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Detection of VAC-β (annexin-8) in human placenta

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A36 kDa calcium/phospholipid binding protein in human placenta was identified as VAC-β (annexin-8) by a combination of immunological and peptide mapping analyses. The protein is a minor product in placenta, accounting for less than 1% of extracted annexins. From 150 g of tissue, only 100 μg of the protein was isolated. By anion-exchange chromatography on diethylaminoethyl-cellulose annexin-8 coeluted with annexin-3. By gel filtration, the protein chromatographed as a broad peak, where half the product eluted as a monomer and half eluted as a heterodimer that was associated with a 10 kDa subunit. The combination of annexin-8 being a minor component in standard annexin preparations and it co-eluting with annexin-3 by ion exchange chromatography are likely to account for the failure of other labs to characterize the product.

Annexin-8; VAC-β; Lipocortin; Calcium/phospholipid binding protein

1. INTRODUCTION

The lipocortins/annexins are a large family of calcium binding proteins that to date have been identified in plants, animals, insects, sponges, and slime molds. While the physiological functions of annexin-like proteins are uncertain, their potential involvement in signal transduction, membrane trafficking, coagulation, adhesion, and various facets of inflammation have made them key targets for many research programs (for references see refs. 1–3). In humans, 9 distinct genes have been identified [4–8]. All share approx. 50% identity. Most conserved is the core structure which is made up of four repeats (eight in the case of annexin-6) and is derived from a 70 amino acid unit. The existing gene sequences for annexins support that the generic structure is generated through duplication. Each protein also contains a unique amino terminus (5–167 amino acids in length) that confers functional specificity. The recently published crystal structure for one member of the family annexin-5 [9], has provided further insight into our understanding of the properties of the family.

VAC-β (annexin-8) was first identified in 1989 where the original authors in identifying the gene for annexin-5 (VAC-α), detected another gene product, cloned it, and expressed the protein in *E.coli* [5]. Since the annexin-8 mRNA for cloning was obtained from human placenta, placenta seemed to be a likely candidate for producing annexin-8. Here we have identified annexin-8 in placenta by a combination of Western blotting and peptide mapping. The protein (100 μg annexin-8/150 g tissue) represents less than 1% of total annexins in the placenta, explaining the difficulty in identifying the product.

Abbreviations: VAC, vascular anticoagulant; DEAE, diethylaminoethyl; CNBr, cyanogen bromide; DSS, disuccinimidyl suberate; PLBP, calcium-dependent phospholipid binding protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Precast 8 × 10 cm Daiichi gradient SDS-polyacrylamide gels were from Integrated Separation Systems. Immobilon transfer membranes were from Millipore, goat anti-rabbit IgG alkaline phosphatase conjugate from BioRad, and alkaline phosphatase color development kit (NBT, BCIP) from Bethesda Research Labs. Disuccinimidyl suberate was from Pierce and iodoacetamide from Sigma. Anti-lipocortin-3 antisera was produced in rabbits using bovine lipocortin-3 as immunogen. The anti-lipocortin-5 rabbit polyclonal antibody (no. 890) was raised with recombinant human lipocortin-5. Rabbit anti-VAC-β polyclonal antiserum raised against recombinant human VAC-β and recombinant human VAC-β were kindly provided by G.R. Adolf (Ernst-Boehringer Institute; Vienna, Austria).

2.2. *Isolation of annexin-like proteins from placenta*

Calcium/phospholipid binding proteins were extracted from human placenta as previously described [10]. A fresh term human placenta was quick frozen on dry ice and stored at −70°C. 150 g of tissue was thawed on ice in 50 mM Tris-HCl pH 7.7, 1 mM $CaCl_2$, 100 μg/ml soybean trypsin inhibitor, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, skinned, and cut into chunks. 250 ml of the buffer was added and the tissue disrupted with a polytron. The homogenate was centrifuged for 1 h at 4°C in a SA600 rotor (14,000 rpm). Pellets were resuspended with a polytron into 250 ml of the same buffer and again centrifuged. After an additional wash, the calcium binding proteins were extracted with 240 ml of 15 mM *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonic acid pH 7.5, 4 mM EDTA, 1 mM EGTA. Debris was removed by centrifugation and the extract loaded onto a 20 ml DEAE-cellulose column equilibrated with 25 mM Tris-HCl pH 7.7. The column was subjected to sequential salt steps (10, 30, 100, 150,

