Volume 223, number 2, 267-272 FEB 05241 November 1987

A 32-kDa protein associated with phospholipase A_2-inhibitory activity from human placenta

Hideki Hayashi, M. Koji Owada*, Seiji Sonobe+, Takeo Kakunaga, Hisaaki Kawakatsu° and Junichi Yano°

*Departments of Oncogene Research and *Tumor Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamada-oka, Suita, Osaka 565* and °*Research Laboratories, Nippon Shinyaku Co., Ltd, Nishiohji Hachijo, Minamiku, Kyoto 601, Japan*

Received 7 September 1987

Two monomeric 32-kDa proteins, termed 32K-I (p*I* 5.8) and 32K-II (p*I* 5.1), were isolated from human placenta, which was solubilized by a Ca^{2+}-chelator. Only 32K-I was associated with PLA_2-inhibitory activity. CNBr peptide mapping indicated that 32K-I was distinct from 32K-II and two 36-kDa proteins, called calpactin I and II or lipocortin II and I, which have been shown to possess PLA_2-inhibitory activity. 32K-I bound to PS in a Ca^{2+}-dependent manner. 32K-I was detected in many tissues except brain, cardiac and skeletal muscle.

32-kDa protein; Phospholipase A_2; Enzyme inhibitor; Ca^{2+}-dependent phospholipid binding; Calpactin; Lipocortin; (Human placenta)

1. INTRODUCTION

The actions of hormones, growth factors or oncogenes evoke the acceleration of phospholipid turnover and the elevation of intracellular free Ca^{2+}-concentrations [1,2]. Calcium and phospholipids as second messengers transfer their effects through specific binding proteins to specific targets of membrane, cytoplasmic and nuclear components. Recent works have revealed the presence of new families of Ca^{2+}- and phospholipid-binding proteins distinct from protein kinase C [3-7]. Lipocortins or calpactins which are the substrates of the *src*-gene product and EGF-receptor tyrosine kinases, were identified as major members of the family. Lipocortins were shown to inhibit PLA_2 activity [6,8,9], and were induced by steroid hormone and secreted by cells. It has been postulated that they may play crucial roles in controlling the biosynthesis of cyclooxygenase by inhibiting PLA_2 activity [10,11]. Because a variety of proteins similar to lipocortins have been detected in various tissues and species [12-16], it is necessary to find out which protein molecular masses are involved in anti-inflammatory functions.

We have recently purified three distinct forms of 36-kDa proteins with PLA_2-inhibitory activity from human placenta which belong to the new family of Ca^{2+}- and phospholipid-binding proteins [9]. Here we report that a 32-kDa protein purified from human placenta inhibits PLA_2 activity in vitro. This protein is not a proteolytic fragment of calpactins or lipocortins.

Correspondence address: T. Kakunaga, Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamada-oka, Suita, Osaka 565, Japan

+ Present address: Department of Biology, Faculty of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 565, Japan

Abbreviations: PLA_2, phospholipase A_2; CNBr, cyanogen bromide; PS, phosphatidylserine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; EGF, epidermal growth factor

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

2.1. *Purification of 32K-I and 32K-II proteins*

A fresh placenta was dissected free of adjacent tissues and extensively washed with chilled saline containing 5% sucrose. All subsequent steps were carried out at 4°C. The washed placenta was homogenized in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl supplemented with the proteinase inhibitors described in [9]. The homogenate was centrifuged for 60 min at 30000 × *g* and washed twice with 50 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$ and once with 20 mM Hepes, pH 7.5. The resulting pellet was extracted overnight in 20 mM Hepes, pH 7.5, containing 5 mM EGTA and subjected to a DE-52 cellulose column (Whatman) equilibrated in 10 mM imidazole, pH 7.4, and 0.5 mM DTT. The flow-through fraction was concentrated with Pellicon (Millipore) and applied to a hydroxyapatite column (Koken, Co., Tokyo, Japan) equilibrated with 10 mM imidazole, pH 7.4, 0.5 mM DTT and 10% glycerol. It was eluted in a linear gradient of 0–0.5 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The 0.2 M eluate was loaded on a Mono S column (Pharmacia) equilibrated with 20 mM sodium acetate/acetic acid, pH 5.6, 0.5 mM DTT, and then eluted in a linear gradient of 0–0.5 M NaCl. The 0.1 M eluate containing mainly a 32-kDa polypeptide was purified further by gel filtration using a G3000SW HPLC column (Toyosoda) equilibrated with gel filtration buffer (10 mM imidazole, pH 7.4, 100 mM NaCl, 1 mM EGTA, 0.5 mM DTT). The 32-kDa polypeptide (32K-I) was finally purified to 95% purity (see fig. 1, lane 6).

