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IDENTIFICATION AND PARTIAL SEPARATION OF THREE DISTINCT 32-kDa CALCIUM/PHOSPHOLIPID-REGULATED PROTEINS FROM BOVINE SPLEEN

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Summary : We have separated three distinct 32-kDa calcium/phospholipid-regulated proteins from bovine spleen, which we have designated as 32kDa-Ia, 32kDa-Ib and 32kDa-II. By one-dimensional peptide mapping and chromatographic behavior, the three proteins are distinct from each other. Gizzard 35kDa calcimedin antibody recognizes both 32kDa-Ia and 32kDa-II but not 32kDa-Ib. Anti-aorta endonexin II specifically reacts with 32kDa-II. Bovine lens endonexin antibody reacts with 32kDa-Ia and 32kDa-Ib, but not with 32kDa-II. These data suggest that the 32-kDa calcium/phospholipid-regulated proteins purified from various sources can be divided into three distinct classes of proteins.

Intracellular calcium has been shown to play a major role in the regulation of diverse cellular events such as secretion, membrane transport, contraction, and cell division (1). The best known intracellular calcium-regulated proteins are the "E-F hand" proteins, which include calmodulin, troponin C, parvalbumin, and other small, soluble proteins(1). Recently, a second group of calcium-binding proteins has been purified based on its property of calcium-dependent reversible binding to cellular membranes, phospholipid-containing liposomes, or cytoskeletal fractions (for reviews, see refs. 2,3,4,5). The molecular weights of most of these calcium/phospholipid binding proteins fall within two groups, one between 67,000 and 73,000 Da and the other between 32,000 and 40,000 Da. The latter group contains two related proteins, calpactin I (lipocortin II) and calpactin II

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(lipocortin I), which have been extensively studied (2,3,4,5). In this family, other than the calpactins or lipocortins, several related proteins have purified from different sources and reported as 35kDa calcimedin (6), p32.6 calelectrin (7), protein II (8), endonexin (9), and endonexin II (10). However, the interrelationships among these proteins have not been clearly defined. Our laboratory has produced affinity-purified polyclonal antibodies to chicken gizzard 35kDa calcimedin(11), porcine aorta endonexin II (similar to placental anticoagulant protein, refs. 12 and 13) and bovine lens endonexin (similar to protein II, ref. 8).

In this report, we describe the partial purification of three distinct 32-kDa calcium/phospholipid-regulated proteins from bovine spleen. We have utilized our polyclonal antibodies to determine the immunological cross-reactivities of these three 32-kDa proteins. This comparison should provide a clearer understanding of the interrelationships among the 32-kDa calcium/phospholipid-regulated proteins.

Materials and Methods

Ca^{2+}/Phospholipid-dependent proteins: Chicken gizzard calcimedin and bovine aorta endonexin II were purified as described previously, with some modifications (14). Briefly, the isolation scheme consisted of EGTA-containing buffer extraction of the Triton-insoluble fraction which was followed by DEAE-cellulose chromatography, hydroxylapatite chromatography and gel permeation chromatography (Ultrogel AcA 54, LKB). Endonexin, an EDTA-extractable protein, was extracted from bovine lens as described previously (15), and then fractionated by Q-Sepharose chromatography and gel permeation chromatography. Details of the purification and the characterization of the EDTA-extractable proteins will be published elsewhere.

Analytical procedures: Antibodies to chicken gizzard calcimedin, porcine aorta endonexin II and bovine lens endonexin were developed in rabbits and individually affinity-purified as described earlier(16). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the Laemmli method (17). Western blots of the gels were obtained after electrophoretic transfer of proteins onto nitrocellulose paper as described by Towbin et al. (18). Anti-phospholipase A_2 activity was assayed as described by Rothhut et al. (19) using ^{3}[H]-oleic acid-labeled Escherichia coli membranes ($3x10^5$ dpm/nmol phospholipid) as the substrate for porcine pancreatic

phospholipase A_2. One-dimensional peptide mapping was carried out as described by Cleveland et al. (20) using Staphylococcus aureus protease V_8.

