Caldesmon is a major actin-binding protein identified in smooth muscle and many non-muscle cells. It also interacts with calmodulin and a number of other acidic proteins. We have shown previously that the polypeptide stretch from Val^{629} to Ser^{666} near the C terminus contains a calmodulin binding site (Wang, C-L. A., Wang, L.-W. C., Xu, S., Lu, R. C., Saavedra-Alanis, V., and Bryan, J. (1991) J. Biol. Chem. 266, 9166–9172). On the other hand, Bartegi et al. (Bartegi, A., Fattoum, A., Derancourt, D., and Kassab, R. (1989) J. Biol. Chem. 264, 15231–15238) reported a cyanogen bromide fragment beginning at Trp^{596} which is also capable of binding both calmodulin and actin. A comparison of the overlapping sequence between these two peptides suggests that this calmodulin binding site is localized in a 7-residue segment, Trp-Glu-Lys-Gly-Asn-Val-Phe^{856}. We have chemically synthesized an 18-residue peptide (GS17C, from Gly^{651} to Ser^{667} with an added cysteine at the C terminus) that contains this segment. This peptide was purified by high performance liquid chromatography and labeled with fluorescent probes at the terminal cysteine residue. We found that GS17C indeed binds calmodulin in a Ca^{2+}-dependent manner ($K_d = 8 \times 10^{-7}$ M) and appears to compete with caldesmon. Interestingly, this synthetic peptide also cosediments with F-actin, binding to actin and appears to compete with caldesmon. This suggests that this segment may act concurrently with the phosphorylation-activated ATPase activity of myosin (for reviews, see Pritchard and Moody, 1986; Chalovich, 1988). Furthermore, this synthetic peptide also cosediments with F-actin, indicating that this peptide segment does not contain the inhibitory region.

MATERIALS AND METHODS

Caldesmon was purified from fresh chicken gizzard according to published procedures (Lynch and Bretscher, 1986). Actin was isolated from rabbit skeletal muscle (Spudich and Watt, 1971). Smooth muscle myosin was isolated from chicken gizzard (Sobieszek and Small, 1976). Phosphorylation of myosin was achieved by using standard procedures (Ikobe et al., 1978). Calmodulin was purified from bovine brain using phenyl Sepharose (Edman and Kaetzel, 1983). Purified calmodulin was attached to CNBr-activated Sepharose 4B (from Sigma) to make the affinity column according to manufacturer’s procedures.

Synthetic peptides were prepared on an automated peptide synthesizer (a modified version of model S-4 of Milligen) using Fmoc chemistry. Fmoc-amino acids and resins were from Advanced ChemTech (Louisville, KY). Other reagents were from commercial sources.

The abbreviations used are: Fmoc, N-(9-fluorenylmethoxycarbonyl); GS17C, the 18-amino acid residue synthetic peptide (GVRIKSMWEKGNYVFFSSC) that contains the sequence corresponding to the peptide stretch from Gly^{651} to Ser^{667} of caldesmon plus an artificial cysteine residue at the C terminus; C16AA, the 17-amino acid residue synthetic peptide (CAEPLNRSKQGSMKPA) that corresponds to the sequence from Ala^{634} to Ala^{650} of caldesmon plus an artificial cysteine residue at the N terminus; IANBD, iodoacetamido-4-nitrobenz-2-oxa-1,3-diazole; IAEDANS, 5-(iodoacetamidothyl)aminalophthalene-1-sulfonic acid; HPLC, high performance liquid chromatography.
an on-line phenylthiohydantoin analyzer (model 120A). The amino acid composition analyses were performed on a Beckman high performance amino acid analyzer (model 3300).

