Volume 317, number 3, 263–266 FEBS 12110 February 1993
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Enhancing factor protein from mouse small intestines belongs to the phospholipase A2 family

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Received 7 December 1992; revised version received 5 January 1993

Enhancing factor (EF), a growth regulatory molecule, isolated from mouse small intestines, has been well characterized in this laboratory. It increases the binding of epidermal growth factor in a unique manner via its own receptor. In the first 20 N-terminal amino acids sequenced, EF showed 50% homology to human Group II phospholipase A2 (PLA2). Here we propose that EF is yet another, unidentified isoform of PLA2 which regulates cell proliferation via modulation of EGF binding. To our knowledge, this is the first report implicating PLA2-II-like molecules in growth regulation.

Enhancing factor; PLA2; EGF; Receptor; Paneth cell; HPLC

1. INTRODUCTION

Earlier we have reported the isolation, purification and characterization of a unique modulator of epidermal growth factor from mouse small intestines [1,2]. Termed the enhancing factor (EF), the 14 kDa heat- and acid-stable protein was isolated based on its ability to increase the binding of EGF in the radioreceptor assay [1]. We have demonstrated earlier that EF binds to a membrane-associated receptor which is distinct from the EGF receptor [2]. Crude EF, obtained from acid-soluble proteins extracted from small intestines, was purified to homogeneity following gel permeation chromatography on BioGel P100 column and 2 cycles of reverse-phase HPLC on a μ-Bondapak C18 column [3]. In this paper we report the further purification of EF on a cation-exchange column on HPLC and the partial amino acid sequence obtained on an automated gas-phase sequencer. The first 20 amino acids in the sequence from the N-terminal end of the protein showed considerable homology to the phospholipase A2 (PLA2) enzyme, specially to the mammalian group II PLA2 [4,5]. Various laboratories have reported that PLA2s are involved in cellular arachidonic acid release and inflammatory reactions [4–6]. However, the presence of group II PLA2 in small intestines suggests that they may not only be involved in inflammatory reactions but may have an additional, as yet unidentified physiological role.

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2. EXPERIMENTAL PROCEDURES

2.1. *Purification of EF*

Acid-soluble proteins were extracted from mouse small intestines and purified as described earlier [3]. To obtain sequencing grade protein, reverse-phase HPLC (RP-HPLC)-purified EF was further passed over a cation-exchange HPLC (CE-HPLC) column (Protein-Pak SP 5PW, Waters). The column was equilibrated in 0.1 M sodium phosphate buffer, pH 6.6 (SPB) and 100 μg of protein injected. The column was washed for 10 min with SPB and the bound protein was eluted with a 1 M NaCl gradient. 5 μg of protein from each peak eluted from the column was tested for enhancing activity in the A431-^{125}I-EGF binding radioreceptor assay as described earlier [1].

2.2. *Sequencing of EF*

A partial amino acid sequence was obtained on an automated gas phase microsequencer (model 477A, Applied Biosystems). The first 20 N-terminal amino acids sequence was subjected to homology search against protein sequences in the EMBL data bank.

2.3. *Assay for phospholipase A2*

The method of Habermann and Hardt [6] was followed to assay the PLA2 activity. Briefly, 0.6% agarose in 0.05 M sodium acetate buffer, pH 7.5 was mixed with 0.4 ml egg yolk suspension (1 part fresh egg yolk mixed with 3 parts 0.85% saline, centrifuged at 2,000 rpm and 1 ml aliquots stored at −20°C) and 0.25 ml of 0.01 M $CaCl_2$. The mixture was poured in 100 mm plates and wells punched in the agarose. Standard PLA2 and the test samples were suspended in saline containing 0.1% BSA, added to different wells and the plates incubated at 50°C for 20 h. Formation of clear plaques around the wells indicated PLA2 activity.

3. RESULTS

RP-HPLC purified EF, when passed over a cation-exchange column gave 3 minor and one major peak (Fig. 1). 5 μg protein equivalent from each peak was tested in the radioreceptor assay as described earlier [1]. Protein eluting at 18.77 min and 19.75 min showed 235% and 285% EGF binding which means 135% and

