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Localization of phospholipid-binding sites of caldesmon

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Abstract

The interaction of phosphatidylserine with intact smooth muscle caldesmon and caldesmon fragments obtained by bacterial expression was investigated by means of light scattering. Among these fragments only those derived from the C-terminal part of caldesmon (so-called domain 4) were able to interact with phospholipids. Fragments 606C (residues 606–756), H7 (566–710) and H2 (626–710) form tight complexes with phosphatidylserine, whereas fragments H8 (658–737), H9 (669–737) and fragment H4 (566–624) interact with phospholipids less effectively. It is concluded that the phospholipid-binding site is located in the sequence 626–710 of caldesmon. This sequence contains calmodulin-binding sites and serine residues phosphorylated by protein kinase C and pro-directed protein kinases. This could explain the effects of calmodulin and phosphorylation on the caldesmon–phospholipid interaction described earlier.

Key words: Caldesmon; Phospholipid; Calmodulin; Phosphorylation

1. Introduction

Caldesmon is a multifunctional protein involved in the regulation of smooth muscle contraction [1,2] and organization of the cytoskeleton [3,4]. Caldesmon interacts with actin, tropomyosin and myosin, and many caldesmon properties are regulated by calmodulin and some other Ca-binding proteins [1,2,5]. In certain cell types caldesmon is located in the vicinity of the cell membrane [4,6] and seems to be involved in receptor capping [7] and exocytosis [8]. These facts may be indicative of the binding of caldesmon to biomembranes. Indeed, recently we have shown that caldesmon interacts with phospholipids and that this interaction depends on calmodulin and is regulated by phosphorylation [9,10]. Czurylo et al. [11] confirmed these data and in agreement with our results found that caldesmon preferentially interacts with acidic phospholipids. These properties of caldesmon are similar to those of a number of other actin-binding proteins (such as gelsolin, vilin, destrin, cofilin, profilin etc.) which also interact with phopholipids [12]. The present paper deals with the localization of phospholipid-binding sites of caldesmon.

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Abbreviations: CD, caldesmon; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

2. Materials and methods

Duck gizzard caldesmon and bovine brain calmodulin were isolated as described earlier [13,14]. Fragments of chicken gizzard caldesmon were obtained by bacterial expression in the pMW172 plasmid/BL21 (DE3) cell system described by Redwood and Marston [15]. The following mutants were used in the present investigation: N128 (amino acid residues 1–128), central helix (230–419) and 606C (606–756) (numbering corresponds to the chicken caldesmon sequence published by Bryan et al. [16]). Human caldesmon fragments were obtained as described in [17,18]. The following mutants were used in this investigation (numbering corresponds to the chicken gizzard caldesmon sequence): H2 (residues 626–710), H3 (476–510), H4 (566–624), H6 (476–569), H7 (566–710), H8 (658–737) and H9 (669–737). The purity of all proteins was checked by SDS gel electrophoresis [19] and their concentration was determined by using fluorescamine [20]. We used this approach because both the Lowry and the Bradford methods depend on the amino acid composition and the length of peptides and therefore are not completely applicable for determination of peptide concentration. Knowing the amino acid composition and determining the total quantity of amino groups we were able to estimate the real concentration of peptides. Amino groups of peptides were modified by fluorescamine in 40 mM sodium borate buffer (pH 9.1) containing 1% SDS, and the fluorescence of the adducts formed was excited at 390 nm and measured at 475 nm.