250 mM NaCl) in the same buffer, each step consisting of two- 10-ml collections. 0.5 ml from fraction 100-2 was loaded onto a P150 gel filtration column (1 × 40 cm) in 25 mM Tris-HCl pH 7.7, 1 mM EDTA and run at 2 ml/h. Fractions were analyzed for absorbance at 280 nm and by SDS- PAGE.

2.3. *Western blotting*

Proteins or CNBr fragments were subjected to SDS- PAGE and either stained with Coomassie brilliant blue, silver-stained [11] or electrophoretically transferred to Immobilon (1 h, 150 mA) using 10 mM 3-(cyclohexylamino)propanesulfonic acid pH 11.25, 10% methanol as the transfer medium. The blot was blocked with 3% bovine serum albumin, 10 mM Tris-HCl pH 7.5, 150 mM NaCl (30 min), treated with primary antisera at a 1:2000 dilution in blocking buffer (1.5 h), then treated with an alkaline phosphatase goat anti-rabbit IgG conjugate at a 1:2000 dilution in blocking buffer, and visualized with BRL color development reagent in 1 M diethylamine pH 9.1, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. After each antibody step, filters were rinsed 3 times with Tris-saline and washed 3 times for 10 min with Tris-saline.

3. RESULTS AND DISCUSSION

A preparation of calcium binding proteins from human placenta was generated by disrupting the tissue in buffer containing 1 mM $CaCl_2$, washing the particulate fraction with the same buffer, and then selectively extracting the binding proteins with EDTA. Fig. 1A, lane b shows the product from such a treatment. The two prominent bands at 35 and 37 kDa are the four domain annexins and the band at 70 kDa is a mixture of annexins and residual human serum albumin in the preparation. The simple calcium-dependent extraction protocol produced about a 100-fold enrichment of the calcium binding proteins. When the EDTA extract was subjected to DEAE-cellulose anion-exchange chromatography, the major protein species of interest were fractionated into three pools, a flow-through fraction, fraction 100-2+150-1, and fraction 150-2+250-1 (Fig. 1A, lanes c–k). From the flow-through fraction we previously isolated annexins-1 (37 kDa), -2 (37 kDa), -4 (35 kDa), and a dimeric form of annexin-1 that had been generated by transglutaminase-induced cross-linking [10]. 6 mg of annexin-1, 3 mg of annexin-2, 1 mg of annexin-4 and 1.5 mg of the annexin-1 dimer were recovered. Similarly, we purified annexin-3 (35 kDa) from fraction 100-2+150-1 (lanes h and i) and annexins-5 (35 kDa) and -6 (68 kDa) from fraction 150-2+250-1 (lanes j and k). These preparations yielded 1 mg of annexin-3, 2 mg of annexin-6, and 10 mg of annexin-5.

Because of our familarity with the placental preparation, the same set of samples were probed for annexin-8. When the column fractions were subjected to Western blotting and probed with anti-annexin-8 antiserum, the results shown in Fig. 1B were obtained. A prominent 36 kDa reactive species was detected in fraction 100-2 (lane h) that by SDS-PAGE migrated slower than annexin-3 by approx. 1 kDa. This difference in mobility was particularly apparent when the position of the signal was compared to the position of annexin-5 (lanes j and k),