The DE-52-absorbed materials were eluted in a linear gradient of 0–0.5 M NaCl. The 0.1 M eluate was gel filtrated on a Sephacryl S-200 column (2 × 100 cm, Pharmacia) equilibrated with the gel filtration buffer. Another 32-kDa polypeptide (32K-II) was homogeneously purified as a monomeric form (see fig. 1, lane 8).

2.2. *Assay for PLA_2 inhibitory activity*

PLA_2-inhibitory activity was conventionally measured as described in [14], using autoclaved [^{3}H]oleic acid-labeled *Escherichia coli* as the substrate of PLA_2, except for bee venom PLA_2 (Sigma) and the reaction buffer (10 mM imidazole, pH 7.0, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02 mM ATP). The reaction was initiated at 30°C by addition of ^{3}H-labeled *E. coli* to the protein sample which was preincubated with PLA_2 (50 ng) at 4°C for 10 min, and terminated 30 s later by addition of 2 N HCl and 100 mg/ml BSA. After centrifugation, the released radioactivity in the supernatant was counted in a liquid scintillation counter. The reaction was linear under these conditions. About 30–50% radioactivity of ^{3}H-labeled *E. coli* was released by PLA_2 (50 μg/assay).

2.3. *CNBr peptide mapping*

As described [17], the cleaved peptides of the proteins were analyzed by SDS-PAGE (15% gel) and visualized by silver staining.

2.4. *Phospholipid and F-actin binding assay*

The assay was performed as described in [9].

2.5. *Preparation of antiserum*

New Zealand White rabbits were immunized with purified protein (100–200 μg per immunization). The animals were injected with a 1:1 emulsion of the antigen in complete Freund's adjuvant. Three weeks later, two booster immunizations were given every 2 weeks as 1:1 emulsion in incomplete Freund's adjuvant. The antisera were collected 10 days following the second booster.

2.6. *Western blot analysis*

Purified proteins (1 μg each) or tissue homogenates were analyzed in SDS-PAGE, electrically transferred to nitrocellulose paper, and stained with India ink as described by Glenney [18]. The paper was incubated with 1:1000–5000 dilution of antiserum and followed by incubation with ^{125}I-labeled protein A and autoradiography. For preparation of the tissue homogenate (20% v/w), tissues were homogenized in buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, proteinase inhibitors) by a Physcotron homogenizer. The homogenates were prerun on SDS-PAGE (12.5% gel) and stained with Coomassie blue. On the basis of stained gel, the amount of tissue protein used was adjusted to approximately equal.

2.7. *Isoelectric focusing*

Slab-gel isoelectric focusing was performed according to Giulian et al. [19].

3. RESULTS AND DISCUSSION

When the homogenate of whole human placenta was treated with EGTA, the major polypeptides detected by SDS-PAGE were of 68, 45, 36 and 32 kDa (fig.1, lane 2). The 32-, 36- and 68-kDa proteins passed through a DE-52 column (lane 3). The 32-kDa proteins were separated from two other proteins by an FPLC mono S column (lane 5). Finally the 32-kDa protein was homogeneously purified by gel filtration on HPLC (lane 6). The protein was monomeric and designated as 32K-I. Another 32-kDa protein, termed 32K-II, was eluted at 0.1 M NaCl from the DE-52 column (fig. 1, lane 7) and gel-filtrated on Sephacryl S-200 to homogeneity (lane 8). The isoelectric points of 32K-I and -II were 5.8 and 5.1, respectively.