Phospholipids (azolectin (Serva) and phosphatidylserine (bovine brain extract type 5; Sigma) were suspended in 20 mM imidazole-HCl, pH 7.0, containing 0.1 mM EDTA and sonicated on ice under argon 3–5 times (30 s each) with 1 min intervals between sonications in a USDN-A ultrasonic disintegrator (Ukraine) working at maximum output. Phospholipids were diluted with the 20 mM imidazole-HCl, pH 7.0, up to 0.03 mg/ml (or 40 μM taking the average molecular weight of phospholipids as being equal to 750). Light scattering at the right angle was measured in a 1 ml quartz cell at 27°C on a Hitachi F-3000 fluorometer setting the wavelength of excitation and emission at 340 nm. Suspension of phospholipids was titrated by caldesmon or caldesmon fragments (final volume of addition was less than 50 ml). The values obtained were corrected for dilution and initial buffer scattering. Plotting $\sqrt{F/F_0}$ against weight ratio protein/phospholipid and making correction for the scattering induced by unbound protein [21] we deter-

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mined the dependence of the relative molecular weights of the complexes formed on the total concentration of protein added. Assuming this dependence is hyperbolic and using a non-linear regression fit we determined the number of phospholipid molecules involved in caldesmon binding and the concentration of protein ($C_{0.5}$) inducing a half-maximal increase in relative molecular weight. All experiments with calmodulin were performed in the same buffer in the presence of 0.1 mM $CaCl_2$. A 2–4-fold molar excess of calmodulin was added at the end of the titration.

3. Results

A number of different methods have been used for investigation of caldesmon–phospholipid interaction [9–11]. The methods of gel-filtration, ultracentrifugation and native gel-electrophoresis [9,10] are only semiquantitative and require rather large quantities of peptides. The method of intrinsic fluorescence used by Czurylo et al. [11] depends on the presence of Trp residues in peptide and therefore is not universal. We chose the sensitive and quantitative method of light scattering which was earlier successfully used for the investigation of protein–phospholipid interaction [21,22]

As reported earlier [9,10] titration of azolectin or phosphatidylserine with intact caldesmon results in a sharp increase in the light scattering and the half-maximal increase of the relative molecular weight is observed at a caldesmon concentration equal to 0.3–0.5 μM (see Fig. 1 and Table 1). These values agree well with our earlier published results [10]. Using the method of Nelsestuen and Lim [21] we determined the concentrations of free and bound caldesmon and constructed a double reciprocal plot of caldesmon–phospholipid binding. This plot shows considerable downward curvature indicating the complex nature of caldesmon–phospholipid interaction.

We analyzed the dependence of caldesmon–phospholipid interaction on the ionic strength. Increase of the ionic strength up to 150 mM NaCl diminished caldesmon-induced light scattering by 70–80%. This agrees well with earlier published data [10,11]. We found that although caldesmon–phospholipid binding strongly depends on ionic strength it cannot be completely reversed by increasing NaCl concentration up to 0.3 M. We were also unable to reverse caldesmon–phospholipid binding by addition of a 2–4 molar excess of calmodulin over caldesmon. This can mean that the interaction of caldesmon with phospholipids is not completely reversible and that after an initial binding determined by electrostatic interactions, caldesmon induced irreversible changes in the light scattering. A similar mechanism of interaction has been postulated for a number of phospholipid-binding proteins [22].

In order to localize the phospholipid-binding site of caldesmon, we titrated the suspension of phosphatidylserine with three large fragments derived from the N-terminal (N128), central (central helix, residues 230–419) and C-terminal parts (606C) of chicken gizzard caldesmon. N-terminal and central fragments of chicken gizzard caldesmon do not affect the light scattering of phospholipids, whereas the C-terminal fragment induced a large increase in light scattering (Table 1, Fig. 1). We were unable to reach saturation when titrating phospholipids with 606C because addition of large quantities of peptide induced a time dependent decrease of the light scattering, probably caused by aggregation. The data presented indicate that the phospholipid-binding site is located in the C-terminal part of caldesmon.

Table 1
Parameters of interaction of intact caldesmon and its fragments with phosphatidylserine (PS)

Expressed protein	Amino acid numbers (gizzard sequence [16])	Amino acid numbers (human sequence [29])	Molecular weight (kDa)	Maximal F/F_0	Mol PS per mol protein	$C_{0.5}$ (μM)
Caldesmon	1–756	1–793	87	4.04	120	0.4
Chicken caldesmon fragments						
N128	1–128		15.5	1.05	ND	ND
Central helix	230–419		22.1	1.03	ND	ND
606C	606–756		16	>1.9	>50	ND
Human caldesmon fragments						
H2	626–710	683–767	9	1.3	90	0.3
H3	476–510	506–566	7	1.9	25	12.9
H4	566–624	622–681	6.7	1.5	40	1.3
H6	476–569	506–625	14.7	1.06	ND	ND
H7	566–710	622–767	15.6	1.4	100	0.3
H8	658–737	715–793	8.5	1.3	90	0.5
H9	669–737	726–793	7.3	1.4	60	0.6

ND, not determined.