To facilitate chemical modification of the peptides we have put an artificial cysteine residue at either the N terminus or the C terminus. For labeling, these peptides were first reduced with 100 mM dithiothreitol, followed by exhaustive dialysis using M, 1,000 cut-off dialysis tubings (from Spectrum Medical Industries, Inc.); the peptides were then reacted with IANBD or IAEARDS (peptide:labeling reagent = 1:10) for 4-5 h, filtered through Milipore filter, and finally dialyzed against the buffer. The amount of label was quantified by the absorbance at 480 nm using an extinction coefficient of 25,000 cm⁻¹ M⁻¹ for IANBD (Rosenfeld and Taylor, 1986), or at 337 nm using an extinction coefficient of 6,000 cm⁻¹ M⁻¹ for IAEARDS (Hudson and Weber, 1973).

Fluorescence measurements were carried out on a Perkin-Elmer MFP-4 fluorometer. Co-aedimentation with F-actin was done by centrifugation in a Beckman Airfuge. The samples were incubated for 30 min at 25 °C in a solution containing 50 mM KCl, 2 mM Tris (pH 7.5), 2 mM MgCl₂, 0.2 mM CaCl₂, and 0.4 mM ATP, then centrifuged for 30 min at 100,000 × g. ATPase activity assays were performed as described (Wang et al., 1991).

RESULTS AND DISCUSSION

Characterization of Synthetic Peptides—GS17C contains 18 amino acid residues: GVRKIKSMWEGKNVFSSC; the sequence corresponds to the peptide stretch from Gly⁵⁶⁰ to Ser⁶⁶⁷ of caldesmon (Bryan et al., 1989), plus an artificial cysteine residue added at the C terminus to facilitate labeling and monitoring. After trifluoroacetic acid cleavage GS17C was eluted as a single major peak by reverse phase HPLC at 31% of CH₃CN (Fig. 1A). Since both amino acid composition analysis and sequence analysis are consistent with the expected sequence (Table I), this peptide was used without further purification.

Another peptide, C16AA (CAEFLNKSQQGSGKMPA), which corresponds to the sequence from Ala⁶⁰⁴ to Ala⁶⁰⁹ of caldesmon plus an artificial cysteine residue at the N terminus, was also synthesized as a control. Upon separation by HPLC, the synthesized C16AA was eluted as three peaks (Fig. 1B), all having the same amino acid composition (Table I). The major peak, eluted at 24.9% of Solvent B, was collected for IANBD labeling and fluorescence titration experiments, while both the purified fraction and the whole mixture were used for testing binding to calmodulin-Sepharose. Neither the purified major component nor the entire mixture showed any interaction with calmodulin (see below).

Both GS17C and C16AA are readily soluble in aqueous buffer. Since GS17C contains a tryptophan residue, the UV absorption spectrum exhibits characteristic features of tryptophan absorption. The concentration of the peptides was determined by three independent methods: (i) amino acid composition; (ii) quantification of free sulfhydryls by the dithiobis-(2-nitrobenzoic acid) test; (iii) absorption of tryptophan (for GS17C only) using an extinction coefficient for tryptophan of 5,600 cm⁻¹ M⁻¹ (Bailey, 1986). All three measurements gave reasonably close estimates.

Interaction with Calmodulin—Binding of peptides to calmodulin was assayed by calmodulin-Sepharose affinity column chromatography and by fluorescence measurements. In the first approach, synthetic peptides were loaded to a calmodulin affinity column in the presence of Ca²⁺; the column was then washed with a buffer that contained Ca²⁺, and the bound fractions eluted with an EDTA-containing buffer. GS17C was retained to calmodulin-Sepharose (Fig. 2). C16AA, on the other hand, did not bind to the calmodulin column.