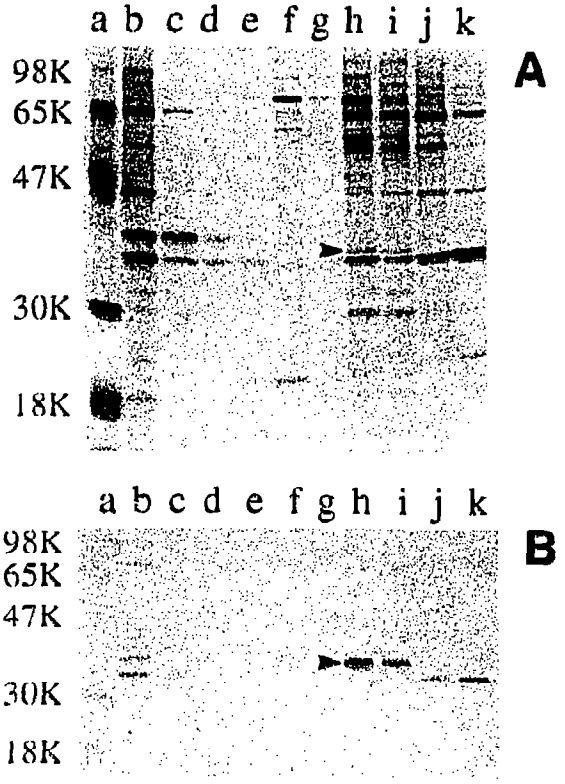

Fig. 1. Detection of human placental annexin-8. Calcium binding proteins from human placenta were fractionated on a DEAE-cellulose column. Aliquots from the column fractions were analyzed by SDS-PAGE and either visualized by staining with Coomassie brilliant blue (A) or by Western blotting with anti-annexin-8 polyclonal antisera (B). Lanes b-k correspond to column load, flow-through, and salt steps containing, 10, 30-1, 30-2, 100-1, 100-2, 150-1, 150-2, and 250 mM NaCl, respectively. In panel A, 40 μl of sample was used for the load and flow-through fractions and 10 μl for each of the salt steps. In panel B, one fiftieth of the corresponding samples were loaded. Arrowheads mark the position of annexin-8. Lane a, prestained molecular weight markers. Numbers at the left of the panels denote molecular weights.

which cross-reacted with the anti-annexin-8 antisera under the blotting conditions used and thus was visualized. The tentative identification of annexin-8 as the minor band above annexin-3 in the coomassie stained gel (Fig. 1A) was verified in subsequent analyses. From relative band intensities, we inferred that the entire preparation contained about 100 μg of annexin-8.

Fraction 100-2 was further fractionated by gel filtration (see Fig. 2). When selected column fractions were subjected to SDS-PAGE and silver stained, the elution profile shown in panel A was obtained. Parallel aliquots also were subjected to Western blotting and developed with antisera to annexin-8 (panel B) and annexin-3 (panel C). Annexin-3 eluted from the column as a single peak with apparent size of 35 kDa and annexin-8 eluted as two overlapping peaks with sizes of 35 and 45 kDa. The distinctive elution profiles of annexins-3 and -8 aided in identifying the corresponding protein bands in the silver stained gel (panel A), which are readily apparent from the analysis.