Results

Calcium/phospholipid-regulated proteins were extracted from bovine spleen using the Gerke-Weber procedure (8). The EGTA extract was dialyzed against 10mM Tris-HCl, pH 7.5, and subjected to Q-Sepharose (Pharmacia) chromatography. Bound proteins were eluted with a NaCl gradient (0-0.75M) in the same buffer. Two major peaks of phospholipase A_2 inhibitory activity were detected(Fig. 1-A). SDS-PAGE analyses indicated that the first peak, which eluted at 0.2M NaCl, contained a 32-kDa protein as its major constituent. The second relatively broad peak, eluting at 0.3-0.4M NaCl, contained another 32-kDa protein and a 68-kDa protein as major constituents (Fig. 1-B). The 32-kDa proteins in peak 1 and peak 2 (referred to as 32kDa-I and 32kDa-II, respectively) on the Q-Sepharose column were screened with the antibodies to chicken gizzard calcimedin, porcine aorta endonexin II and bovine lens endonexin. As shown in Fig. 2, the lens endonexin antibody recognizes 32kDa-I, whereas it does not recognize 32kDa-II. On the other hand, the aorta endonexin II antibody recognizes 32kDa-II, whereas it does not recognize 32kDa-I. The gizzard calcimedin antibody recognizes both 32kDa-I and 32kDa-II. However, the immune gel replicate indicated that only the fast migrating edge of the 32kDa-I band was stained by the antibody. These results suggested that 32kDa-I is a mixture of two immunologically distinct proteins.

The 32kDa-I fraction was further purified by hydroxylapatite chromatography. As shown in Fig. 3-A, the phospholipase A_2 inhibitory activity elutes as a single broad peak. Figure 3-B shows the Coomassie blue-stained profile of proteins from the same column samples after SDS-PAGE analysis. The proteins that

