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Mapping of Caldesmon: Relationship between the High and Low Molecular Weight Forms†

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ABSTRACT: Caldesmon is a widely distributed contractile protein that occurs in both a high molecular weight [120–150-kilodalton (kDa)] and a low molecular weight (71–80-kDa) form, depending on the tissue. The structural relationship between these two forms was examined by mapping techniques. Partial cyanogen bromide cleavage in conjunction with sodium dodecyl sulfate gel electrophoresis was used to construct a map of the cleavage points and determine the relative position of the fragments in a high molecular weight caldesmon from chicken gizzard ($caldesmon_{125}$). By use of this map, markers for different regions of the protein were obtained: Antibodies directed toward certain areas were prepared by affinity purification, and specific ^{125}I-labeled tryptic peptides were found to originate from terminal cyanogen bromide fragments. Mapping of a lower molecular weight form of caldesmon ($caldesmon_{72}$ from chicken liver) revealed the presence of sequences located in both ends of $caldesmon_{125}$. A terminal 38-kDa fragment of both proteins was apparently identical on the basis of arrangement of cleavage sites, antibody reactivity, and iodopeptide mapping. Fragments from the other end of both proteins exhibited an identical pattern of peptides. These results show that it is sequences located in the central area of $caldesmon_{125}$ which are missing in $caldesmon_{72}$, indicating that the smaller molecule is not simply a proteolytic product of the larger. The two forms of caldesmon may be derived from separate genes or by alternative splicing from a single gene.

Caldesmon was originally identified as a major calmodulin- and actin-binding protein in smooth muscle (Sobue et al., 1981). Although its function in vivo is not yet certain, caldesmon has many properties suggestive of a contractile regulator. In the absence of calcium ion, the protein inhibits the myosin ATPase reaction and binds tightly to actin filaments (Sobue et al., 1982; Ngai & Walsh, 1984; Marston & Lehman, 1985; for reviews, see Kakiuchi & Sobue, 1983; Marston & Smith, 1985). When the calcium concentration rises, calcium–calmodulin decreases the affinity of caldesmon for actin and abrogates the inhibition of the ATPase. Hence, caldesmon may play a role in smooth muscle analogous to that of troponin I in skeletal muscle. Caldesmon is also subject to phosphorylation in vitro and in vivo, possibly indicating further levels of control (Ngai & Walsh, 1984; Marston & Lehman, 1985; Umekawa & Hidaka, 1985; Litchfield & Ball, 1987).

Caldesmon was first isolated as a doublet of 150K and 147K from smooth muscle, but it is now clear that it is widely distributed (Ngai & Walsh, 1985a; Owada et al., 1984; Sobue et al., 1985; Kakiuchi et al., 1983; Bretscher & Lynch, 1985). Antibodies to smooth muscle caldesmon recognize proteins in a variety of different tissues and cell lines. These cross-reactive caldesmons fall into two apparent classes on the basis of size, one of 120–150K and the other of 70–80K. Often both types are found in a single cell (Bretscher & Lynch, 1985). The lower M_r proteins share all the known properties of caldesmon including stability to heat. The different sizes of caldesmon are referred to by subscripting their relative molecular masses [e.g., $caldesmon_{120}$, Sobue et al. (1985)].

Details of the relationship between the two forms of caldesmon are currently not known. The most obvious possibility is that the smaller forms are proteolytically cleaved fragments of the larger (Ngai & Walsh, 1985a). Caldesmon is known to be very sensitive to proteases. There is, however, evidence to suggest that the smaller form exists prior to cell lysis: inclusion of protease inhibitors does not influence the profile of reactive proteins (Owada et al., 1984), lower molecular weight caldesmon is not generated from higher molecular weight caldesmon added to extracts (Bretscher & Lynch, 1985), and disruption of cells in 10% TCA[1] or hot SDS still allows subsequent detection of lower M_r forms (Bretscher & Lynch, 1985). In order to investigate this question more directly, we have used mapping techniques to compare $caldesmon_{125}$ and $caldesmon_{72}$ isolated from chicken tissues. Mapping of cyanogen bromide cleavage sites by partial cleavages and SDS gel electrophoresis allowed ordering of the fragments and delineation of markers for both ends of the molecule. The results indicate that the lower M_r form cannot be a simple

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[1] Abbreviations: SDS, sodium dodecyl sulfate; kDa, kilodalton; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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fragment of the larger form, indicating a separate origin for the two sizes of caldesmon.