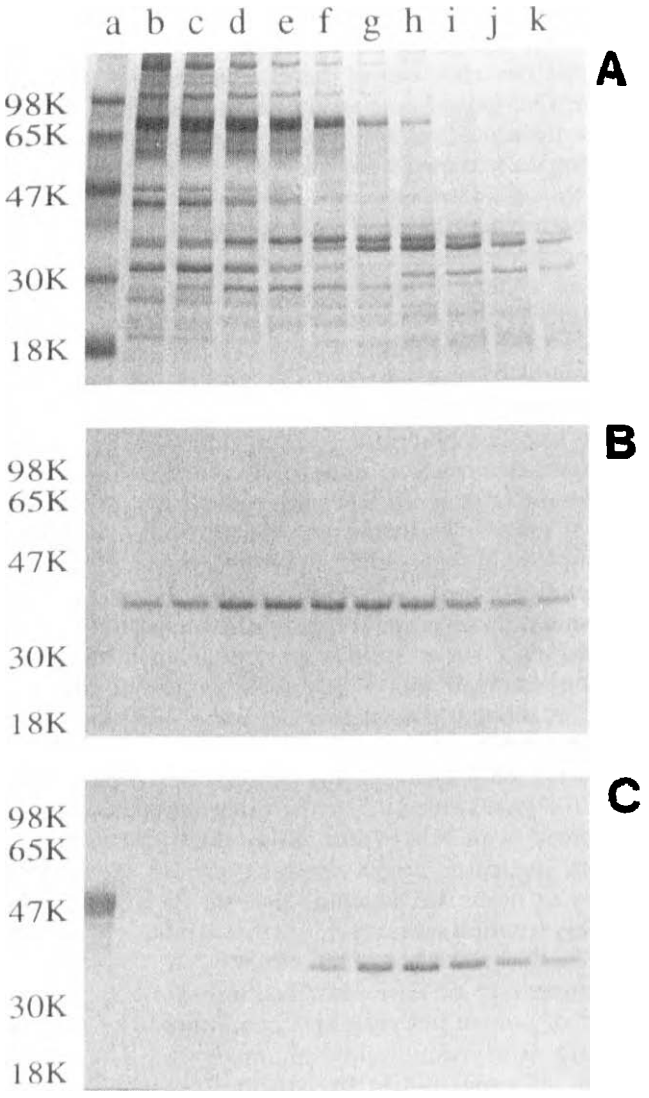

Fig. 2. Analysis of annexin-8 containing fraction by gel filtration. DEAE fraction 100-2 was subjected to gel filtration on a P150 column. Column fractions were analyzed by SDS-PAGE and either visualized by silver staining (A) or by Western blotting. Western blots were visualized with annexin-8 (B) or annexin-3 (C) antisera. Lanes b–k correspond to alternating P150 column fractions from fraction 19 to fraction 37. 30 μl samples were analyzed by silver staining and 10 μl samples for the blots. Lane a, prestained molecular weight markers. Numbers at the left of the panel denote molecular weights.

The identity of the 36 kDa immunoreactive band as annexin-8 was confirmed by peptide mapping. When gel slices containing the 36 kDa placental product and recombinant human annexin-8 were treated with CNBr and the cleavage products analyzed by SDS-PAGE, the cleavage profiles were identical (see Fig. 3, lanes d and e). The 11 cleavage products that had been detected were identical both with respect to position and intensity. In the same analysis, annexin-5 was included as a control. Only three cleavage products in the annexin-5 cleavage profile (lane f) were recognized by the anti-annexin-8 antibody, none were common to annexin-8.

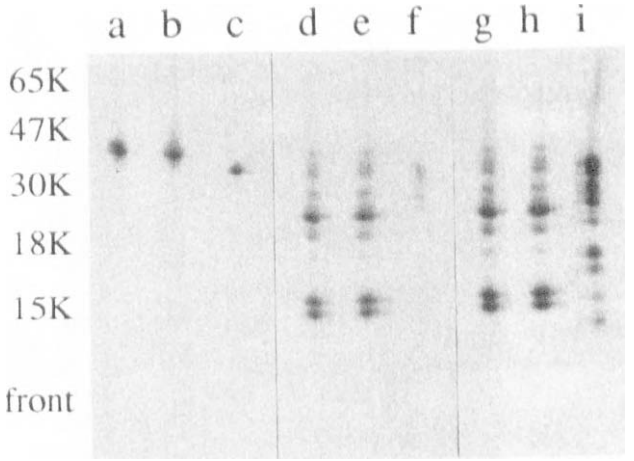

Fig. 3. Identification of placental annexin-8 by peptide mapping. The indicated products were subjected to CNBr peptide mapping as previously described [15,16]. Gel slices containing 30 ng of placental annexin-8 (lanes a, d and g), 30 ng of recombinant human annexin-8 (lanes b,e and h) and 200 ng of human annexin-5 (lanes c, f and i) were treated for 1 h at 23°C with 0.1 N HCl, 0.2% β-mercaptoethanol alone (lanes a–c) or in the presence of 21 mg/ml CNBr (lanes d–i). The gel slices were then washed and equilibrated in electrophoresis sample buffer. Cleavage products were electrophoresed into a 4–20% gradient gel using the Schagger buffer system [17] and transferred to immobilon. The same blot was first developed with anti-annexin-8 antibody (lanes a–f), photographed, and then developed with an anti-annexin-5 antibody (lanes g–i). The position of molecular weight standards are indicated at the left of the panel.