RETENTION TIME(min)	% EGF BINDING
2·52	111
14·50	105
18·77	235
19·75	285
CONTROL	100

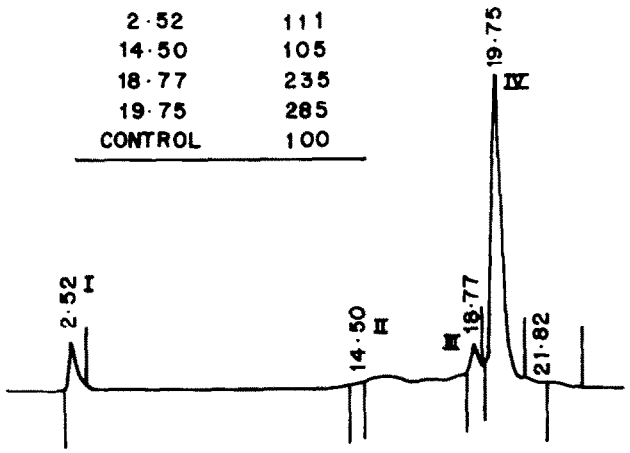

Fig. 1. HPLC profile of purified EF run on a cation-exchange column, SP 5PW (Waters), as described. The retention time (min) is marked on every peak and the roman numbers designate the peak numbers. 5 μg equivalent protein from each peak was tested in the radioreceptor assay using ^{125}I-EGF and fixed A431 cells [1]. Percentage of specific bound ^{125}I-EGF is shown in the Table (inset). (Counts obtained in the control wells where cells were incubated with ^{125}I-EGF alone were considered as 100% bound ^{125}I-EGF.)

185% enhanced activity, respectively (since control is considered as 100% EGF binding, Fig. 1, inset). When run on an SDS-PAGE and silver-stained, proteins in both peaks showed identical bands with the same mobility (data not shown). Thus, both proteins appear to be the same with a slightly different ionic charge. The reason for this is not known. However, more than 90% of the protein eluted in the 19.75 min peak. This was sequenced on an automated protein sequencer.

The sequence of the first 20 N-terminal amino acids is shown in Fig. 2 along with the sequences of the related proteins. EF showed 50% homology to human synovial fluid PLA2 [4], human cartilage PLA2 [5] and *Vipera aspis* snake venom PLA2 [7]. Other mammalian PLA2 including porcine intestinal PLA2 [8], bovine pancreatic PLA2 [9] and rat membrane-associated PLA2 [10] showed 30–45% homology to EF.

Source	Sequence	% homology
mouse EF	N I A Q F G E M I R L K T G R L A E L S	—
human	N L V N F H R M I K L T T G K E A A L S	50
snake venom	N L Y Q F G M M I F K M T K K S A L L S	50
rat	S L L E F G Q M I L F K T G K R A D V S	45
porcine	D L L N F R K M I K L K T G K A P V P V	35
bovine	A L W Q F N G M I K C K I P S S E P L L	30

Fig. 2. Comparison of first 20 N-terminal amino acid sequence of EF with human PLA2 [4,5], snake venom PLA2 [7], rat PLA2 [10], porcine PLA2 [3] and Bovine PLA2 [8]. Residues which are identical between EF and any of the other proteins are boxed.

EF was tested for PLA2 enzyme activity using egg-lecithin embedded in agarose as the substrate. EF showed PLA2 activity as seen by the clear plaques around the sample wells (Fig. 3). The activity was much lower than that compared to snake venom PLA2. Bovine pancreatic PLA2 (Sigma) did not show comparable activity. However, since this was a crude assay, its activity was checked in the PLA2 assay using mixed micellar substrate of [^{14}C]phosphatidyl choline and deoxycholate, in Dr. Anil Mukherjee's laboratory at NICHD/NIH, Bethesda, USA. CE-HPLC-purified EF was found to have high PLA2 activity at a dilution of 600 ng/ml (personal communication). Snake venom PLA2, when tested in the radioreceptor assay showed enhanced activity comparable to EF (data not shown). Neither bovine nor porcine pancreatic PLA2 showed enhanced activity.

4. DISCUSSION

EF is a 14 kDa molecule isolated from mouse small intestines based on its ability to increase the binding of EGF in the radioreceptor assay [1–3]. Our earlier studies, using antibodies raised against EF, have shown that partially purified EF binds to labelled EGF in a cell-free system, in a dose-dependent manner [2]. Data on the Scatchard plot analysis have shown that pretreatment of cells with EF results in an increase in the number of binding sites for EGF without an apparent change in the affinity [2]. We have also demonstrated that EF binds to a 100 kDa cell membrane receptor on A431 cells which is distinct from the EGF receptor [2]. Preliminary studies in our laboratory indicate that the receptor