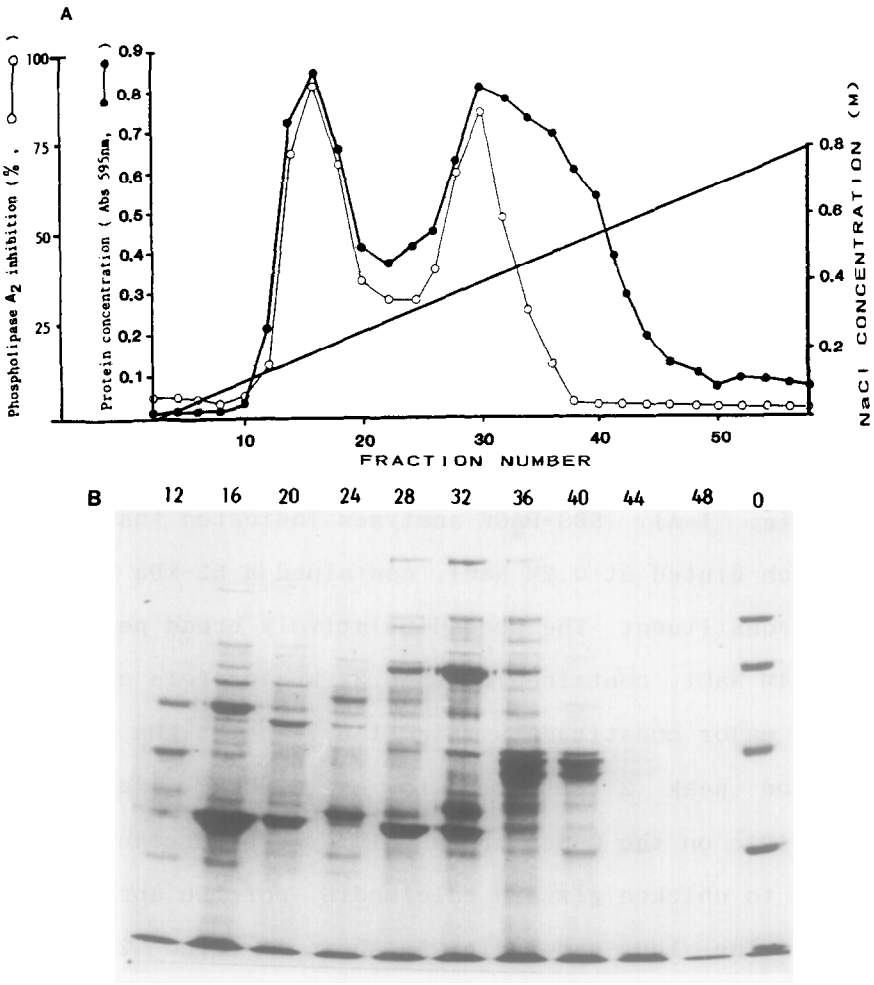

Fig. 1 Fractionation of bovine spleen EGTA extracts on a Q-Sepharose column. A. Approximately 300mg of EGTA extract in 10mM Tris-HCl buffer, pH 7.5, was loaded on a 1.5 x 10-cm column of Q-Sepharose. Elution was performed by a linear NaCl gradient (0-0.75M). Fractions of 3ml were collected and analyzed for phospholipase A_2 inhibitory activity (○) and protein concentration (●). B. Selected fractions from the column were analyzed on 10% SDS-polyacrylamide gels. The number in the panel indicates fractions from the column. Lane 0 indicates molecular mass markers (phosphorylase b, 94kDa; bovine serum albumin, 68kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa).

appeared as a closely-spaced doublet on SDS-polyacrylamide gels were partially resolved by hydroxylapatite chromatography. Fractions 17 to 20 contained a slightly smaller variant of 32kDa-I (referred to as 32kDa-Ia). Fractions 40 to 45 contained a slightly larger variant of 32kDa-I (referred to as 32kDa-Ib),

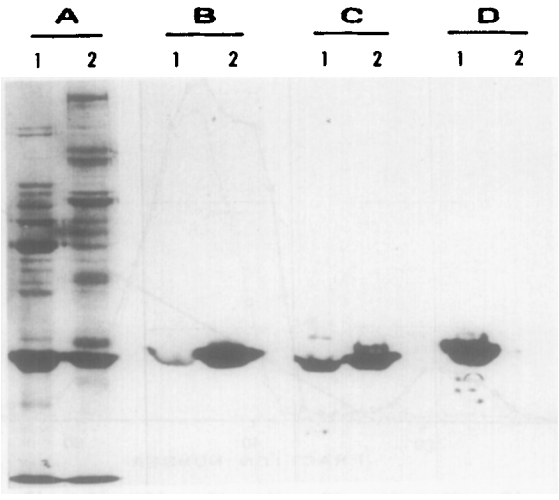

Fig. 2 Immunoblot analysis of 32kDa-I (fractions 20-27 of the Q-Sepharose column) and 32kDa-II (fractions 40-45) using anti-32kDa antibodies. Ten-percent SDS-polyacrylamide gels were run and stained with Coomassie blue (A) or transferred to nitrocellulose (B, C, and D). The nitrocellulose transfers were incubated with an antibody to aorta endonexin II (B), gizzard calcimedin (C), and lens endonexin (D). Lane 1, 32kDa-I from bovine spleen ; Lane 2, 32kDa-II from bovine spleen.

whereas fractions 21 to 39 contained both 32kDa-Ia and 32kDa-Ib. As shown in Fig. 4, the antibody to lens endonexin reacts with both 32kDa-Ia and 32kDa-Ib, whereas anti-gizzard calcimedin reactivity is largely confined to 32kDa-Ia.

To evaluate a possible structural relationship between 32kDa-Ia, 32kDa-Ib and 32kDa-II, we compared these proteins using the one-dimensional peptide mapping technique of Cleveland et al. (20). Figure 5 shows the results from the analyses. Cleavage profiles of 32kDa-Ia (Fig. 5, lane 2), 32KDa-Ib (Fig. 5, lane 3) and 32kDa-II (lane 1) are different from each other, confirming that they are distinct proteins.

Discussion

We have partially purified three distinct 32-kDa calcium/phospholipid-regulated proteins from bovine spleen that inhibit phospholipase A_2 activity. These proteins bind to the membrane and other particulate fractions in the presence of

