Some properties of duck gizzard caldesmon

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The domain structure of duck gizzard caldesmon was investigated. A single thiol group is located in the vicinity of the C-terminus of the protein. A simple method for the purification of a short (21 kDa) C-terminal peptide formed after chemical cleavage of caldesmon at cysteine residues was evolved. The C-terminal peptide of caldesmon interacts with calmodulin with an affinity one order of magnitude higher than that of native caldesmon. The Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C) transfers about 2 mol of phosphate per mol of caldesmon. All sites phosphorylated by protein kinase C are located in the short (21 kDa) C-terminal peptide of caldesmon. Phosphorylation does not affect the interaction of caldesmon with calmodulin.

INTRODUCTION

Caldesmon is one of the major actin- and calmodulin-binding proteins of smooth muscle (Sobue *et al.*, 1981; Marston & Smith, 1985). In the complex with calmodulin or another Ca^{2+} -binding protein, caldesmon seems to be involved in the regulation of actin–myosin interaction (Marston & Smith, 1985; Lash *et al.*, 1986; Smith *et al.*, 1987). Caldesmon is phosphorylated by protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase and casein kinase II (Umekawa & Hidaka, 1985; Abougou *et al.*, 1989; Vorotnikov *et al.*, 1988). A hypothesis has been put forward that caldesmon phosphorylation can influence its interaction with other proteins of the contractile apparatus as well as the ability of caldesmon to regulate the actin–myosin interaction (Ngai & Walsh, 1987; Sutherland & Walsh, 1989). In order to understand the mechanism of action of caldesmon, it is desirable to obtain detailed information on the structure and domain organization of this protein. In recent years there have been several published papers devoted to the investigation of chicken gizzard caldesmon structure (Szpacenko & Dabrowska, 1986; Fujii *et al.*, 1987; Sutherland & Walsh, 1989; Wang *et al.*, 1989; Riseman *et al.*, 1989; Makuch *et al.*, 1989; Bryan *et al.*, 1989). The aim of the present work was to investigate the structure of gizzard caldesmon with the main emphasis on the location of phosphorylation sites and cysteine residues in the structure of the protein as well as on calmodulin–caldesmon interaction.

MATERIALS AND METHODS

The initial steps in duck gizzard caldesmon purification [extraction, heating and $(\text{NH}_4)_2\text{SO}_4$ fractionation] were performed as described by Lynch & Bretscher (1986). After dialysis, the protein was concentrated on a DE-Toyopearl 650 M column dialysed against 20 mM-Mops/NaOH buffer, pH 7.2, containing 0.1 mM-EGTA, 0.1 mM-EDTA, 5 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride, and chromatographed on a phosphocellulose column by the method of Lash *et al.* (1986). After dialysis against 10 mM-imidazole/HCl buffer, pH 7.0, containing 30 mM-NaCl, 5 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride, the fractions containing caldesmon were concentrated on a DE-Toyopearl 650 M column and stored in 10 mM-imidazole/HCl buffer, pH 7.0, containing 0.3 M-NaCl, 5 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride at -20°C . The

molecular mass of caldesmon as determined by SDS/PAGE was 140 kDa.

Protein kinase C and casein kinase II were isolated from rat brain and liver respectively by previously described methods (Vorotnikov *et al.*, 1988; Meggio *et al.*, 1977). Assays of protein kinase activity and caldesmon phosphorylation were performed as described previously (Vorotnikov *et al.*, 1988).

The method of Fuji *et al.* (1987) was used for the cleavage of caldesmon by chymotrypsin. The thiol-group content of caldesmon was determined in 5 mM-imidazole/HCl buffer, pH 7.7, containing 5 M-urea and 0.15 M-NaCl by the Ellman (1959) method. Chemical cleavage of caldesmon at cysteine residues was performed in 100 mM-Tris/HCl buffer, pH 8.5, in the presence of 6 M-urea. The reduced protein (8 mg/ml) was modified by a 4-fold molar excess of 5,5'-dithiobis-(2-nitrobenzoic acid), and the mixed disulphide formed was cleaved by a great excess of KCN (Jacobson *et al.*, 1973). The composition of the peptide mixture was analysed by SDS/PAGE (Laemmli, 1970). To obtain preparative quantities of the C-terminal peptide of caldesmon, 10 mg of protein was cleaved at cysteine residues, and the hydrolysate (1.5 ml) was mixed with a dense suspension (1 ml) of DEAE-cellulose (Whatman DE 52) equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. After a 20 min incubation at room temperature, the cellulose was removed by centrifugation, and the supernatant was mixed with a new portion (1 ml) of DEAE-cellulose. The supernatant obtained after centrifugation was acidified by acetic acid to pH 4.6 and loaded on a small column packed with CM-cellulose (Whatman CM 52) and equilibrated with 15 mM-sodium acetate buffer, pH 4.6. After the column had been washed with water, adsorbed material was eluted by 0.1 M-HCl and freeze-dried. The yield was 0.8 mg of homogeneous 21 kDa peptide from 10 mg of caldesmon.