In vitro phospholipid-binding experiments showed that 32K-I bound to PS but not to phosphatidylinositol and phosphatidylcholine at 50 μM of free Ca^{2+} (not shown). This binding was blocked by EGTA. In contrast to 32K-I, no phospholipid-binding was observed with 32K-II.

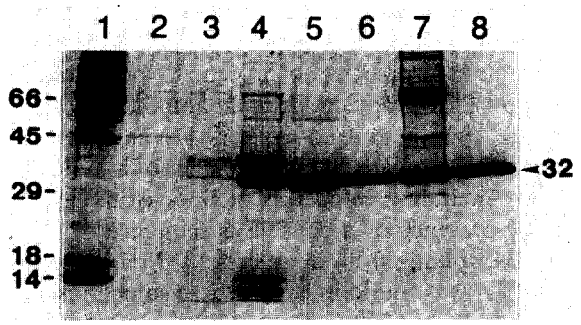


Fig.1. SDS-PAGE of 32-kDa proteins from human placenta at each purification step. Two monomeric 32-kDa proteins were purified as described in section 2. The homogenate of whole human placenta (lane 1), the extract of the homogenate with 5 mM EGTA (lane 2), the concentrated flow-through fraction of the EGTA extract applied to a DE-52 column (lane 3), the 0.2 M potassium phosphate eluate from a hydroxyapatite column (lane 4), the 0.1 M NaCl eluate from a Mono S column (lane 5), purified 32K-I after gel filtration (lane 6), the 0.1 M NaCl eluate from a DE-52 column (lane 7) and purified 32K-II after gel filtration on a Sephacryl S-200 column (lane 8). The numbers on the left side represent the molecular mass $\times 10^{-3}$. The arrowhead on the right indicates 32K-I and -II. Note that faint bands seen in all lanes near 68 kDa were artificial.

Neither 32K-I nor 32K-II showed F-actin-binding ability in the presence of 0.1 M KCl. Whereas they bound to F-actin in a Ca^{2+}-dependent manner in the absence of 0.1 M KCl. We do not know yet whether 32K-I and -II are potassium-sensitive F-actin-binding proteins.

The PLA_2-inhibitory activity was examined for 32K-I and -II. As shown in fig.2, only 32K-I showed a dose-dependent PLA_2-inhibitory activity. Almost indentical results were obtained when different assay conditions [9] were employed. The magnitude of the PLA_2-inhibitory activity of 32K-I appeared to be 2-fold lower than that of calpactins (not shown).

We have recently reported [9] that three distinct forms of 36-kDa proteins purified from the membranes and the total human placenta homogenate showed PLA_2-inhibitory activity as well as Ca^{2+}-dependent phospholipid- and F-actin-binding abilities. We identified the 36-kDa proteins as human calpactins or lipocortins [7-9]. 32K-I was copurified with the 36-kDa proteins until further purification using a hydroxyapatite column (see fig.1, lanes 3 and 4; [9]). Therefore, we

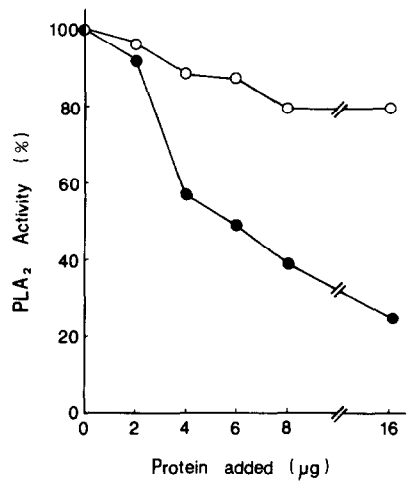


Fig.2. Dose-response of 32K-I for PLA_2-inhibitory activity. The reaction was initiated at 30°C by adding [^{3}H]-labeled *E. coli* (25 000 cpm) as substrate to aliquots of purified proteins preincubated with PLA_2 (50 ng) at 4°C for 10 min, and terminated 30 s later. The released radioactivity from the *E. coli* membrane was counted in a liquid scintillation counter. In all analyses, samples were assayed in duplicates and adjusted for nonspecific release by subtracting a control value. 32K-I (●) and 32K-II (○).