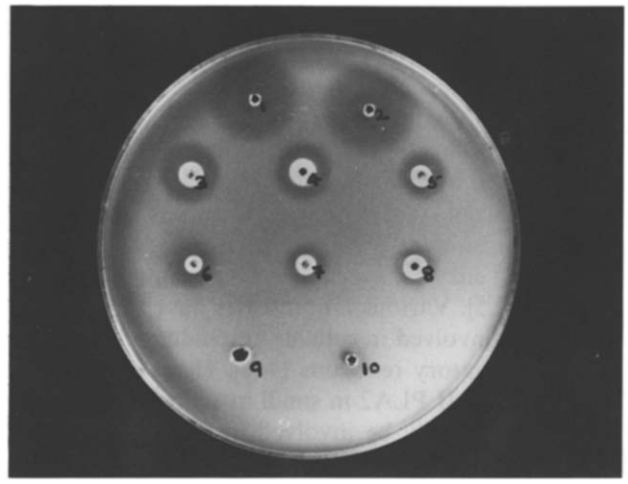

Fig. 3. Assay for PLA2 activity in agarose plates was carried out as described. Samples loaded in the wells were as follows: 1, snake venom PLA2 (10 ng); 2, snake venom PLA2 (20 ng); 3, intestinal crude extract (170 μg); 4, Biogel purified-EF* (100 μg); 5, RP-HPLC (first cycle)-purified EF* (10 μg); 6, RP-HPLC (second cycle)-purified EF* (10 μg); 7 and 8, CE-HPLC- purified EF (two different batches, 15 μg each); 9, distilled water (control); 10, bovine pancreatic PLA2 (Sigma, 30 μg).

*Prepared as described in reference 1 and 3.

could be a heparan-sulphate proteoglycan located on the cell membrane.

Immunohistochemically, EF was found to be localized specifically in the Paneth cell, situated at the base of the villi, adjacent to the stem cell population, in the small intestines [11–13]. Immunoreactive EF was not found to be present in large amounts in any other tissue. Small amounts of EF were found in stomach, proximal colon and immature hair follicles in newborn mouse skin [12,13]. In vitro, EF induces anchorage-independent growth of normal rat fibroblasts in the presence of EGF and also stimulates [^{3}H]thymidine incorporation in NR6 cells (devoid of functional EGF receptors) in the presence of EGF [2]. In vivo, EF gene appears to be expressed in mouse regenerating liver as well as in hyperplastic oesophageal epithelium (unpublished observations). From these data, we have concluded that EF plays a role in cell proliferation by increasing the binding of EGF to the cells and thereby modulating its action.

In the present study we report that the EF protein is yet another member of the PLA2 family as the first 20 N-terminal amino acids sequence shows 40–50% homology to PLA2 from different sources (Fig. 2). Also purified EF showed lipid hydrolysing activity (Fig. 3). On the other hand, snake venom PLA2, but not bovine or porcine PLA2 showed enhanced activity in the radioreceptor assay.

PLA2 are widely distributed enzymes which catalyse fatty acid release from the *sn*-2 position of glycerophospholipids [14]. Mammalian PLA2 have been divided into 2 main groups based on their primary structure [15]. Mammalian group I PLA2 (PLA2-I) is mainly secreted from pancreas as a digestive enzyme [16,17]. Recently, Arita et al. [18] have reported that PLA2-I has a novel proliferative effect in Swiss 3T3 cells via specific binding sites, possibly unrelated to its phospholipid hydrolysing activity. They have also reported that PLA2-I plays a role in the migration of vascular smooth muscles via specific binding sites [19]. PLA2-I has also been shown to be present in bovine adrenal medulla [20], rat gastric mucosa and lung [21] and rat glandular stomach [22]. PLA2s from group II (PLA2-II) are involved in cellular arachidonic acid release and inflammation [4,5,23]. PLA2-II have been reported to be present in rat platelets [22], rat spleen [10], human placenta, synovial fluid [4,24,25] as well as in human articular cartilage [5]. Presence of PLA2-like EF in the small intestines suggests that it has additional, yet unidentified physiological function.

Based on our studies we propose that EF is an isoform of PLA2-II, present in large amounts in the Paneth cells and it plays a crucial role in cell proliferation by modulating the binding of EGF. EF could bind to its own membrane receptor and in turn offer a binding site for EGF. Other PLA2-IIs like snake venom PLA2, rat platelet PLA2 and human synovial fluid PLA2 may function in a similar manner. Such molecules which regulate growth factor binding to cells may be important in tissues with high cell turnover. Lokeshwar et al. [26] have reported that basic proteins such as protamine increase EGF binding to high- as well as low-affinity receptors on cells by activating cryptic EGF receptors. EF could act via a similar mechanism. However, that would not explain the increase in DNA synthesis brought about by EF and EGF added together in NR6 (EGF receptorless) cells as reported earlier [2]. Whether the lipid hydrolysing enzymatic action is necessary and sufficient for the enhancing activity is now being investigated.