Experimental Procedures

Materials. Chromatographic media were from Whatman (DE52 DEAE-cellulose, P11 phosphocellulose) or Pharmacia (Sepharose 4B). Sodium [^{125}I]iodide was obtained from Amersham. Cellulose-coated thin-layer chromatography plates for peptide mapping were from Merck. Acrylamide and gel electrophoresis chemicals were from Bio-Rad; buffers and other chemicals were of reagent grade or better and were purchased from Sigma, BDH Canada (Toronto), or Canlab (Toronto).

Proteins. For most experiments, the high M_r form of caldesmon was purified from chicken gizzard by a modification of the method of Bretscher (1984). Briefly, after heat treatment the 30–50% ammonium sulfate fraction was redissolved in and dialyzed against buffer A (10 mM Tris–acetate, pH 7.5, 1 mM EDTA, and 4 mM mercaptoethanol). The dialyzed material was applied to a DE52 column (1.8 × 7 cm) and caldesmon eluted with a 0–0.3 M gradient of NaCl in buffer A. The caldesmon-containing fractions were pooled, diluted with one half-volume of buffer A, and applied to a phosphocellulose column (1.8 × 7 cm). Caldesmon was eluted with a 0.1–0.4 M NaCl gradient in buffer A. The eluted caldesmon was dialyzed against buffer A and concentrated by step elution with 0.3 M NaCl from a small (10-mL) DE52 column. Fractions were stored at −80 °C until used.

Although the reported M_r for caldesmon from gizzard has ranged from 120 000 (Fujii et al., 1987) to 150 000 (Sobue et al., 1981), in our gels the apparent M_r was 125 000. This caldesmon is therefore referred to as caldesmon$_{125}$.

A fragment of caldesmon$_{125}$ of approximate M_r 85 000 was also purified by chromatography on DE52. It eluted as the major band at very low salt concentration (~10 mM). This protein was identified as a fragment of caldesmon$_{125}$ rather than a different form of preexisting caldesmon, since no cross-reacting protein of this size is seen in Western blots of SDS-disrupted gizzard (Kakiuchi et al. 1983) and it occurs in larger amounts if the purification is slower.

Caldesmon purified without heat treatment (Sobue et al., 1981) was also utilized in some experiments; no difference was apparent between caldesmon$_{125}$ purified in the different ways. Caldesmon$_{72}$ was obtained from fresh chicken liver as described (Litchfield & Ball, 1987), although the yield was low (less than 1 mg from 50 g of liver).

CNBr Cleavage. For initial cleavage in solution, caldesmon$_{125}$ was precipitated with acetone (4 volumes, −20 °C), resuspended at 3–5 mg/mL in 70% formic acid, and treated with 5 mg/mL CNBr at room temperature. The reaction was stopped by dilution with 20 volumes of distilled water, freezing, and lyophilization.

Proteins in stained gel slices were cleaved by successive incubation in the following solutions (30 min each, room temperature, with gentle agitation): water, 0.1 N HCl, 0.1 N HCl containing 20 mg/mL CNBr, water, water, 0.15 M Tris–HCl, pH 6.8, and sample buffer (Laemmli, 1970); 20 mL volumes of each solution were used.

Antibodies. Antibodies to caldesmon$_{125}$ were raised in rabbits and affinity purified on a column of antigen linked to CNBr-activated Sepharose. For the purification of antibodies specific for certain fragments of caldesmon$_{125}$, 2 mg of CNBr-digested caldesmon$_{125}$ (24 h) was electrophoresed on a flat-topped preparative SDS gel, transferred to nitrocellulose, and stained with amido black. Bands of interest were excised, blocked with 4% casein in Tris-buffered saline, and incubated with the affinity-purified antibodies. After unbound antibody was washed away, bound antibody was eluted with pH 2.2 glycine hydrochloride buffer, immediately neutralized, and dialyzed against PBS.