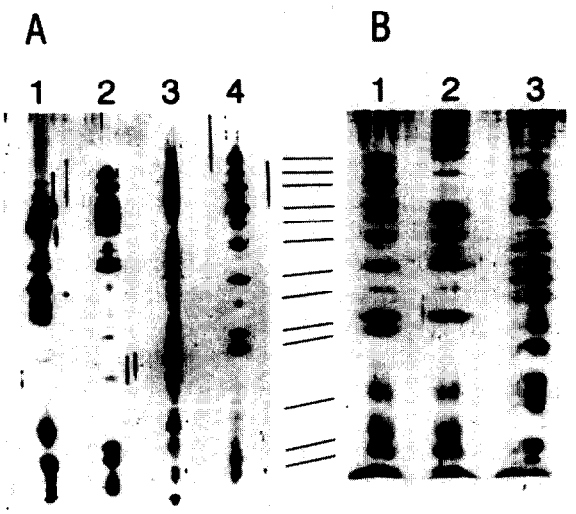


Fig.3. Comparison of CNBr peptide maps between 32-kDa proteins and calpactins. Purified proteins (10 μg each) were separated on SDS-PAGE (15% gel). Each band that stained with Coomassie blue was excised from the gel, incubated with CNBr (60 mg/ml) at 20°C for 2 h and washed. CNBr-cleaved fragments were visualized by silver staining after electrophoresis on an SDS-PAG (15%) containing 8 M urea. In panel A, 32K-I (lane 1); 32K-II (lane 2); calpactin I (36K-I)(lane 3); and calpactin II (36K-II)(lane 4). In panel B, calpactin II (lane, 1); calpactin II-related, Ca^{2+}-dependent phospholipid-binding 68-kDa protein specific for human placenta (68K-I)(lane 2); another Ca^{2+}-dependent phospholipid-binding 68-kDa from human placenta (68K-II)(lane 3).

compared the structure among 32K-I, 32K-II and calpactins. CNBr peptide mapping analysis indicated that 32K-I was distinct from 32K-II, calpactin I, II and 68-kDa proteins (fig.3). However, this result does not rule out the possibility that they possess common epitopes.

To investigate the immunological relationships among these proteins, rabbit antibodies against the purified proteins were prepared and then their cross-reactivities were examined by Western blotting (fig.4). Anti-32K-I antibody cross-reacted weakly with 32K-II and 68K-II. It also detected 36K-I (calpactin I) at a level 10-fold lower. Anti-32K-II antibody reacted strongly with 32K-I and weakly with 68K-II. 32K-I and -II were reactive with anti-68K-II antibody. Antisera against calpactins were not able to react with 32K-I, 32K-II and 68K-II. These results suggest the presence of common antigenic determinant(s) in 32K-I, -II and 68K-II even though their CNBr peptide maps were different.

The 68-kDa protein in the human placenta homogenate recognized by the anti-32K-II antibody but not by anti-68K-I and -II antibodies will require further investigation (fig.4B, lane 1). The purification and characterization of 68K-I which cross-reacted with the anti-calpactin II antibody will be published elsewhere (in preparation).

The tissue-distribution of 32K-I or 32K-II in adult rat was examined by using the Western blotting method. The results are summarized in table 1. 32K-I was expressed in many tissues except cardiac muscle, skeletal muscle and brain. The distribution of 32K-I is similar to that of protein II, porcine intestinal Ca^{2+}-dependent phospholipid-binding 32-kDa protein [7,20–22]. Since 32K-I has similar properties to protein II including PS-binding, 32K-I may be a human homologue of protein II. Weber and co-workers [7] have recently reported that protein II is a member of a multigene family including calpactins or lipocortins because of the presence of a consensus sequence of Ca^{2+}- and phospholipid-binding sites [3–7]. Interestingly, 32K-I was found in liver, where calpactins were undetectable. Endonexin (p32.5), a mammalian Ca^{2+}-binding protein related to *Torpedo* calelectrin [3,23,24] is also located in liver and has a p*I* value and a molecular mass similar to 32K-I. To elucidate the relationships among these proteins, it will be necessary to define their chemical structures.