Calmodulin was isolated from bovine brain by the method of Gopalakrishna & Anderson (1982). The protein was modified by dansyl chloride as described by Kincaid *et al.* (1982). Under the conditions used no more than 2 mol of dansyl groups was incorporated/mol of calmodulin. The fluorescence of dansyl-calmodulin was excited at 330 nm and recorded within the range 400–600 nm on a Hitachi 3000 fluorimeter. All fluorescence measurements were performed at 20°C in 50 mM-imidazole/HCl buffer, pH 7.0, containing 1 mM- MgCl_2 and 2 mM- Ca^{2+} /EGTA to maintain a desirable value of pCa in the incubation medium.

Protein concentration was determined spectrophotometrically by taking $A_{278}^{0.1\%}$ to be 3.3 for caldesmon (Graceffa *et al.*, 1988) and 2.0 for calmodulin (Szpacenko & Dabrowska, 1986) or by

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the method of Spector (1978) for the 21 kDa peptide of caldesmon. In the last-mentioned case intact caldesmon was used as a standard. The molecular mass of caldesmon was taken to be 87 kDa (Bryan *et al.*, 1989).

RESULTS

Location of thiol groups and phosphorylation sites

Titration of duck gizzard caldesmon with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of 5 M-urea, we determined 1.0 mol of thiol groups/mol (87 kDa) of caldesmon. This value is roughly half the corresponding value for chicken gizzard caldesmon (Horiuchi & Chacko, 1988; Riseman *et al.*, 1989; Bryan *et al.*, 1989). In full accord with the data obtained, we found that the chemical cleavage of duck gizzard caldesmon at cysteine residues leads to the formation of only two peptides, with molecular masses 115 kDa and 21 kDa (Fig. 1). The data obtained indicate that duck gizzard caldesmon contains only one thiol group.

To locate the sites phosphorylated by protein kinase C and casein kinase II, we cleaved the phosphorylated caldesmon at cysteine residues. Of the two peptides formed after the cleavage of protein kinase C-phosphorylated caldesmon, only the smaller one (21 kDa) contained sites of phosphorylation. At the same time, if caldesmon was phosphorylated by casein kinase II, of two cleavage products only the larger one (115 kDa) was phosphorylated. After chymotrypsinolysis of caldesmon phosphorylated by protein kinase C, the sites of phosphorylation were detected in 40–45 kDa and 19–21 kDa peptides (Vorotnikov *et al.*, 1988). Both peptides were identified as C-terminal peptides of caldesmon (Makuch *et al.*, 1989). Thus both the single thiol group and the sites phosphorylated by protein kinase C are located in the C-terminus of caldesmon in the vicinity of the site involved in the interaction with calmodulin.

Isolation of the 21 kDa C-terminal peptide of caldesmon and investigation of its properties

The fact that duck gizzard caldesmon contain only one thiol group enables the development of a simple and rapid method for purification of the short C-terminal peptide of caldesmon that is able to interact with actin and calmodulin. Chemical cleavage of caldesmon proceeds with a high yield (Fig. 1). The C-terminal part of caldesmon is rich in positively charged residues (Bryan *et al.*, 1989); therefore it is not adsorbed on DEAE-cellulose and is tightly bound to CM-cellulose. In contrast, the isoelectric points of the native caldesmon and of its large N-terminal peptide lie at acidic pH values, and therefore they are tightly bound to DEAE-cellulose and are weakly adsorbed on CM-cellulose. Thus, by using stepwise ion-exchange chromatography on DEAE-cellulose

Fig. 1. (a) Electrophoretograms of native caldesmon phosphorylated by protein kinase C (lane 1) or casein kinase II (lane 3) and of peptides obtained after chemical cleavage of these proteins at cysteine residues (lanes 2 and 4 respectively), and (b) autoradiograms of the gels depicted in (a) (lanes 1–4)

A scale of apparent molecular masses is given on the right.

Fig. 2. (a) Fluorescence spectra of isolated dansyl-calmodulin at pCa 8 (curve 1) and pCa 4 (curve 2) and of an equimolar dansyl-calmodulin-C-terminal caldesmon peptide complex at pCa 8 (curve 3) and pCa 4 (curve 4), and (b) titration of dansyl-calmodulin with the C-terminal peptide of caldesmon at pCa 8 (curve 1) and pCa 4 (curve 2)

The incubation mixture (20 °C) contained 50 mM-imidazole/HCl buffer pH 7.0, 1 mM-MgCl₂ and 2 mM-Ca²⁺/EGTA with the desired pCa value. Calmodulin (0.6 μM) was added. The fluorescence was excited at 330 nm and was measured within the range 400–600 nm (in a) or at 485 nm (in b).

and CM-cellulose (see the Materials and methods section), we could effectively purify the C-terminal peptide of caldesmon. The yield of homogeneous peptide was about 35% of theoretical (taking molecular masses of caldesmon and its C-terminal peptide to be 87 kDa and 21 kDa respectively).