As GS17C contains a tryptophan residue whereas calmodulin contains none, binding of the peptide to calmodulin was also studied by monitoring the change of tryptophan fluorescence. Upon addition of calmodulin in the presence of Ca²⁺, there was an increase in the fluorescence intensity accompanied by a blue shift of the peak maximum, the change being fully reversible with addition of EDTA (Fig. 3A). Since Ca²⁺ alone did not have any effect on the emission spectrum, the observed fluorescence enhancement must have resulted from binding of calmodulin to the peptide, thereby changing the environment of the indole ring to a more hydrophobic one. It

| Amino acid | GS17C | C16AA |
|-----------|-------|-------|
| Asx       | 2.02  | 2.0   | 1.2 | 1.0 |
| Ser       | 3.0   | 1.0   | 1.8 | 2.0 |
| Glx       | 1.1   | 1.0   | 2.0 | 2.0 |
| Pro       | 2.1   | 2.0   | 1.0 | 1.0 |
| Gly       | 2.9   | 3.0   |     |     |
| Ala       | 1.9   | 2.0   | 1.0 | 1.0 |
| Val       | 0.5   | 1.0   | 1.0 | 1.0 |
| Met       | 1.1   | 1.0   | 1.0 | 1.0 |
| Ile       | 0.9   | 1.0   | 1.0 | 1.0 |
| Leu       | 2.6   | 3.0   | 3.0 | 3.0 |
| Trp       | 1.2   | 1.0   | 1.0 | 1.0 |

Values shown here are the numbers of residues in each peptide.
is noteworthy that the tryptophan fluorescence of intact caldesmon undergoes a calmodulin-induced increase (Shirinsky et al., 1988), suggesting that at least 1 tryptophan residue is affected by binding of calmodulin, either directly or allosterically. The present result is consistent with the idea that the tryptophan residue is actually located within or near the calmodulin binding site.

From the titration data a dissociation constant of \(7.9 \times 10^{-7} \) M was determined by curve fitting, assuming a 1:1 stoichiometry (Fig. 3B). This affinity is comparable to, although slightly higher than, the reported value of the intact caldesmon toward calmodulin (\(K_C = 1.25 \times 10^{-6}\)) (Smith et al., 1987). The same binding parameter was obtained when the titration was carried out using GS17C labeled with IANBD at the cysteine residue, in which case a Ca\(^{2+}\)/calmodulin-dependent, 5-fold increase of the NBD emission was observed (Fig. 3C). In contrast, the emission spectrum of the NBD-labeled C16AA was insensitive to addition of calmodulin.

Thus it is clear that GS17C interacts with calmodulin whereas C16AA does not. The C-terminal calmodulin binding site of caldesmon is therefore localized in the peptide stretch between Gly\(^{651}\) and Ser\(^{697}\), not between Ala\(^{594}\) and Ala\(^{600}\). Although neither of the two peptides bears any resemblance with the consensus sequence of other known calmodulin-binding peptides, the latter segment has been suggested earlier to be a potential candidate for calmodulin binding because of its amphipathic character when fitted onto a helical wheel (Leszyk et al., 1989). Such a prediction turned out to be incorrect, as shown in this study, suggesting that either the proposed helix does not exist or the amphipathic characteristics are not applicable to the calmodulin-caldesmon interactions. In either case binding of calmodulin to caldesmon is quite different from that to the other known calmodulin targets such as myosin light chain kinase and phosphodiesterase. This is reflected by the fact that calmodulin has a much lower affinity for caldesmon (\(K_C \sim 10^{-8}\) M) than for the other target proteins (typically \(K_C \sim 10^{-4}\) M). Caldesmon may therefore represent a different class of calmodulin targets in the cell.
was used as an indicator for the binding of calmodulin to caldesmon. We found that with the presence of GS17C, the calmodulin-induced enhancement of NBD fluorescence was attenuated (Fig. 4), suggesting that binding of the peptide prevents calmodulin from interacting with caldesmon, most likely via direct competition. As expected, the lost fluorescence enhancement of caldesmon-NBD was restored by addition of more calmodulin. ClGAA, which does not interact with calmodulin, did not appreciably change the calmodulin-enhanced fluorescence intensity of caldesmon-NBD. In the absence of calmodulin and/or Ca^{2+}, there was no increase in the NBD emission, and the peptides had no effect on the fluorescence intensity. Thus the interaction between GS17C and calmodulin appears to be quite specific, suggesting that both the synthetic peptide and the intact caldesmon in fact bind at the same site on the surface of the calmodulin molecule.