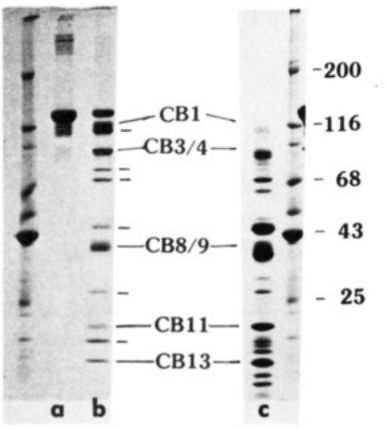

FIGURE 1: CNBr cleavage of caldesmon$_{125}$. Purified caldesmon$_{125}$ was cleaved for 0 min (lane a), 20 min (lane b), or 24 h (lane c) and 10 μg run in each lane. The SDS gel was stained with Coomassie brilliant blue. The positions of the fragments are marked with bars, and some are labeled. Lanes at the far left and right have standard proteins and their relative molecular mass in kilodaltons is marked on the right: myosin, 200; β-galactosidase, 116; bovine serum albumin, 68; actin, 43; and immunoglobulin G light chain, 25.

Other Procedures. SDS–polyacrylamide gel electrophoresis was performed with 6–15% linear gradient gels and the buffer system of Laemmli, 1970. Two-dimensional tryptic peptide mapping was conducted by the method of Elder et al. (1977) as modified by Zweig and Singer (1979). Protein determination was by the Hartree modification (Hartree, 1971) of the method of Lowry et al. (1951). Western blotting was by the method of Towbin et al. (1979).

Results

Partial Cleavage Map of Caldesmon$_{125}$. Mapping the positions of cleavage points in a protein requires limited cleavage to generate an overlapping set of fragments. Small numbers of fragments (i.e., few cleavage points) make maps more readily interpretable. The digestion of caldesmon$_{125}$ by cyanogen bromide resulted in ~30 fragments visible on heavily loaded gels. However, only 13 of these were present in large amounts at early times and could be readily visualized in SDS gels (Figure 1). [A virtually identical cleavage pattern has been shown by Lynch et al. (1987).] These were named CB1–CB13 in order of decreasing M_r. Two pairs of peptides (CB3/4 and CB8/9) exhibited very similar M_r and were only slightly separable on SDS gels. After longer digestion times more fragments were visible (Figure 1, lane c), but it is notable that only fragments CB7, CB11, and CB13 appeared to accumulate, suggesting resistance to further attack by cyanogen bromide. The relatively small number of fragments observed indicated few major cleavage points.

In order to construct a map of the relative distance between cleavage points, the relationship among the various fragments must be known. This information is derived from a second partial cleavage of the first fragments generated. This is conveniently accomplished by subjecting an entire gel lane (containing partially cleaved protein) to CNBr and running it on a second gel (Figure 2) (Pepinsky, 1983). Spots on the diagonal represent products of the first cleavage that are unmodified in the second. Spots below the diagonal are cleavage products derived from spots on the diagonal. This type of gel allows the subfragmentation pattern of each peptide to be determined. Table I displays the data obtained from gels such

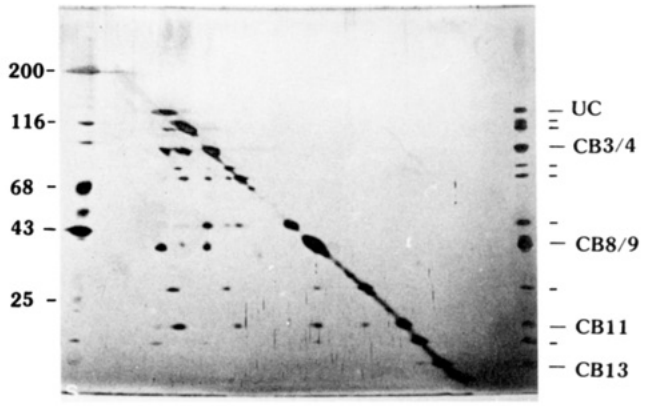

FIGURE 2: Two-dimensional SDS–PAGE of CNBr-cleaved caldesmon$_{125}$. A lane from a 6–15% SDS gel containing 10 μg of partially cleaved caldesmon was excised, subjected to CNBr treatment, and placed horizontally on a second 6–15% SDS gel. After electrophoresis, the gel was silver stained and photographed. Numbers at left mark relative molecular mass in kilodaltons. A lane of partially cleaved caldesmon$_{125}$ appears at the right edge with the CNBr fragments marked; UC, uncleaved.

