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Specific binding of CAP-50 to calcyclin

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CAP-50, a calcyclin-associated protein with an apparent molecular mass of 50 kDa, was purified and proved to be a novel annexin [Tokumitsu, H. et al. (1992) J. Biol. Chem. 267, 8919–8924]. We examined the binding of CAP-50 to other Ca^{2+}-binding proteins which have two or four EF-hand structures, by a co-precipitation assay with phospholipid (phosphatidylserine). Among nine Ca^{2+}-binding proteins (calcyclin, S-100 proteins, p11, calgizzarin, calvasculin, calmodulin and troponin C) examined, only calcyclin interacted with CAP-50. These results clearly show that the interaction of CAP-50 to calcyclin is specific, i.e. other Ca^{2+}-binding proteins with the EF-hand structure could not substitute for calcyclin, thereby suggesting the possible role in specific regulation of the function of CAP-50 by Ca^{2+}/calcyclin.

EF-hand protein; Calcyclin; Annexin; CAP-50

1. INTRODUCTION

Intracellular calcium ion participates in various biochemical phenomena and calcium-binding proteins have an important role in these events. Calmodulin, S-100 proteins etc. contain homologous calcium binding structural elements, so called EF-hand structures [1].

Calcyclin is a product of a growth-regulated gene (2A9) and was found to be an EF-hand protein [2,3]. While much interest has been directed to calcydlin, its physiological function is unclear. Recently we reported evidence that calcyclin associated proteins present in various tissues [4]. One of these proteins, CAP-50 was purified and proved to be a newly identified member of the annexin family [5], these proteins bind to phospolipid in a Ca^{2+}-dependent manner and are structurally related. These proteins are formed by the short N-terminal domain and the C-terminal core domain which have a 4- or 8-fold repeat of conserved ca. 70 amino-acid segments. Annexins inhibit phospholipase A_2 activity and blood coagulation. Among these proteins, much attention was directed to CAP-50 because of its behavior, distribution in nuclei unlike other annexin family proteins [6]. As it binds to phospholipid in a Ca^{2+}-dependent manner, the interaction of CAP-50 with calcyclin or EF-hand proteins were examined by co-precipitation assay with phospholipid- vesicles.

Abbreviations: CAP-50, calcyclin-associated protein of M_r 50,000; W-77, (*S*)-*p*-(2-aminoethyloxy)-*N*-[2-(4-benzyloxycalbonylpiperazinyl)-1-(*p*-methyloxybenzyl)]-*N*-methylbenzenesulfonamide dihydrochloride; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphtalene-sulfonamide; W-66, *N*-(2-aminoethyl)-*N*-[2-(4-chlorocinnamylamino)-ethyl]-5-isoquinoline-sulfonamide; PS, phosphatidylserine; DTT, dithiothreitol; BPB, Bromophenol blue; Tricine, Tris(hydroxymethyl)methylglycine.

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2. MATERIALS AND METHODS

2.1. *Purification of proteins*

CAP-50 was purified from rabbit lung by the method of Tokumitsu et al. [5]. Calcyclin and calmodulin were purified from rabbit lung, using W-77 affinity column chromatography. Calcyclin and calmodulin were separated by Q-Sepharose column chromatography [4]. S-100α and β were purified from bovine brain, using W-7 affinity column chromatography [7]. S-100α and β were separated by Q-Sepharose column chromatography. S-100L was purified from bovine lung as described by Glenney et al. [8]. Protein I, p11 and the Annexin II complex, were purified from bovine lung by the method of Glenney et al. [9]. P11 and Annexin II subunit were dissociated with 9 M urea [10] and separated by Q-Sepharose column chromatography. Calvasculin was purified from bovine aorta, using W-66 affinity column chromatography [11]. Calgizzarin was purified from rabbit lung, using W-7 affinity column chromatography [12]. Troponin C was purified from rabbit skeletal muscle as described by Potter [13].

2.2. *Binding assay*

The binding assay was carried out as follows: 0.06 nmol of purified CAP-50 and 5 μl of PS-containing vesicles (12.5 μg of PS and 25 μg of cholesterol) were mixed in 50 μl of solution containing 0.1 M NaCl, 20 mM imidazole-HCl (pH 7.5), 2 mM $MgCl_2$, 0.5 mM DTT and 2 mM $CaCl_2$. The mixture was left to stand for 30 min at room temperature, then 0.2 nmol of EF-hand protein (50 μl) containing 2 mM $CaCl_2$ was added to the mixture and was left to stand for 30 min at room temperature. The solution was then ultracentrifuged at 100,000 × *g* for 15 min at 25°C. The pellet was separated from the supernatant and resuspended in 100 μl of sample buffer (50 mM Tris-HCl (pH 6.8), 8 M urea, 2% SDS and 0.04% BPB). A 30-μl aliquot of the supernatant and a 45 μl aliquot of the suspended solution were applied to SDS-PAGE as described elsewhere [14].