Fig.4. Immunological cross-reactivity of 32K-I with antisera against 32K-II, calpactins and 68-kDa proteins. Samples (1 μg of each purified proteins and 1 μg of human placenta homogenate (20%, v/w)) were separated on SDS-PAGE (12.5%), transferred to nitrocellulose paper, reached with 1:5000 dilution of rabbit antisera, incubated with ^{125}I-protein A and visualized by autoradiography. Rabbit antisera were anti-32K-I (A), anti-32K-II (B), anti-calpactin I (C), anti-calpactin II (D), anti-68K-I (E) and anti-68K-II (F). Human placenta homogenate (lane 1), 32K-I (lane 2), 32K-II (lane 3), calpactin I (lane 4), calpactin II (lane 5), 68K-I (lane 6) and 68K-II (lane 7). Arrowheads indicate 32-kDa, 36-kDa (calpactins) and 68-kDa proteins, respectively.

Table 1

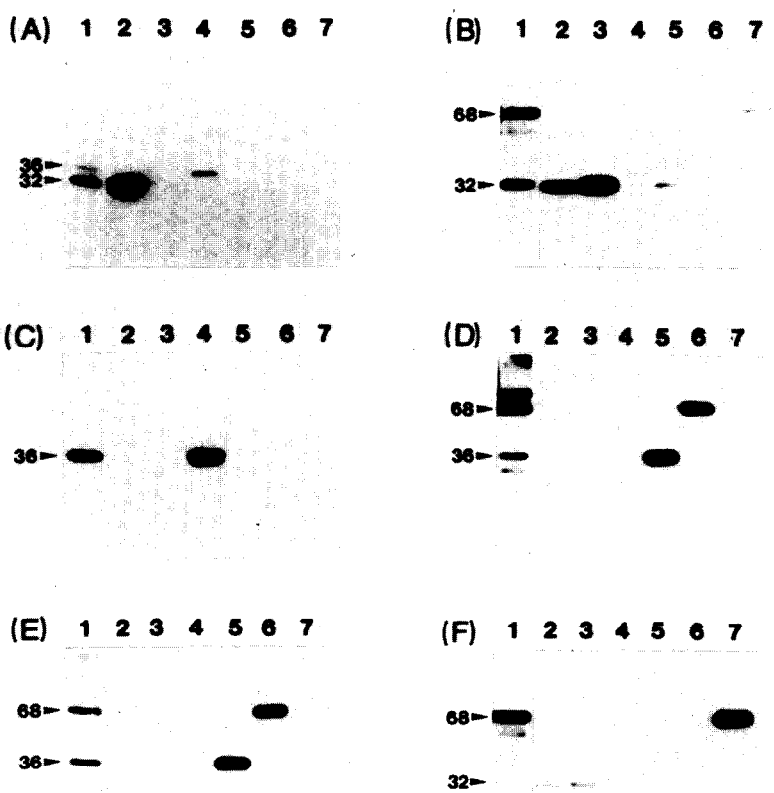
Distribution of 32K-I and -II proteins in adult rat[a]

Tissue	Proteins					
	32K-I	32K-II	36K-I[b]	36K-II[b]	68K-I[c]	68K-II[c]
Lung	+ +	+ +	+ + +	+ + +	−	+ + +
Intestine	+ +	+ +	+ +	+	−	+ + +
Liver	+	−	−	−	−	+
Spleen	+	−	+	+ +	−	+
Kidney	+	+	−	+	−	+ +
Cardiac muscle	−	−	−	−	−	+
Aorta	+	+	+ +	+ +	−	+ + +
Skeletal muscle	−	−	−	−	−	+
Brain	−	−	−	−	−	+

