycoprotein of the plasma membrane from a filamin-mediated association with the actin-based cytoskeleton (Fox, 1985). Whether similar associations occur in HeLa cells and whether the Ca protease peptides have specific functions in any cell are not yet known.

Acknowledgments

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Expression of a Human α-Tubulin: Properties of the Isolated Subunit†

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ABSTRACT: We examined the in vitro expression and biochemical properties of the isolated α subunit of tubulin both in rabbit reticulocyte lysates and in *Escherichia coli* extracts. Both systems produce soluble, full-length human α-tubulin polypeptide. When α-tubulin mRNA is translated in rabbit reticulocyte lysates, the isolated α subunit is *fully* functional as assayed by coassembly with bovine brain tubulin using temperature-dependent or taxol/salt assembly procedures. The conformation of the isolated α subunit was probed by limited proteolytic digestion with chymotrypsin and by reductive methylation. Limited proteolysis studies indicated that the "monomeric" α subunit is highly susceptible to chymotrypsin digestion and becomes resistant to chymotrypsin cleavage following incorporation into the heterodimer. Reductive methylation indicated that the unassociated α subunit has a highly reactive lysyl residue essential for microtubule assembly similar to that observed in the heterodimer. In contrast, α-tubulin expressed in *E. coli* lysates was incapable of coassemblying with bovine brain tubulin. Differences in assembly competence of the two α-tubulin products appear to be related to formylation of the N-terminal methionine in the procaryotic synthesized subunit. These findings suggest that the amino-terminal methionine of α-tubulin plays an essential role in the isolated subunit and/or in the heterodimer, a hypothesis supported by chemical reactivity studies [Sherman, G., Rosenberry, T. L., & Sternlicht, H. (1983) *J. Biol. Chem. 258*, 2148–2156] which imply that this residue is in a salt-bridge interaction in the dimer.

Microtubules are filamentous polymer structures involved in a variety of diverse functions in eucaryotic cells. Mitosis, morphogenesis, and maintenance of cell shape are some of the processes dependent on the ordered assembly and disassembly of microtubules (Dustin, 1984). A molecular understanding of these functions will involve in part a detailed knowledge of the properties of the constituent heterodimer protein of the microtubule, tubulin, and its two homologous subunits, α- and β-tubulin. These two subunits, each ~450 amino acid residues in length (Luduena et al., 1977; Ponstingl et al., 1981; Krauhs et al., 1981), are held together by noncovalent interactions (Lee et al., 1973) with an apparent K_D of ~1 μM at 4 °C (Detrich & Williams, 1978). Despite this relatively high K_D, it has not yet been possible to isolate the native subunits from the heterodimer (Kirchner & Mandelkow, 1985; H. Sternlicht et al., unpublished observations), and, therefore, even such basic properties as stability and assembly competence of the isolated subunits are not known.

As an alternative approach to probe the biochemical properties of the individual subunits, we have used expression vectors to obtain native, isolated α- and β-tubulin. In previous work, we expressed a human α-tubulin cDNA (Cowen et al., 1983) in *Escherichia coli*, thereby producing significant amounts of α-tubulin (>1% of total protein) (Yaffe et al., 1986, 1987). However, the protein was insoluble and accumulated in "midbodies". [Similar findings were also obtained by Wu and Yarbrough (1986) in their investigation of *Trypanosoma rhodesiense* α- and β-tubulin expression in *E. coli*.] In this study, we describe the use of in vitro expression systems for the synthesis and characterization of the isolated α-tubulin subunit. In vitro systems typically generate soluble proteins (albeit at low level concentrations, ≤10 nM). This is an important consideration in the case of tubulin as no procedure currently exists to generate the native polypeptides from insoluble or denatured products. In this paper, we examine and evaluate two systems for the in vitro expression of human α-tubulin: a eucaryotic translation system using rabbit reticulocyte lysates and a procaryotic coupled transcription–translation system using *E. coli* lysates. Both systems produce full-length α-tubulin subunit. We demonstrate that the α subunit synthesized in reticulocyte lysates is stable and able to form assembly-competent heterodimers with bovine brain tubulin. In contrast, the α subunit synthesized in *E. coli* lysates is unable to coassemble into microtubule polymer, an effect which we attribute to *N*-formyl modification of the amino-terminal methionine residue in the *E. coli* lysates.

We have a long-standing interest in the C-terminal domain of α-tubulin, a region potentially involved in dimer–dimer contacts in the microtubule (Kirchner & Mandelkow, 1985). In the free heterodimer, this region contains a highly reactive lysyl residue, Lys-394, whose methylation renders tubulin assembly-incompetent (Sherman et al., 1983; Szasz et al., 1986). The enhanced reactivity of this residue as a nucleophile is attributed to its location in a cluster of basic residues (Blank

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