Table I: Cleavage of Cyanogen Bromide Fragments of Caldesmon$_{125}$

fragment	M_r	products[a]
CB1	110	CB2, CB3/4, CB5, CB8/9, CB10
CB2	104	CB3/4, CB6, CB8/9, CB11
CB3/4	86	CB5, CB6, CB7, CB8/9, CB12
CB5	72	CB6, CB7, CB10
CB6	65	CB7, CB11
CB7	44	none
CB8/9	38	CB10, CB11, CB12
CB10	26	CB11
CB11	21	none
CB12	19	CB13
CB13	16	none

[a] Refers to fragments found directly below the parent fragment. The slash separating CB3/4 and CB8/9 indicates that these pairs could not be distinguished, and it should be read as "either/or".

as that shown in Figure 2. From this information, together with the apparent M_r of the various fragments, a map of the relative positions of the major cleavage points can be derived. This is accomplished by examining overlapping fragments and fitting them together. For example, CB6 can be derived from CB5 and each gives rise to CB7, apparently by a single cleavage (since the molecular weights of CB7 plus CB10 or CB11 add up to the molecular weights of CB5 and CB6, respectively). Hence, both CB5 and CB6 contain CB7 at one end but have CB10 and CB11, respectively, at the other. This arrangement would require that CB10 could be cleaved to CB11, and this is indeed the case (see Figure 2 or Table I). This type of reasoning allows the ordering of the various fragments to give a map.

The simplest and most logical arrangement of cleavage points is shown in Figure 3. Only four major sites are required to account for the observed pattern of cyanogen bromide fragments. Three of these are clustered at one end of the molecule, giving rise to the low M_r fragments CB10–CB13. The original N- and C-termini of caldesmon$_{125}$ are present in CB13 and CB9 (although it is not known which is which) and larger fragments that contain these. The map is consistent with the resistance of CB7, CB11, and CB13 to further cleavage (as seen in Figure 1). Some ambiguities do arise, however, from the difficulty in separating CB8/9 and CB3/4, from inaccuracies in relative molecular mass, and from the absence of expected fragments in some cleavages. For example, CB13 is expected as a constituent of CB8 and CB3 but is not visible in the gel (Figure 2). This may be due simply

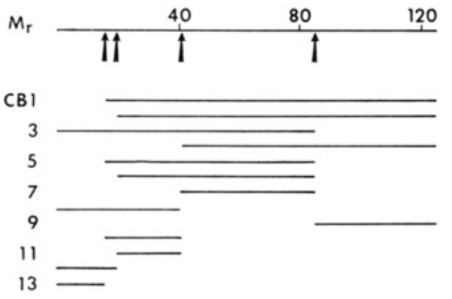

FIGURE 3: Cleavage site positions in caldesmon$_{125}$. A map showing the sites of CNBr cleavage, marked with arrows, and the derivation of the various CNBr fragments. Numbers represent molecular mass in kilodaltons measured from one end of the molecule. Odd-numbered CNBr fragments are labeled.

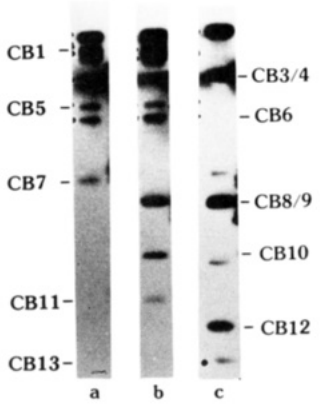

FIGURE 4: Immunoblot of CNBr-cleaved caldesmon$_{125}$ with fragment-specific antibodies. Partially CNBr-cleaved caldesmon$_{125}$ was electrophoresed on a 6–15% SDS gel and transferred to nitrocellulose; lanes were probed with affinity-purified antibodies to CB7 (lane a), CB11 (lane b), or CB13 (lane c). The positions of the CNBr fragments are marked.

to problems with staining small amounts of material; nevertheless, a method to verify the ordering was sought.

Antibodies specific to various fragments were selected from antibodies to whole caldesmon$_{125}$ (see Experimental Procedures). These antibodies were then used to probe Western blots of the cyanogen bromide cleavage products (Figure 4). This method has its own problems stemming from its sensitivity and differences in immunological recognition based on size (i.e., the antibodies apparently reacted more strongly with larger fragments). Nevertheless, the results confirm the assignment of fragments shown in Figure 3. No unexpected cross-reactivities were observed; antibodies to CB13 do not react with CB10 or CB11, for example. [They do react with minor peptides just above CB7 and just below CB10 (Figure 4, lane c).] Antibodies to the terminal fragment CB13 do not react with the high M_r peptides CB1, CB2, CB5, and CB6, indicating that CB13 is located at one end of the protein and that these large peptides lack this end, as expected. All of these large peptides retain reactivity with antibodies specific for the CB11 fragment (lane b), in accord with the assignments shown in Figure 3. Antibodies to the central region CB7 do not react with lower M_r fragments (lane a), again suggesting the resistance of this area to further cleavage.