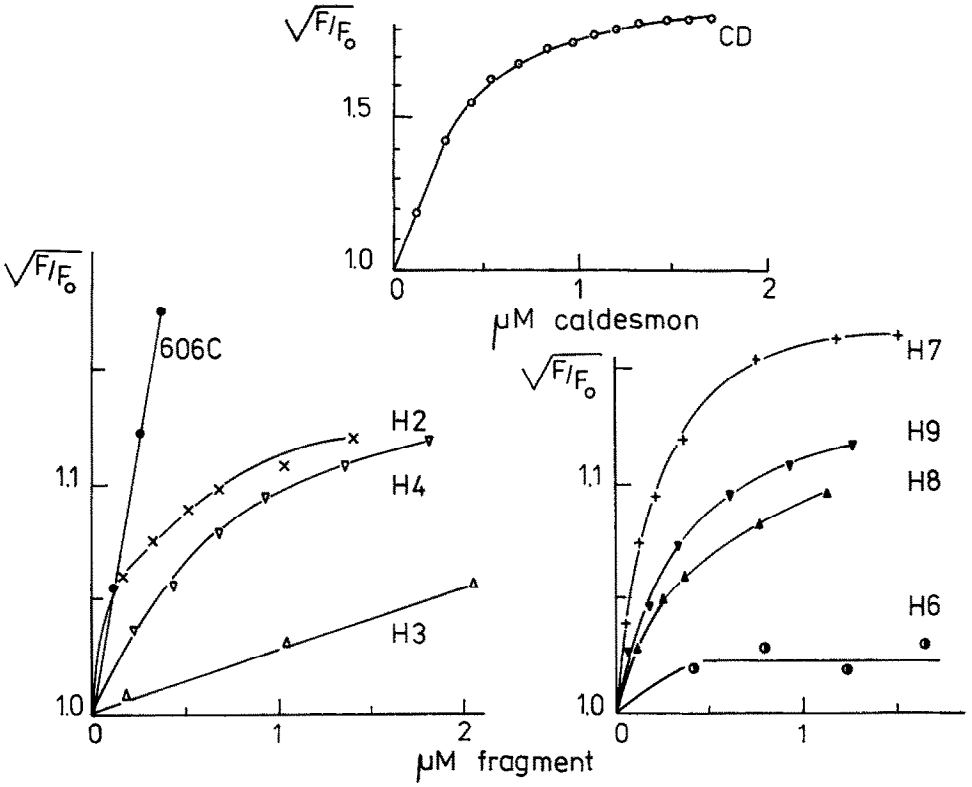

Fig. 1. Effect of intact duck gizzard caldesmon (CD), fragments 606C, H2, H3, H4, and fragments H6, H7, H8 and H9 on the light scattering of a suspension of phosphatidylserine. 0.03 mg/ml of phosphatidylserine in 20 mM imidazole-HCl, pH 7.0, was titrated by caldesmon or caldesmon fragments and the relative molecular weight of the complexes formed which is equal to $\sqrt{F/F_0}$ was plotted against total protein concentration.

We verified this assumption by using a large collection of fragments derived from domains 3 and 4 of human caldesmon. A large fragment (H6, residues 476–569) representing domain 3 as well as a short peptide H3 (residues 476–510) located on the N-terminal end of domain 3 (Fig. 2) induced only a small increase in the light scattering, and this increase was observed at rather high peptide concentration (Fig. 1, Table 1). This means that domain 3 does not contain the phospholipid-binding site.