[a] The preparation of tissue homogenates is described in section 2. Approximately equal amounts of tissue proteins were used for Western blot analysis using rabbit antisera against indicated proteins as exactly described in the legend of fig.4. After incubation of the blots with ^{125}I-protein A, autoradiographs were done with short (3 h) and long (3 days) exposure time. Presence at high (+ + +), medium (+ +) or low (+) levels or absence (−) of the proteins was judged from the autoradiogram

[b] 36K-I and -II correspond to calpactin I and II, respectively (see [9])

[c] 68K-I and -II are Ca^{2+}-dependent phospholipid-binding proteins isolated from human placenta (in preparation)



ACKNOWLEDGEMENTS

We are grateful to Drs T. Hashimoto and M. Ohmichi at Ikeda and Minoh Municipal Hospitals for supplying human placentas. We also express our thanks to Miss Y. Sugiyama for typing the manuscript. This work was supported by Grant-in-Aid for special project research on Cancer-Bioscience (No. 61210019) to T.K. from the Ministry of Education, Science and Culture of Japan.

REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Geisow, M.J., Fritsche, U., Hexham, J.M., Pash, B. and Johnson, T. (1986) Nature 320, 636–638.
[4] Geisow, M.J. and Walker, J.H. (1986) Trends Biochem. Sci. 11, 420–423.
[5] Huang, K.S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E. and Pepinsky, R.B. (1986) Cell 46, 191–199.
[6] Saris, C.J.M., Tack, B.F., Kristensen, T., Glenney, J.R., jr and Hunter, T. (1986) Cell 46, 201–212.
[7] Weber, K., Johnson, N., Plessmann, U., Nguygen Van, P., Sölling, H.D., Ampe, C. and Vandekerckhove, J. (1987) EMBO J. 6, 1599–1604.
[8] Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R., jr (1987) J. Biol. Chem. 262, 1698–1705.
[9] Hayashi, H., Sonobe, S., Owada, M.K. and Kakunaga, T. (1987) Biochem. Biophys. Res. Commun., in press.
[10] Flower, R.J., Wood, J.N. and Parente, L. (1984) Adv. Inflamm. Res. 7, 61–70.
[11] Hirata, F. (1984) Adv. Inflamm. Res. 7, 71–78.
[12] Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2533–2636.
[13] Hirata, F. (1981) J. Biol. Chem. 256, 7730–7733.
[14] Rothhut, B., Russo-Marie, F., Wood, J., DiRosa, M. and Flower, R. (1983) Biochem. Biophys. Res. Commun. 117, 878–884.
[15] Gupta, C., Katsumata, M., Goldman, A.S., Herold, R. and Piddington, R. (1984) Proc. Natl. Acad. Sci. USA 81, 1140–1143.
[16] Pepinsky, R.B., Sinclair, L.K., Browing, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Falbael, T., Ribolini, A., Garwin, J.L. and Wallner, B.P. (1986) J. Biol. Chem. 261, 4239–4246.
[17] Pepinsky, R.B. (1983) J. Biol. Chem. 258, 11229–11235.
[18] Glenney, J. (1986) Anal. Biochem. 156, 315–319.
[19] Giulian, G.G., Moss, R.L. and Greaser, M. (1984) Anal. Biochem. 142, 421–436.
[20] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[21] Shadle, P.J., Gerke, V. and Weber, K. (1985) J. Biol. Chem. 260, 16354–16360.
[22] Shadle, P.J. and Weber, K. (1987) Biochim. Biophys. Acta 897, 502–506.
[23] Südhof, T.C., Ebbecke, M., Walker, J.H., Fritsche, U. and Boustead, C. (1984) Biochemistry 23, 1103–1109.
[24] Geisow, M., Childs, J., Dash, B., Harris, A., Panayotou, G., Südhof, T. and Walker, J.H. (1984) EMBO J. 3, 2969–2974.