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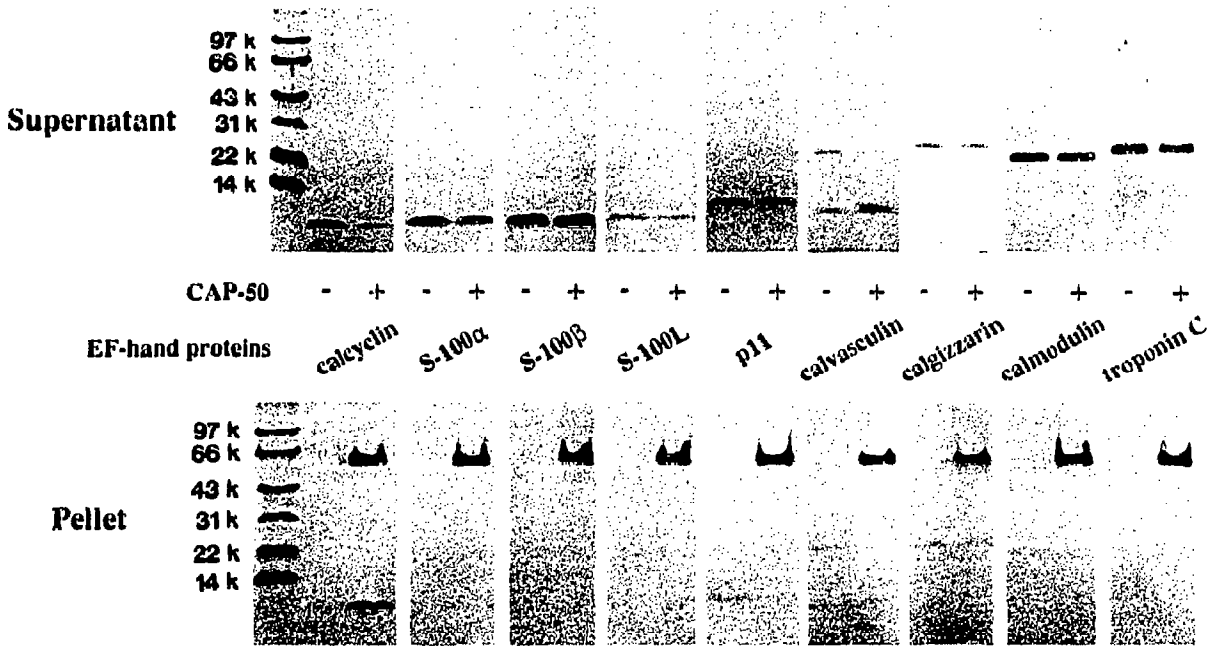

Fig. 1. Binding assay of CAP-50 and EF-hand proteins using co-precipitation with phosphatidylserine. Various EF-hand proteins (0.2 nmol) were mixed with (+) or without (−) CAP-50 (0.06 nmol) and with PS-containing vesicles. After ultracentrifugation (100,000 × g for 15 min at 25°C), the aliquot of supernatant (upper panel) and pellet (lower panel) were analyzed by tricine SDS-PAGE [14].

Protein concentration was determined by the method of Bradford [15] using bovine albumin as a standard. All chemicals were of reagent grade or better. W-77, W-7 and W-66 were synthesized by the method of Hidaka et al. [16,17].

3. RESULTS AND DISCUSSION

We reported the purification of CAP-50 (calcyclin associated protein, 50 kDa), a newly identified annexin and its biochemical properties were characterized. The binding assay of calcyclin with annexins using the co-precipitation assay with PS-containing vesicles and the ^{125}I-labeled calcyclin-gel overlay method showed that calcyclin bound to only CAP-50, is a Ca^{2+}-dependent manner and not to annexin II, V, VI or VII [5]. The functional region peculiar to CAP-50 is probably the N-terminal domain because the amino acid sequence of the core domain of annexins is highly homologous and the domain may be responsible for the association with Ca^{2+}/phospholipid [10]. We considered that the co-precipitation assay reflects the functional binding of annexins to its associated protein.

In the present work, we examined whether CAP-50 binds only to calcyclin or to other Ca^{2+}-binding proteins. We selected EF-hand proteins including S-100α, S-100β, S-100L, p11, calvasculin, calgizzarin, calmodulin and troponin C as substitutions for calcyclin, and assayed their binding ability using the co-precipitation assay (Fig. 1). The apparent difference in protein content was probably not related to the amount but to the staining efficiency of Coomassie blue. The amount of calcyclin in the supernatant decreased in a CAP-50 dependent manner and that in pellet appeared to correspond. This would suggest that calcyclin bound with CAP-50 which associated with PS-containing vesicles. Other EF-hand proteins did not co-precipitate in the absence or presence of CAP-50. The non-specific binding of EF-hand proteins to PS-containing vesicles was detected in the presence or absence of CAP-50. However, we obtained no positive evidence that these proteins bound to CAP-50 which associated with PS-containing vesicles. These results suggest that the various EF-hand proteins that we tested cannot substitute for calcyclin, demonstrating that the binding CAP-50 to calcyclin is apparently specific.

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