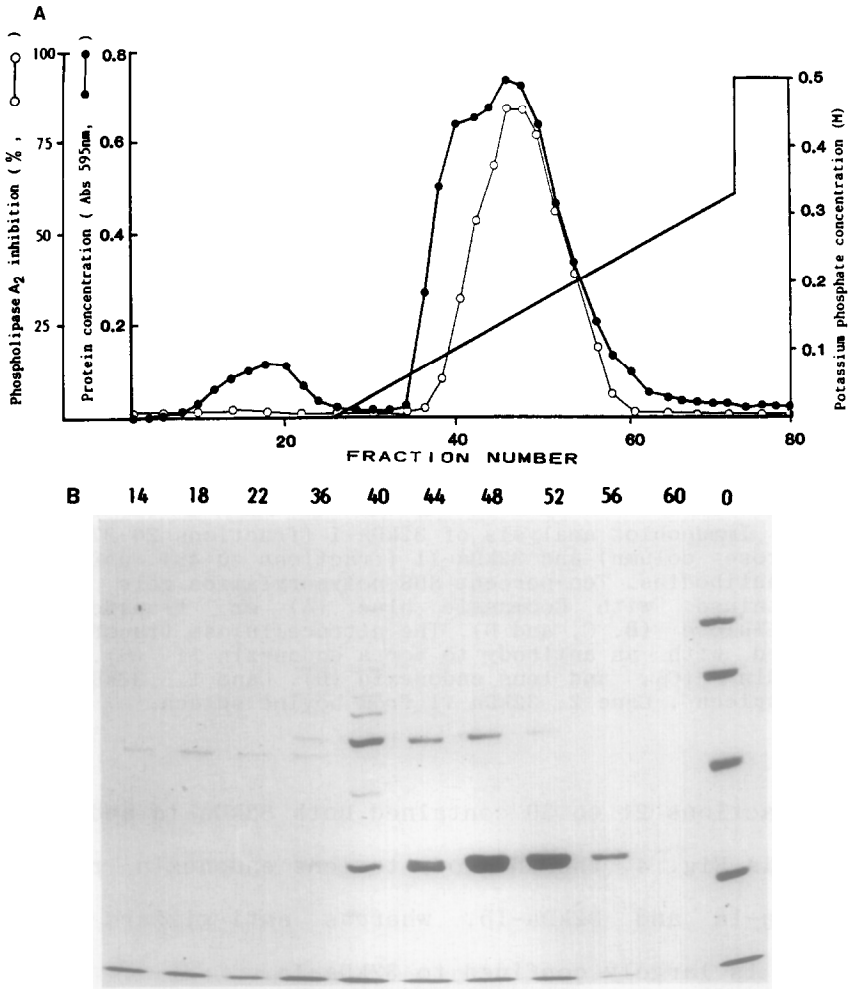

Fig. 3 Fractionation of bovine spleen 32kDa-I on a hydroxylapatite column. A. Partially purified 32kDa-I in 10mM potassium phosphate buffer, pH 6.8, was loaded on a 1.2 x 10-cm column of hydroxylapatite. After washing with 100ml of the buffer, elution was performed by a linear gradient of potassium phosphate, pH 6.8 (10 - 300mM). Fractions of 3ml were collected for the analyses of phospholipase A_2 inhibitory activity (○) and protein concentration (●). B. Selected fractions from the column were analyzed on 10% SDS-polyacrylamide gels. The number in the panel indicates fractions from the column. Lane 0 indicates molecular mass markers.

calcium. Their extractability is very similar to other calcium/phospholipid-regulated proteins from various tissues (6-10,14,15).

Based on the published characteristics of p32.5 calelectrin (7), protein II (8), calcimedin (6,14), endonexin (9), endonexin II (10), and the anticoagulant protein (12,13), these proteins