Three large peptides derived from domain 4 of caldesmon induced a large increase in the light scattering. In addition to the above mentioned fragment 606C (residues 606–756) peptides H7 (566–710) and H2 (626–710) also effectively interacted with phosphatidylserine (Fig. 1, Table 1). This fact completely agrees with our assumption that the phospholipid-binding site is located in domain 4 of caldesmon. Splitting of domain 4 affects the ability of peptides to interact with phosphatidylserine. The fragment H4 (566–624) containing the N-terminal part of domain 4 had low affinity for phospholipids. H8 (658–737) and H9 (669–737) representing the C-terminal part of domain 4 interacted with phospholipids less effectively than 606C, H7 or H2 (Table 1, Fig. 1). Summing up we may conclude that domain 4 of caldesmon contains the phospholipid-binding site which is located between residues 626 and 710.

4. Discussion

Our preliminary attempts to locate the phospholipid-binding site by the method of ultracentrifugation led to the conclusion that the C-terminal chymotryptic peptides of caldesmon with M_r 20, 22 and 23 kDa interact with azolectin [10]. Due to the low sensitivity of the method used we failed to detect the interaction of short caldesmon peptides with phospholipids. Analyzing the published data [23,24] we may suppose that the peptide with M_r 23 kDa contains residues 581–756, that with M_r 22 kDa consists of residues 597–756 or 581–710 and the peptide with M_r 20 kDa is restricted by residues 629–756. In other words, only peptides derived from domain 4 of caldesmon (Fig. 2) interacted and were cosedimented with azolectin [10]. This conclusion was confirmed in the present investigation. Indeed the peptides derived from domain 1 (1–128), domain 2 (230–419) or domain 3 (476–570) practically do not interact with phospholipids. At the same time peptides belonging to domain 4 of caldesmon (606–756 (606C), 626–710 (H2) and 566–710 (H7) formed tight complexes with phosphatidylserine. The phospholipid binding properties of H7 (566–710) and H2 (626–710) are very similar (Table 1, Fig. 1). This means that the residues 566–626 are not important for phospholipid binding. This correlates with the fact that

