Enhancing activity and phospholipase A₂ activity: two independent activities present in the enhancing factor molecule

Shilpa KADAM and Rita MULHERKAR¹

Genetic Engineering Unit, Cancer Research Institute, Tata Memorial Centre, Parel, Mumbai 400012, India

Enhancing factor (EF), a molecule that increases the binding of epidermal growth factor (EGF) to A431 cells, was first isolated in our laboratory from mouse intestines, and subsequently shown to be a secretory form of phospholipase A₂ (PLA₂) [Mulherkar, Rao, Wagle, Patki and Deo (1993) *Biochem. Biophys. Res. Commun.* **195**, 1254–1263]. We had proposed earlier that EF increases the binding of EGF by first binding to its own cell-surface receptor [identified as a 100 kDa molecule; Mulherkar and Deo (1986) *J. Cell. Physiol.* **127**, 183–188], and then by creating a binding site for EGF. However, due to its PLA₂ activity, there was a possibility that EF, by its phospholipase activity could be unmasking cryptic EGF receptors on the cell surface, thereby increasing the number of binding sites for EGF. To test whether enhancing activity and phospholipase activity

are independent of each other, a series of mutations were created using the full-length EF cDNA as a template, expressed in 293 cells and the mutant recombinant proteins checked for EF as well as PLA₂ activities. Our studies have shown that one of the mutant EF proteins, lacking PLA₂ activity, retains EF activity. This demonstrates unambiguously that EF and PLA₂ activities are two independent activities in the same molecule. Mutation in the Ca²⁺-binding loop resulted in loss of EF activity, thereby demonstrating that EF activity is Ca²⁺-dependent. The N-terminal region of the EF molecule appears to be crucial for the enhancing activity.

Key words: Ca²⁺-binding loop, epidermal growth factor, heparin-binding protein, mouse intestine, secretory phospholipase A₂.

INTRODUCTION

Enhancing factor (EF), a secretory phospholipase A₂ (sPLA₂), was first isolated in our laboratory from mouse intestines as a growth-factor modulator. EF increases the binding of epidermal growth factor (EGF) to A431 cells in the radio-receptor assay [1–3]. It was proposed that EF binds to a 100 kDa receptor on the cell surface, traps EGF and makes it available to the cell, thereby giving it a growth advantage [2]. Immunohistochemical studies have demonstrated that EF is localized predominantly in the Paneth cells of small intestine [4–6] and has been used as a marker for Paneth cells [7]. Sequence analysis [8,9] and phylogenetic analysis have shown that EF belongs to the class of membrane-associated PLA₂s [10].

PLA₂s have been isolated from various sources and studied extensively, although their function has yet to be determined fully. X-ray analyses from several PLA₂s show that the enzymes are structurally similar. The low-molecular-mass (14 kDa) PLA₂s are characterized by a rigid three-dimensional structure held together by disulphide bridges and require Ca²⁺ for their catalytic activity [11]. PLA₂s have also been shown to bind heparin, and the catalytic activity of porcine pancreatic PLA₂ has been shown to be inhibited by heparin [12]. However, Murakami et al. [13] have reported that neither mouse nor rat sPLA₂ activities were inhibited by heparin in their assay system. We have reported that heparin inhibits enhancing activity as well as PLA₂ activities [14].

The increased binding of EGF to the cells by EF could be explained in two ways. EF, by its inherent ability to hydrolyse phospholipids, could expose cryptic EGF receptors on the cell surface, or EF could bind to its own cell-surface receptor and in

turn offer a binding site for EGF. Whereas EF protein has been found to have PLA₂ activity [8], it was not evident whether the enhancing activity is dependent on the PLA₂ activity. Since the structures of PLA₂ molecules have been well established and the different active sites defined, it was proposed to mutate the enzyme active site(s) and examine the activities of the mutated molecules. In the present study we report cloning and expression of wild-type and mutant EF cDNAs expressed in 293 cells and functional characterization of the recombinant protein.

MATERIALS AND METHODS

Amplification and cloning of wild-type and mutant EF cDNAs

The full-length EF cDNA was obtained by rapid amplification of cDNA ends (RACE)-reverse transcriptase (RT)-PCR and cloned into pUC vector as described by Kadam et al. [10]. The 733 bp full-length EF cDNA insert from pUC was excised with *HindIII*/*XbaI* and religated to the mammalian expression vector, pRc/CMV (Invitrogen), cut with the same enzymes. During RACE-RT-PCR, one of the full-length cDNA products amplified using primers P5 and P6 (Table 1), encompassing 87 bp of the 5'-untranslated region (UTR), 441 bp of the complete open reading frame and 264 bp of the 3'-UTR (total 792 bp), was found to have a point mutation at the nucleotide position 149 after the first ATG codon, which resulted in substitution of A in place of G. This mutant cDNA was designated SK-8m.