Additional evidence in support of the given ordering of fragments comes from the fragmentation pattern of a prominent 85K fragment of caldesmon$_{125}$ arising during purification. Cleavage of this molecule by cyanogen bromide gave rise to a series of fragments apparently identical with CB10–CB13 (Figure 5, compare lanes a and b). CB8 is also produced, but no fragment corresponding to CB9 is observed (note the comparative lack of material just below CB8 in lane b). These

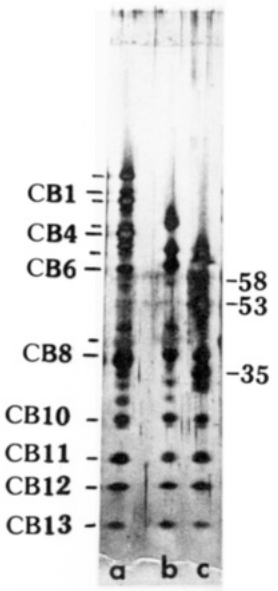

FIGURE 5: SDS–PAGE of partially CNBr-cleaved caldesmons. A silver-stained gel showing the pattern of fragments derived from caldesmon$_{125}$ (lane a), the 85-kDa fragment of caldesmon$_{125}$ (lane b), and caldesmon$_{72}$ (lane c). Protein bands were excised from an initial gel before CNBr cleavage. The positions of CB8–CB13 are marked on the left. Numbers on the right refer to the relative molecular mass of several major fragments of caldesmon$_{72}$ (lane c).

assignments were confirmed by immunoblotting with the fragment-specific antibodies (data not shown). On this basis, it is clear that the 85K is derived from the left-hand end of caldesmon$_{125}$, as shown in Figure 3, and is very similar to the CB3 fragment. As would be predicted from the map, the 85K also gives rise to CB5–CB7. Taken together, the various types of evidence all indicate the essential correctness of the map shown in Figure 3.

Mapping of Caldesmon$_{72}$. In order to compare the low M_r form of caldesmon (caldesmon$_{72}$) with caldesmon$_{125}$, we wished to map the shorter molecule. Since it was only available in much smaller quantities, a single cleavage combined with immunoblotting was used. Figure 5, lane c, shows the CNBr cleavage pattern of caldesmon$_{72}$ after silver staining. This protein gave rise to the same set of low M_r fragments (CB10–CB13) seen in caldesmon$_{125}$ and its 85-kDa fragment, showing that it contained at least this end of the larger molecule. Additional major fragments of M_r 58 000, 53 000, and 35 000 are also observed: these can be accounted for as complementary to CB13, CB12, and CB8 by their apparent molecular mass. The results with immunoblotting were consistent with this (Figure 6); antibodies to CB13 recognized CB8 and the uncleaved molecule (lane d). CB12 and CB13 could also be seen after longer exposure of the autoradiograph. These antibodies did not recognize the major fragments of M_r 58, 53, or 35 kDa. Anti-CB11 reacted with CB8 and the two large peptides of M_r 58 and 53 kDa (lane c). It also recognized a third fragment, which was much less readily seen in the stained gel (M_r of 49 kDa). This seems to be due to a minor cleavage site in CB11 since the fragment is not recognized by anti-CB13. A fragment of apparently similar origin also arises in cleavage of the 85-kDa molecule. From this information it is clear that caldesmon$_{72}$ has a map that is consistent with its being a portion of caldesmon$_{125}$. No additional or different cleavage sites need be postulated to explain the fragmentation pattern.

It was, however, surprising that the antibodies to the central part of caldesmon$_{125}$ (CB7) showed only a trace of reactivity with caldesmon$_{72}$ (lane b). Such a result might arise if the

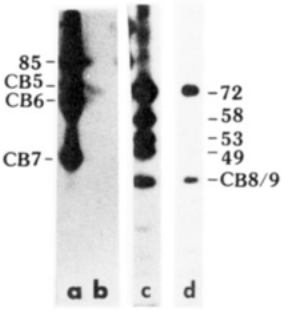

FIGURE 6: Immunoblot of partially cleaved caldesmon$_{72}$ with fragment-specific antibodies. CNBr-cleaved caldesmon$_{72}$ (lanes b–d) or 85-kDa fragment (lane a) after electrophoresis in a 6–15% gel was transferred to nitrocellulose and probed with antibodies specific for CB7 (lanes a and b), CB11 (lane c), or CB13 (lane d). The position of the uncleaved 85-kDa fragment is marked on the left. Numbers on the right right refer to the apparent molecular mass in kilodaltons of uncleaved caldesmon$_{72}$ and three fragments that react with anti-CB11 (lane c).