To study the interaction of calmodulin with native caldesmon and its C-terminal peptide, we used fluorescence spectroscopy. Dansyl-calmodulin retains its ability to bind Ca²⁺, which is reflected by a 1.5–2-fold increase in the fluorescence intensity and in a 10–15 nm blue-shift of the fluorescence maximum. In the presence of Ca²⁺ an addition of native caldesmon or its C-terminal (21 kDa) peptide induced a 10–15 nm blue-shift of the fluorescence maximum and a 1.5–1.7-fold increase of the dansyl-calmodulin fluorescence (Fig. 2). Trifluoperazine (5 μM) or EGTA (2 mM) completely abolishes the effect of caldesmon or its C-terminal peptide on the fluorescence of dansyl-calmodulin. These data indicate that the change in fluorescence reflects the specific interaction between calmodulin and caldesmon or its short C-terminal peptide.

Titration of dansyl-calmodulin with caldesmon and using the method of Wilkinson (1961), we determined the apparent dissociation constant for the complex formed between these two proteins. Under the conditions used this constant was equal to 1–2 μM. Phosphorylation catalysed by protein kinase C up to 1.5–2 mol of phosphate/mol of caldesmon did not affect the apparent *K_d* for the dissociation of the complex between calmodulin and caldesmon. The half-maximal fluorescence changes of both the isolated calmodulin and equimolar calmodulin-caldesmon complex occurred at pCa 6.6–6.7, i.e. caldesmon did not affect the apparent Ca²⁺-binding constant of calmodulin. At the same time, the Hill plots indicate that caldesmon slightly increases the co-operativity of Ca²⁺ binding by calmodulin. The apparent *K_d* for the dissociation of the complex between calmodulin and the 21 kDa C-terminal peptide of caldesmon is 0.1–0.3 μM, i.e. it is one order of magnitude higher than in the case of native caldesmon. All other changes in the fluorescence of dansyl-calmodulin induced by the C-terminal peptide of caldesmon were similar to those induced by the native

caldesmon. Thus the rather short 21 kDa peptide retains an undamaged calmodulin-binding site of caldesmon.

DISCUSSION

The literature data (Leszyk *et al.*, 1989; Bryan *et al.*, 1989) indicate that one of the two thiol groups of chicken gizzard caldesmon is located 176 residues from the C-terminus. This cysteine residue seems to be very close to one of the calmodulin-binding sites of caldesmon that is located in a 35 kDa C-terminal part of the molecule (Fujii *et al.*, 1987; Yazawa *et al.*, 1987; Leszyk *et al.*, 1989; Riseman *et al.*, 1989). Wang *et al.* (1989) claimed to find the second calmodulin-binding site located close to the N-terminus of caldesmon. This site contains the second thiol group. Thus the two thiol groups of chicken gizzard caldesmon are close to or included in the calmodulin-binding domains.

Duck gizzard caldesmon contains one thiol group located 21 kDa from the C-terminus. The locations of the C-terminal cysteine residues seem to be identical for duck and chicken gizzard caldesmons. However, duck gizzard caldesmon does not contain any other cysteine residues. The C-terminal peptide obtained after chemical cleavage of duck gizzard caldesmon at cysteine residues contains an undamaged calmodulin-binding site. The dissociation constant of the complex calmodulin-C-terminal peptide of caldesmon is one order of magnitude lower than the corresponding value for the calmodulin-caldesmon complex. These results are similar to those obtained by Riseman *et al.* (1989) showing that the 35 kDa C-terminal peptide of chicken gizzard caldesmon forms a tighter complex with calmodulin than does native caldesmon. According to Yazawa *et al.* (1987), the C-terminal fragment of caldesmon is able to increase the affinity of calmodulin for Ca^{2+} . In our case both native caldesmon and its C-terminal peptide only slightly increase the co-operativity and do not affect the apparent constant of Ca^{2+} binding to calmodulin.

The results presented indicate that all sites phosphorylated by protein kinase C are located in the short (21 kDa) C-terminal peptide of caldesmon. This C-terminal peptide of caldesmon is enriched in serine and threonine residues, and therefore it was suggested (Leszyk *et al.*, 1989; Bryan *et al.*, 1989) that this peptide contains the sites phosphorylated by different protein kinases. Although the sites phosphorylated by protein kinase C are located in the vicinity of the calmodulin-binding site of caldesmon, we failed to detect any changes in the interaction between calmodulin and caldesmon induced by its phosphorylation. Casein kinase II is also able to phosphorylate caldesmon, and transfers about 1 mol of phosphate/mol of caldesmon. The sites phosphorylated by this enzyme are located in the N-terminal part of caldesmon (Vorotnikov *et al.*, 1988). It has been proposed

that phosphorylation of certain sites located in the N-terminal part of caldesmon may affect its interaction with smooth-muscle myosin (Sutherland & Walsh, 1989).

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