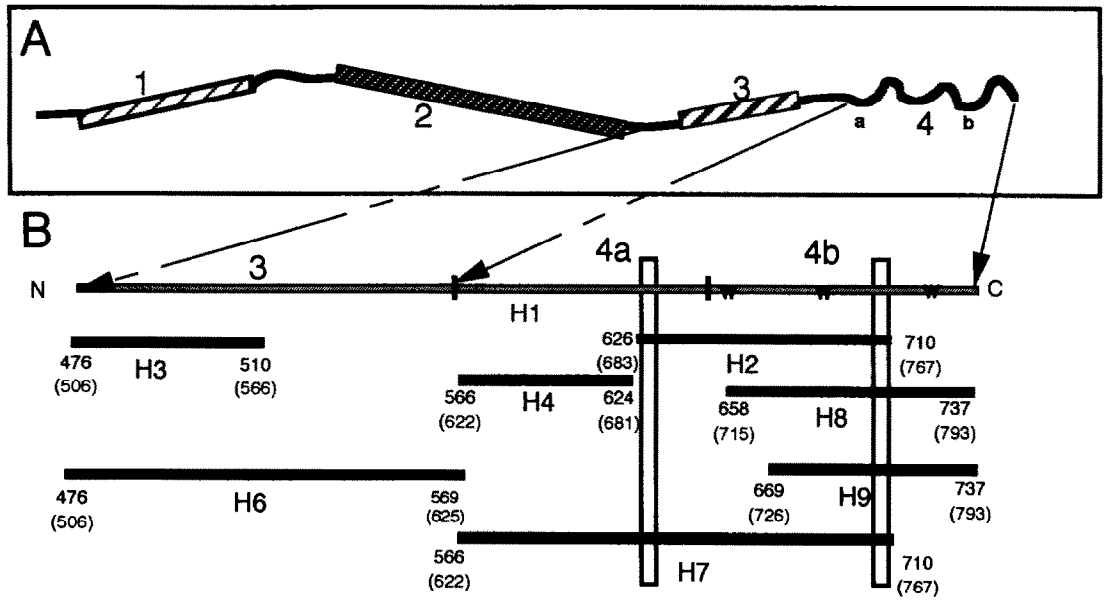

Fig. 2. Domain model of smooth muscle caldesmon (A) and localization of some of human caldesmon fragments (B) used in the present investigation (the numbering corresponds to the chicken caldesmon sequence [16] with human sequence in brackets [29]). Position of Trp residues is marked by W; putative phospholipid-binding region is marked by vertical lines.

fragment H4 (566–624) weakly interacted with phosphatidylserine (Table 1). At the same time the fragments H8 (658–737) and H9 (669–737) bind phospholipids less effectively than H2 (626–710). Therefore we may suppose that there are two regions providing for the interaction of caldesmon with phospholipids. The first one consists of residues 626–658 (in domain 4a) and the second of residues 669–710) (domain 4b [1]) (Fig. 2). At present we do not know whether these sites operate independently or form a single phospholipid-binding site. As already mentioned many actin-binding proteins interact with phospholipids [12]. The primary structure of the phospholipid-binding sites of gelsolin, gCap39, cofilin and profilin can be represented as $K(X)_{3-6}KXKK$ and replacement of 2 lysines individually with alanine did not significantly affect phospholipid-binding [25]. The first putative phospholipid-binding region of caldesmon contains the sequence $K^{633}(X)_2K(X)_2K^{639}$ which seems to be similar to the above mentioned consensus sequence. It is worthwhile mentioning that Wang et al. [24] detected a weak sequence similarity between regions containing residues 631–681 and 699–749 of caldesmon. This sequence similarity may explain the presence of the second phospholipid-binding site of caldesmon. If this suggestion is correct, then the second putative phospholipid-binding region of caldesmon contains the sequence $K^{701}(X)_4K(X)_4R^{711}$ which also resembles the consensus motif. These sequences also contain many Pro residues. The two putative phospholipid-binding regions of caldesmon are located close to Trp^{659}, Trp^{692} and Trp^{722}. This explains the strong effect of phospholipids on the intrinsic Trp fluorescence of caldesmon [11]. At least one of these putative sequences overlaps a proposed actin binding inhibitory site (Lys^{692} to Trp^{722}) [28].

The data in the literature indicate that under certain experimental conditions calmodulin and phospholipids may interfere with caldesmon binding [10,11]. Caldesmon has two calmodulin-binding sites [18]. The first site (site A) is restricted to residues 658–668 and the second (site B) is located somewhere in the sequence 669–710, probably between Ser^{687} and Lys^{695} [18]. Both these sites are located close to the putative phospholipid-binding sites of caldesmon. This explains why calmodulin binding may affect caldesmon–phospholipid interactions and vice versa. It is worthwhile mentioning that the mechanism of caldesmon–phospholipid interaction is rather complicated and binding of caldesmon to phospholipids seems to be at least partially irreversible. Therefore it is difficult to expect simple competition between calmodulin and phospholipids for caldesmon binding. For example we were unable to reverse the light scattering induced by caldesmon by addition of a 2–4-fold molar excess of calmodulin over caldesmon.

The C-terminal domain of caldesmon contains a number of sites phosphorylated by Pro-directed protein kinases and protein kinase C [26,27]. Some of these sites (i.e. Ser^{667}, Ser^{702}, Thr^{673}, Thr^{696} for Pro-directed protein kinases and Ser^{657}, Ser^{686} and Ser^{726} for protein kinase C) are located in the close proximity to the putative phospholipid-binding sites of caldesmon. Phosphorylation results in the introduction of a negative charge and by this means may affect the initial electrostatic interaction of caldesmon with phospholipids. This suggestion agrees with our earlier published results indicating that phos-

phorylation by protein kinase C or its Ca-phospholipid-independent fragments decreases the interaction of caldesmon with phospholipids [10].