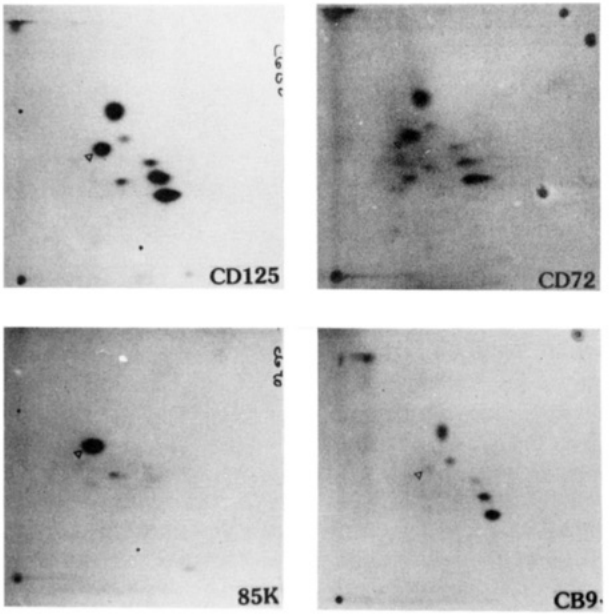

FIGURE 7: [^{125}I]Iodopeptide maps of caldesmons and fragments. Tryptic peptides of iodinated proteins were electrophoresed in the horizontal dimension and chromatographed in the vertical. The origin is in the lower left-hand corner of each plate. CD125 refers to caldesmon$_{125}$, CD72 to caldesmon$_{72}$, and 85K to the 85-kDa fragment of caldesmon$_{125}$. Spots in the upper right corner of the CD72 and CB9 maps are radioactive ink used for orientation. The position of the major peptide found in the 85-kDa fragment is marked with an arrowhead.

antibodies recognized only a portion of CB7 that was missing in the caldesmon$_{72}$, but this seemed a remote possibility with polyclonal antibodies. Another possibility is that the central sequences of caldesmon$_{125}$ were absent in caldesmon$_{72}$.

^{125}I-Peptide Mapping. To investigate this question other markers for the CB9 fragment were required. (No antibodies that recognized this region of caldesmon$_{125}$ were present in the polyclonal antisera; hence, the immunoblot method used above could not be applied.) Tryptic peptide mapping of iodinated proteins or peptides offered a method of obtaining markers for the different fragments. When applied to the whole molecule, four major iodinated peptides were found in caldesmon$_{125}$ (Figure 7). These spots originate from the two ends of the molecule, as revealed by mapping the separated fragments. The CB8 and CB9 fragments could be slightly separated on a 15% gel (not shown) and excised individually for mapping. Three of the four major iodinated peptides arise

from CB9 (Figure 7) located at one extreme of caldesmon$_{125}$, and the other one (marked with arrowhead in Figure 7) from the remainder of the molecule, represented by the 85K fragment in Figure 7. A trace of the latter peptide is visible in the CB9 map, probably due to contamination by CB8. This peptide has been further localized to CB13 (not shown). Hence, [^{125}I]iodopeptide maps by themselves give markers for both ends of caldesmon$_{125}$.

Application of this method to caldesmon$_{72}$ gave rise to a pattern of spots virtually identical with those of caldesmon$_{125}$ (compare top panels in Figure 7). This result clearly indicates the presence of sequences in caldesmon$_{72}$ that are found at the two ends of caldesmon$_{125}$. Since the iodinations were performed on caldesmon$_{72}$ excised from gels, the results cannot be due to contamination by caldesmon$_{125}$. That these peptides were present in the expected fragments of caldesmon$_{72}$ was verified by iodopeptide mapping of the 38-kDa (CB8) and 35-kDa fragments that arise from a single cleavage of caldesmon$_{72}$.p The three spots found together in CB9 of the caldesmon$_{125}$ molecule are also found together in caldesmon$_{72}$, but in the 35-kDa fragment. The single remaining spot is found in the 38-kDa CB8. Hence, the 35-kDa fragment corresponds to CB9 in the larger form of caldesmon. The extent of the identity is not clear from these experiments but includes at least the iodinated peptides.