**Binding of F-actin**—The interaction between GS17C and actin was examined by co-sedimentation with actin filaments. When GS17C was incubated with F-actin, followed by centrifugation, the synthetic peptide was found both in the pellet and in the supernatant. The control peptide, C16AA, however, was present only in the supernatant fraction. Thus the observed partial co-sedimentation was interpreted as resulting from a weak-to-moderate binding of GS17C to F-actin. Such binding was inhibited by Ca^{2+}/calmodulin; in the presence of Ca^{2+}, the addition of calmodulin prevented GS17C from co-sedimenting with actin (Fig. 5).

Binding of GS17C to F-actin was unexpected. Although previous studies on the caldesmon fragments tentatively put one of the two actin binding sites between Leu^{652} and Val^{659} (Wang et al., 1991), the boundary of this site at the C-terminal end was in fact rather uncertain (see “Discussion” in Wang et al., 1991). It is possible that the actin binding site I contains some of the residues in GS17C. It is however not clear whether the actin binding site actually overlaps the calmodulin binding site. Studies on various synthetic peptides around this region are currently under way.

**Effect on the ATPase Activity**—Since GS17C can interact with calmodulin and F-actin, and binding to F-actin is abolished by Ca^{2+}/calmodulin in the same manner as the intact caldesmon, it was of interest to see whether the peptide had any effect on the actomyosin ATPase activity. As shown in Table II, GS17C does not inhibit actomyosin ATPase activity.

![Graph](image1)

**Fig. 4. Competition between caldesmon and GS17C for calmodulin binding.** Emission spectra (λ_ε = 490 nm) of NBD-labeled caldesmon (1 μM) in 20 mM Tris-HCl (pH 7.5), 30 mM KCl, and 2 mM CaCl₂ (lower solid line), plus 5 μM calmodulin (upper solid line), with additional 5 μM GS17C (heavy line), and with more calmodulin (5 μM) added (dashed line).

**Table II**

| Addition | ATPase activity (μmol/mg/min) |
|----------|-----------------------------|
| None     | 32.9                        |
| Caldesmon (1.3 μM) | 21.4                   |
| Caldesmon (1.3 μM) + calmodulin (16 μM) | 33.5                |
| GS17C (12 μM) | 33.1                     |
| Calmodulin (16 μM) | 34.1                     |

^aStock solutions contained 2.6 mg/ml caldesmon or 0.5 mg/ml GS17C.

^bTo reverse the inhibition 20 μl of calmodulin (3.5 mg/ml) was added.

This is consistent with the suggestion that the inhibitory site is located more toward the C-terminal end of caldesmon (Wang et al., 1991).

**CONCLUSIONS**

A 17-residue peptide (not counting the added cysteine residue), GS17C, which corresponds to the segment from Gly^{651} to Ser^{667} of chicken gizzard caldesmon, was shown to interact with calmodulin in a Ca^{2+}-dependent manner. GS17C directly competes with the intact caldesmon for binding to calmodulin, suggesting that both GS17C and caldesmon are interacting with the same region of the calmodulin molecule and that the synthetic peptide is in a configuration similar to that of the corresponding peptide stretch in the intact caldesmon. This segment apparently also interacts with actin, as evidenced by the finding that GS17C co-sediments with actin filaments. It is not clear whether (or to what extent) the actin binding site overlaps with the calmodulin binding site, but because the entire segment contains only 17 amino acid residues, these two sites must be very close to each other. This may explain why calmodulin displaces GS17C from F-actin. The displacement, however, is only partial when the intact caldesmon is present.
used, suggesting that other parts of the caldesmon molecule must also interact with actin. This is consistent with the idea that there exists a second (and probably weaker) actin binding domain (Wang et al., 1991). Since GS17C does not inhibit the actomyosin ATPase activity, the simplest interpretation is that the inhibitory domain is localized in the other actin binding site.

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