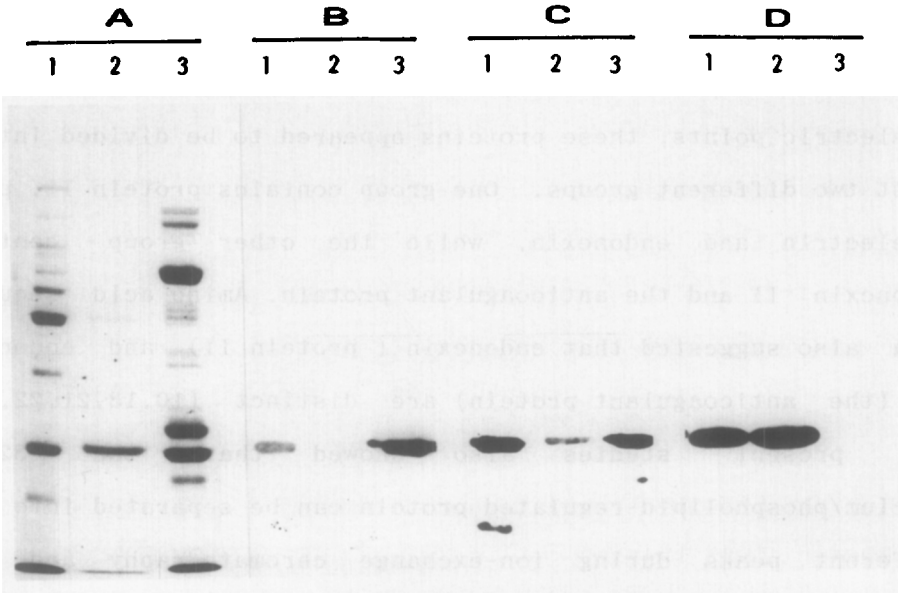

Fig. 4 Immunological comparison of 32kDa-Ia, 32kDa-Ib and 32kDa-II using various antibodies. Coomassie blue stained proteins (A). Immunological detection of 32kDa-Ia, 32kDa-Ib and 32kDa-II by aorta endonexin II antibody (B), gizzard calcimedin antibody (C) and lens endonexin antibody (D). Lane 1, 32kDa-Ia ; lane 2, 32kDa-Ib ; lane 3, 32kDa-II.

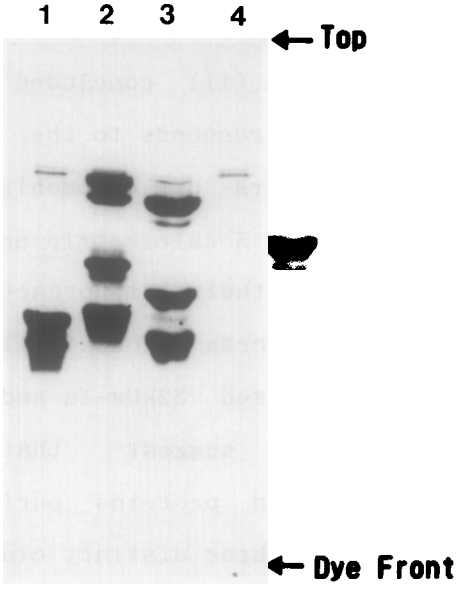

Fig. 5 One-dimensional peptide mapping of 32kDa-Ia, 32kDa-Ib and 32kDa-II by limited proteolysis with S. aureus V_8 protease. About 10ug of proteins were incubated with 0.1μg of V_8 protease at 37°C for 30min. The cleavage products were electrophoresed on a SDS-polyacrylamide (15%) gel. Lane 1, 32kDa-II ; lane 2, 32kDa-Ia ; lane 3, 32kDa-Ib; lane 4, 0.1μg of V_8 protease.

are generally very similar to each other. However, based on behaviors during ion-exchange chromatography and their isoelectric points, these proteins appeared to be divided into at least two different groups. One group contains protein II, p32.5 calelectrin and endonexin, while the other group contains endonexin II and the anticoagulant protein. Amino acid sequence data also suggested that endonexin (protein II) and endonexin II (the anticoagulant protein) are distinct (10,13,21,22,23). The present studies also showed that the 32-kDa calcium/phospholipid-regulated protein can be separated into two different peaks during ion-exchange chromatography and our immunochemical studies demonstrated that the two protein peaks (32kDa-I and 32kDa-II) were distinct from one another. These results strongly support the hypothesis that the 32-kDa calcium/phospholipid-regulated protein is a mixture of proteins rather than a single protein. In addition, 32kDa-I was further separated into two different classes of proteins (32kDa-Ia and 32kDa-Ib) by hydroxylapatite chromatography and immunochemical criteria. Smith and Dedman (11) concluded by immunochemical criteria that calcimedin corresponds to the p32.5 calelectrin, protein II and the clusters of chromobindin. Our results indicate, however, that p32.5 calelectrin and protein II are distinct proteins based on their immunoreactivity to the lens endonexin antibody and the aorta endonexin II antibody. Gizzard calcimedin antibody recognized 32kDa-Ia and 32kDa-II but not 32kDa-Ib. These data suggest that the 32-kDa calcium/phospholipid-regulated proteins purified from various sources can be divided into three distinct classes of proteins.

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