Discussion

The results of this investigation demonstrate that caldesmon$_{72}$ contains sequences found at both termini of caldesmon$_{125}$. It is clear that caldesmon$_{72}$ is too small to span sites at both ends of the 125-kDa molecule, since even the larger 85K fragment does not contain the ^{125}I peptides of the caldesmon$_{125}$ CB9 terminus. Therefore, some of the central sequences of caldesmon$_{125}$ are absent in caldesmon$_{72}$. This conclusion also explains the very poor reaction of antibodies directed to the central area (CB7) with caldesmon$_{72}$. The results also establish that caldesmon$_{72}$ is not a fragment of caldesmon$_{125}$, a conclusion reached by others on the basis of indirect arguments (Owada et al., 1984; Bretscher & Lynch, 1985).

The mechanisms used by the cell to produce two or three related forms of caldesmon are not known, but the two most obvious possibilities are that both proteins derive from a single gene by an alternative splicing event or each protein comes from a separate but related gene. Alternative splicing has been detected in a variety of genes but is particularly common among contractile protein genes, e.g. tropomyosin, troponin, and myosin light chains [reviewed in Breitbart et al. (1987)]. In these cases, there are significant differences in the properties of the alternate proteins produced. Similarly, it would be expected that the different forms of caldesmon will also exhibit differences in activities, although these are not evident so far. Investigation at the nucleic acid level will be required to distinguish how the different forms are made and may also shed light on the function of the central sequences.

The map of cyanogen bromide cleavage points in caldesmon$_{125}$ is much simpler than might be anticipated. Since caldesmon$_{125}$ has 17 methionine residues/mol (Ngai & Walsh, 1985b), an equal number of cleavage sites was expected. It is possible that these methionines are located in clusters, giving rise to only one apparent cleavage per group, but it is more likely that different methionine residues differ greatly in their susceptibility to reaction with cyanogen bromide (Pepinsky, 1983). When larger amounts of protein are loaded on SDS gels, a total of at least 30 fragments can be distinguished, consistent with a larger number of cleavage points. An additional complexity was expected to arise from the heterogeneity of caldesmon$_{125}$ as isolated. Gizzard caldesmon appears as a closely spaced doublet (see Figure 1) on SDS gels and also focuses in multiple bands in isoelectric focusing gels (Bretscher, 1984). These variations did not interfere with construction of the map, however, probably because the upper band of the caldesmon$_{125}$ doublet was relatively minor (Figure 1) and pI would not affect migration in SDS gels.[2]

The several known activities of caldesmon—actin and calmodulin binding and inhibition of myosin ATPase—have recently been found to reside in a terminal proteolytic fragment of ~20 kDa (Szpacenko & Dabrowska, 1986; Fujii et al., 1987). There is some evidence to suggest this end is the carboxyl terminal. We have found that the 85-kDa fragment retains all of the activities. Thus, the CB13 end of caldesmon$_{125}$ found in the 85K fragment must be the "active" end, and CB12 probably corresponds fairly closely to the tryptic fragment. We have found that one major site of phosphorylation by protein kinase C is also located in CB13 (unpublished results). The proximity of the various sites may allow for direct interactions among them, leaving the rest of the protein to engage in other, presently unknown, activities.

Once established, cleavage site maps are useful for a variety of purposes in addition to structural comparisons of proteins as done here. For example, they can be used to determine the relative location of any covalent marker (e.g., glycosylation or phosphorylation sites) or for comparison with DNA-derived protein sequences. Of course, the resolving power of the map is limited by the distance between cleavage points. Nevertheless, a great deal of useful information is potentially available. It is anticipated that the maps of caldesmon$_{125}$ and caldesmon$_{72}$ will be valuable in further investigation of this apparent family of proteins.

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[2] As shown by Lynch et al. (1987), CNBr cleavages of the upper and lower bands of the caldesmon$_{125}$ doublet give rise to the same number of fragments but those corresponding to CB1–CB7 differ in apparent molecular weight depending on the source. Cleavage of the higher M_r caldesmon band gives rise to fragments of 2 kDa greater M_r. Fragments CB8–CB13 are, however, identical [see Figure 2 in Lynch et al. (1987)]. From the map of CNBr cleavage points we have developed (Figure 3) this difference in pattern must arise from extra mass in the central area (CB7) since it is only fragments containing this area that are larger. The doublet of caldesmon must therefore represent two different forms of the protein, created either by synthesis from different mRNAs or by post-translational modification. In view of our findings with caldesmon$_{72}$, the former seems more likely.