Summing up we may conclude that the C-terminal domain of caldesmon contains actin-, calmodulin- and phospholipid-binding sites [15,17,18] and is phosphorylated by a number of protein kinases [26,27]. We plan to analyze the effects of calmodulin, actin and phosphorylation on caldesmon–phospholipid interaction in our future investigations.

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References

[1] Marston, S.B. and Redwood, C.S. (1991) Biochem. J. 279, 1–16.
[2] Sobue, K. and Sellers, J. (1991) J. Biol. Chem. 266, 12115–12118.
[3] Sobue, K., Kanda, K., Tanaka, T. and Ueki, N. (1988) J. Cell Biochem. 37, 317–325.
[4] Takeuchi, K., Takahashi, K., Abe, M., Nishida, W., Hiwada, K., Nabeya, T. and Maruyama, K. (1991) J. Biochem. 109, 311–326.
[5] Bogatcheva, N.V., Panaiotov, M.P., Vorotnikov, A.V. and Gusev, N.B. (1993) FEBS Lett. 335, 193–197.
[6] Yamakita, Y., Yamashiro, S. and Matsumura, F. (1990) J. Cell Biol. 111, 2487–2498.
[7] Walker, G., Kerrick, W.G.L. and Bourguignon, L.Y.W. (1989) J. Biol. Chem. 264, 496–500.
[8] Burgoyne, R.D., Cheek, T.R. and Norman, K.M. (1986) Nature 319, 68–70.
[9] Vorotnikov, A.V. and Gusev, N.B. (1990) FEBS Lett. 277, 134–136.
[10] Vorotnikov, A.V., Bogatcheva, N.V. and Gusev, N.B. (1992) Biochem. J. 284, 911–916.
[11] Czurylo, E.A., Zborowski, J. and Dabrowska, R. (1993) Biochem. J. 291, 403–408.
[12] Isenberg, G. (1991) J. Muscle Res. Cell Motil. 12, 136–144.
[13] Vorotnikov, A.V. and Gusev, N.B. (1991) Biochem. J. 273, 161–163.
[14] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[15] Redwood, C.S. and Marston, S.B. 91993) J. Biol. Chem. 268, 10696–10976.
[16] Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R.G. and Lin, W.-G. (1989) J. Biol. Chem. 264, 13873–13879.
[17] Huber, P.A.J., Redwood, C.S., Avent, N.D., Tanner, M.J.A. and Marston, S.B. (1993) J. Muscle Res. Cell Motil. 14, 385–391.
[18] Marston, S.B., Fraser, I.D.C., Huber, P.A.J., Pritchard, K., Gusev, N.B. and Torok, K. (1994) J. Biol. Chem. (in press).
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.
[21] Nelsestuen, G.L. and Lim, T.K. (1977) Biochemistry 16, 4165–4171.
[22] Horkovics-Kovala, S. and Traub, P. (1990) Biochemistry 29, 8652–8657.
[23] Katayama, E. (1989) J. Biochem. 106, 988–993.
[24] Wang, C.-L.A., Wang, L.-W.C., Xu, S., Lu, R., Saavedra-Alanis, V. and Bryan, J. (1991) J. Biol. Chem. 266, 9166–9172.
[25] Yu, F.-X, Sun, H.-Q., Janmay, P.A. and Yin, H.L. (1992) J. Biol. Chem. 267, 14616–14621.
[26] Vorotnikov, A.V., Gusev, N.B., Hua, S., Collins, J.H., Redwood, C.S. and Marston, S.B. (1994) J. Muscle Res. Cell Motil. 15, 37–48.
[27] Redwood, C.S., Marston, S.B. and Gusev, N.B. (1993) FEBS Lett. 327, 85–89.
[28] Mezgueldi, M., Derancourt, J., Calas, B., Kassab, R. and Fattoum, A. (1994) J. Biol. Chem. (in press).
[29] Humphrey, M.B., Herrera-Sosa, H., Gonzalez, G., Lee, R. and Bryan, J. (1992) Gene 112, 197–205.