To our knowledge this is the first report implicating PLA2-II-like molecules in growth regulation.

Acknowledgements: This work was carried out as part of the project funded by Department of Science and Technology, Government of India to M.G.D. and R.M. (DST no. SP/SO/D79/89). We are grateful to Dr. A.S. Kolaskar, Bioinformatics Centre, Poona University for his help in screening the protein database and Dr. Anil Mukherjee, NICHD, NIH Bethesda, USA for carrying out the PLA2 assay on our samples.

REFERENCES

[1] Deo, M.G., Mulherkar, R. and Mane, S.M. (1983) Ind. J. Biochem. Biophys. 20, 228–231.
[2] Mulherkar, R. and Deo, M.G. (1986) J. Cell. Physiol. 127, 183–188.
[3] Mulherkar, R., Saraf, A., Wagle, A.S. and Deo, M.G. (1986) FEBS Lett. 207, 142–144.
[4] Kramer, R.M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E.P., Tizard, R. and Pepinsky, R.B. (1989) J. Biol. Chem. 264, 5768–5775.
[5] Recklies, A.D. and White, C. (1991) Arthritis and Rheumat. 34, 1106–1115.
[6] Habermann, E. and Hardt, K.L. (1972) Anal. Biochem. 50, 163–173.
[7] Gonzalez-Buritica, H., Khamashta, M.A. and Hughes, G.R.V. (1989) Ann. Rheum. Dis. 48, 267–269.
[8] Verger, R., Ferrato, F., Mansbach, C.M. and Pieroni, G. (1982) Biochemistry 21, 6883–6889.
[9] Fleer, E.A.M., Verheij, H.M. and DeHaas, G.H. (19??) Eur. J. Biochem. 82, 261–269.
[10] Ishizaki, J., Ohara, O., Nakamura, E., Tamaki, M., Kanda, A., Yoshida, N., Teraoka, H. and Okamoto, M. (1989) Biochem. Biophys. Res. Commun. 162, 1030–1036.
[11] Wagle, A.S., Desai, S. and Deo, M.G. (1988) Cell Biol. Int. Rep. 13, 309–312.
[12] Mulherkar, R., Desai, S.J., Rao, R.S., Wagle, A.S. and Deo, M.G. (1991) Histochemistry 96, 367–370.
[13] Desai, S.J., Mulherkar, R., Wagle, A.S. and Deo, M.G. (1991) Histochemistry 96, 371–374.
[14] Verheij, H.M., Slotboom, A.J. and DeHaas, G.H. (1981) Rev. Physiol. Biochem. Pharmacol. 91, 91–203.
[15] Heinrikson, R.L., Krueger, E.T. and Keim, P.S. (1977) J. Biol. Chem. 252, 4913–4921.
[16] DeHaas, G.H., Postema, N.M., Nieuwenhuizen, W. and Deenen, L.L.M. (1968) Biochim. Biophys. Acta 159, 118–129.
[17] Matsuda, Y., Ogawa, M., Shibata, T., Nakaguchi, K., Nishijima, J., Wakasuji, C. and Mori, T. (1987) Res. Commun. Chem. Path. Pharmacol. 58, 281–284.
[18] Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H. and Matsumoto, K. (1991) J. Biol. Chem. 266, 19139–19141.

[19] Kanamasa, T., Hanasaki, K. and Arita, H. (1992) Biochim. Biophys. Acta 1125, 210–214.
[20] Petit, K., Miserez, B., De Block, J., Van Dessel, G. and De Potter, W. (1992) Biochim. Biophys. Acta 1125, 150–156.
[21] Sakata, T., Nakamura, E., Tsuruta, Y., Tamaki, M., Teraoka, H., Tojo, H., Ono, T. and Okamoto, M. (1989) Biochemistry 1007, 124–126.
[22] Dimberg, J., Gustafson-Svard, C., Westrom, B., Tagesson, T. and Soderkvist, P. (1992) Biochim. Biophys. Acta 1130, 47–51.
[23] Hayakawa, M., Kudo, I., Tomita, M., Nojima, S. and Inoue, K. (1988) J. Biochem. (Tokyo) 104, 767–772.
[24] Lai, C.Y. and Wada, K. (1988) Biochem. Biophys. Res. Commun. 157, 488–493.
[25] Seilhammer, J.J., Pruzanski, W., Vadas, P., Plant, S., Miller, J.A., Kloss, J. and Johnson, L.K. (1989) J. Biol. Chem. 264, 5335–5338.
[26] Lokeshwar, V.B., Huang, S.S. and Huang, J.S. (1989) J. Biol. Chem. 264, 19318–19326.