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Molecular Characterization of the AP_{180} Coated Vesicle Assembly Protein†

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ABSTRACT: Recently, a new clathrin assembly protein (AP_{180}) has been purified from coated vesicles of bovine brain (Ahle & Ungewickell, 1986). This protein has been shown to promote polymerization of clathrin into a homogeneous population of baskets under conditions where pure clathrin does not polymerize by itself. We have purified this protein from coated vesicles by a simpler method than has been reported. The method involves a gel filtration step on a Sephacryl S-300 column, in 0.5 M Tris-HCl, pH 8.0, and a hydroxylapatite column eluted with 10 mM sodium phosphate/0.5 M Tris-HCl, pH 7.0. By running SDS gels over an extended period of time (5-15% gradient gel, 10 mA for the first 12 h followed by 20 mA for the next 3-4 h) after the marker dye entered the electrode buffer, we have been able to separate AP_{180} from clathrin heavy chain on the gels. This enabled us to determine its stoichiometry to clathrin heavy chains in isolated coated vesicles and assembled baskets, and was helpful in the purification procedure. The apparent molecular weight of the pure protein on SDS gels was about 180000, yet gel filtration yielded values of about 120000. Thus, we undertook the molecular weight determination by another independent method, sedimentation equilibrium analysis, and found a molecular weight of 115000 and a sedimentation coefficient of 3.50 ± 0.05 S. Circular dichroism data revealed that it has 30% helical structure, 14% β-structure, 27% β-sheet, and the rest random peptides. A scan of the Coomassie Blue stained electrophoretic gel pattern of the polymerized baskets showed that every AP_{180} protein molecule can polymerize approximately one clathrin triskelion into baskets, at 0.1 M Mes-NaOH, pH 6.5. AP_{180} is also involved in binding of clathrin to stripped vesicles, and in this respect it resembles the other assembly proteins of 110-100-50-47 kDa of coated vesicles.

Clathrin-coated vesicles are involved in a variety of cellular processes, namely, membrane recycling, receptor-mediated endocytosis, and transfer of proteins across and between membranes (Brown et al., 1983; Goldstein et al., 1985; Keen, 1985; Heuser & Reese, 1973). The principal protein of the coat of coated vesicles is clathrin with three identical subunits of 180 kilodaltons (kDa)[1] and three light chains of two sizes, i.e., 33 and 36 kDa (Kirchhausen & Harrison, 1981; Pretorius et al., 1981). Clathrin can be dissociated from coated vesicles by a variety of processes, namely, treatment with 0.5 M Tris-HCl, pH 8.0, dialysis against 0.01 M Tris-HCl, pH 8.5, and treatment with 2 M urea (Keen et al., 1979; Pearse & Robinson, 1984; Pretorius et al., 1981; Schook et al., 1979; Woodward & Roth, 1979). Depending on the procedure used to obtain the clathrin-containing fraction, a certain proportion of a group of proteins of 110-100-50-47 kDa remain partly associated with the vesicle and partly with clathrin. Clathrin can then be separated from the above associated proteins by gel filtration in 0.5 M Tris-HCl, pH 8.0, on a Sephacryl S-300 or Sepharose 4B-Cl column (Keen et al., 1979; Prasad et al., 1986). Clathrin thus purified can be polymerized by reducing the pH to 6.0-6.2 in the presence of 0.1 M Mes-NaOH or in the pH range of 6.2-6.5 by the addition of millimolar Ca^{2+} concentrations (Keen et al., 1979; Prasad et al., 1985). The polymerized clathrin resembles the lattice coat of the isolated coated vesicles but with a variety of sizes of basket-like

† Dedicated to the memory of the late Dr. Harold Edelhoch, in whose laboratory the authors have begun their work on clathrin.

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[1] Abbreviations: CV(s), coated vesicle(s); UV(s), uncoated vesicle(s); ANM, AN-maleimide or *N*-(1-anilinonaphthalenyl)maleimide; AN, anilinonaphthalene; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); CHC, clathrin heavy chain; LCs, light chains.