When the same blot was probed with annexin-5 antisera, 12 annexin-5 cleavage products were observed (lane i). The distinct profiles for annexins-5 (lane i) and -8 (lane h) clearly demonstrate that they are unrelated. Previously, we analyzed annexins 1–6 by CNBr mapping and showed that the CNBr cleavage profile for each annexin was distinct [4]. The combination of limited proteolysis and Western analysis creates a further level of selectivity to the analysis, thereby providing a simple diagnostic method for identifying individual annexins.

To investigate the nature of the 45 kDa form of annexin-8 that was detected by gel filtration, the 45 kDa product was analyzed by SDS-PAGE in the presence and absence of reducing agent and after cross-linking (Fig. 4). Whereas annexin-8 in the presence of reducing agent migrated on SDS-gels with an apparent mass of 36 kDa (lane b), the protein's apparent mass was 45 kDa in the absence of reducing agent (lane e). The same result was obtained if the preparation was treated with iodoacetamide prior to analysis (lane f). The shift in apparent molecular weight indicated that annexin-8 might be disulfide-linked to a low molecular weight adduct. The 45 kDa band also was observed when the sample was treated with the homobifunctional amine-specific cross-linker Disuccinimidyl suberate (DSS), supporting an association between annexin-8 and a 10 kDa protein. Since the cytoplasmic compartment of cells is a reducing environment, it is unlikely that an-

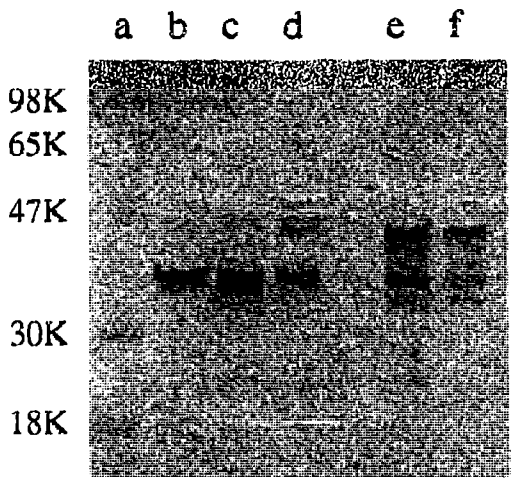

Fig. 4. The 45 kDa form of annexin-8 is a heterodimer. Annexin-8 samples at 1.5 μg/ml were analyzed by cross-linking either with UV light (lane c), DSS (lane d) or oxidation (lanes e and f). After the indicated treatments samples were suspended in electrophoresis sample buffer in the presence (lanes a–d) or absence (lanes e and f) of 2% 2-mercaptoethanol. Samples were subjected to SDS-PAGE on a 10–20% gradient gel, transferred to immobilon, and probed with anti-annexin-8 antibody. For DSS cross-linking, samples in 50 mM triethanolamine HCl pH 8.2 were treated with 1 mM DSS for 1 h at 23°C. For UV cross-linking samples were exposed to a 254 nm UV light (8×10^4 μJ). For oxidation samples were either directly analyzed in the absence of reducing agent or treated with 50 mM iodoacetamide for 30 min at 23°C and then analyzed.

nexin-8 is disulfide-linked to another protein. The prominence of the 45 kDa form suggests that annexin-8 may be associated with the 10 kDa protein, but then becomes disulfide-linked during the purification. It remains to be determined if the association of annexin-8 with the 10 kDa protein is physiologically relevant. Despite a similar content of cysteines in the other annexins, we have never seen disulfide-linked forms of any of the other family members. Interestingly, during the isolation of recombinant annexin-8 from *E. coli* [5], part of the protein had formed a disulfide-linked homodimer. Although the relevance of the 45 kDa annexin-8 is unknown, the presence of multiple forms of annexin-8 leads to low yields of the product during purification.

In the DSS-treated annexin-8 (Fig. 4, lane d), about 5% of the signal was associated with a 70 kDa band that was absent from parallel samples without DSS treatment or in samples generated by oxidation and is likely to be an annexin-8 homodimer. Similar findings have been described for other annexins [10,12,13] and suggest that the functional unit for annexin-like proteins may be a multimeric adduct. Since for annexin-1 we previously showed that the major contact point for the association was the N-terminal tail region [10], it was of interest to determine if annexin-8 also was associated through the tail region. Since three of the first six amino acids of annexin-8 are tryptophans, UV cross-linking was employed for this analysis [14]; however, there was no identification of a UV light-induced dimer (lane c).

Recently, Tsao et al. [18] published a partial amino acid sequence for a 33 kDa protein from rabbit lung that they claimed was a new member of the annexin family. A comparison of this sequence with sequences from the nine known human annexins revealed that the best match, 88% identity, was with the first repeat from annexin-8 (see Table I). For the other annexins, identities ranged from 36% to 59%. Since the conservation of annexin sequences across species typically shows 79% identity or more, we conclude that the 33 kDa protein probably is rabbit annexin-8. Further studies are needed to verify this point.

In summary, we have identified annexin-8 as a component of human placenta by a combination of peptide mapping and immunological analyses. The small amount of annexin-8 in the tissue, its coelution with annexin-3 on DEAE-cellulose, and its presence in mul-

Table I

Alignment of rabbit 33 kDa PLBP and human annexin sequences.

Source	Sequence	Identity
33 kD PLBP	MKGIGTNEQAIIDVLTRRSSAQRQQIAKSFKAQFGSDLTED	
ANNEXIN-1	IMVK.VD.AT...I..K.NN.....KAAYLQET.KP.D.T	41.5%
ANNEXIN-2	I.TK.VD.VT.VNI..N..N....D..FAYQRRTKKE.ASA	36.6%
ANNEXIN-3	IR....D.KML.SI..E..N....L.V.EYQ.AY.KE.KD.	48.8%
ANNEXIN-4	...L..D.D...S..AY.NT....E.RTAY.STI.R..ID.	53.7%
ANNEXIN-5	...L..D.ES.LTL..S..N....E.SAA..TL..R..LD.	56.1%
ANNEXIN-6a	...F.SDKE..L.II.S..NR...EVCQ.Y.SLY.K..IA.	46.3%
ANNEXIN-6b	...L..D.DT...II.H..NV.....RQT..SH..R..MT.	58.5%
ANNEXIN-7	...F..D....V..VAN..ND...K.KAA..TSY.K..IK.	56.1%
ANNEXIN-8	.....K..NT.....K....T	87.8%
ANNEXIN-(IS)	C..M....A...EI.SG.T.DE....KQKY..TY.KE.E.V	51.2%

The 41 amino acid sequence from 33 kDa PLBP [18] was aligned with the corresponding segment of the first repeat unit from each of the known human annexins [4–8]. Dots (.) indicate conserved amino acids. Percent identities between the peptide and the different annexins are given. For annexin-6, the first (6a) and fifth (6b) repeats are shown. Annexin-(IS) refers to the newly discovered intestine-specific annexin [6]. The PLBP sequence also was compared to 55 kDa bovine annexin-XI [19] to which it showed 63% identity.

tiple forms are likely to have contributed to it being missed in previous studies. While the true function of annexin-8 is unknown, the recent discovery of tissue specific annexins [6], raises the possibility that it to may also be specific for a certain cell type. Thus it will be important to determine the cell types that express annexin-8. Annexins in exhibiting potent activities in diverse biological systems have and continue to be important targets for a wide range of research programs.

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