Mutant EF cDNAs were obtained by nested PCR strategy, using wild-type full-length EF cDNA as a template and combinations of EF-specific nested primers (as shown in Table 1). For one of the mutants, SK-3.5m, point mutations were intro-

Abbreviations used: EF, enhancing factor; PLA₂, phospholipase A₂; sPLA₂, secretory PLA₂; EGF, epidermal growth factor; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; UTR, untranslated region; DMEM, Dulbecco's modified Eagle's medium; rEF, recombinant EF.

¹ To whom correspondence should be addressed (e-mail cri3@scochak.ncst.ernet.in).

Table 1 Composition of primers used in the study

Primers (Genset, France) were designed based on the published EF cDNA sequence [9]. Sequences in lower case indicate linkers with restriction sites to facilitate cloning into vectors.

Primer	Sequence	Size
EF ₍₂₄₄₋₂₂₇₎	5'-TCTGGTTTGCAGAACAGG-3'	17 mer
EF(H5')	5'-cccaagcttgggcTGACAGCATGAAGGTCC-3'	30 mer
HEF ₍₂₂₉₋₂₄₀₎	5'-cccaagcttgggaccatGGATGCCACAGACC-3'	31 mer
EF(X3')	5'-GCTCTAGAGCCCAGGACTCTCTTAGGTACG-3'	30 mer
EF(P ₅)	5'-CCTCTGAAGGTTCCAGAATCGATAG-3'	25 mer
EF(P ₆)	5'-ccgaattCCCAGGACTCTCTTAGGTACGAAGC-3'	32 mer
EF(FC3)	5'-gctctagagcTACGATTTGGGCTTCTCCCTTCGC-3'	36 mer

duced in the antisense primer [primer EF(FC3), Table 1] such that Lys¹¹⁹ and Lys¹²¹ were mutated to Glu¹¹⁹ and Glu¹²¹. PCR amplifications were carried out using *Taq* DNA polymerase (Amersham) for 30 cycles at 95 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min. After the last cycle, extension was carried out at 72 °C for 10 min. After confirming the size of the amplified products on an ethidium bromide-stained agarose gel, they were cloned into a pUC vector for sequencing using the Sure Clone ligation kit (Pharmacia), and sequenced using Sequenase version 2 (USB Amersham). After confirming the nucleotide sequences, the inserts were excised using *HindIII/XbaI* and ligated into pRc/CMV cut with the same enzymes. Plasmid DNA was prepared on a large scale from positive clones by standard procedures and purified on columns (Qiagen). After quantification, the DNA was used for transfections.

Transfection of HEK-293 cells with cDNAs for wild-type and mutant EF genes

HEK-293 cells (1×10^5) were seeded in 60 cm tissue culture plates (Nunc) in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (Hyclone). The cells were transfected with lipofectin (Gibco-BRL) using standard procedures. Briefly, 2 µg of each construct DNA was mixed with 10 µl of lipofectin in 200 µl of DMEM and allowed to stand at room temperature for 15 min. The cells were washed twice in DMEM and incubated with 1.8 ml of DMEM. The DNA-lipofectin complex was then added dropwise to the cells and incubated for 6 h at 37 °C in a 5% CO₂ incubator. After 48 h the transfectants were selected on G-418 (800 µg/ml). The antibiotic-resistant clones were screened for the recombinant protein by immunostaining using antibodies to EF (results not shown). One representative clone, positive by immunostaining, from each of the individual transfection reactions was characterized further. The designations of the different transfectants and their properties are shown in Table 2.

Extraction and purification of acid-soluble proteins from HEK-293 transfected cells

Acid-soluble proteins were extracted from wild-type and transfected 293 cells using protocols standardized in our laboratory for extraction of EF from mouse intestines [1]. Cells from 10 subconfluent 100 mm Petri plates (Nunc) were washed thrice with PBS and harvested for extraction. Recombinant EF protein (rEF) was purified from total acid-soluble proteins on a heparin-affinity HPLC column (TSK, Tosoh), as described by Wagle et al. [14]. Briefly, 2 mg of total acid-soluble protein was dissolved in 500 µl of 0.02 M phosphate buffer, pH 7.5, and injected on to

the column pre-equilibrated with the same buffer. The proteins were eluted using a gradient of 0–2 M NaCl in phosphate buffer, at a flow rate of 1 ml/min. EF was shown to elute at 0.8 M NaCl from earlier studies [14]. Hence, peaks in that range were collected individually, lyophilized and tested.

Immunoblotting

Acid-soluble proteins from transfected 293 and wild-type cells were separated on SDS/PAGE (17.5% gel) and transferred to nitrocellulose membrane (Hybond-N, Amersham). Blocking was carried out overnight using 3% BSA in Tris-buffered saline at room temperature. The blot was incubated with rabbit polyclonal anti-EF antiserum (1:10000) as described by Mulherkar et al. [5]. For visualization of the protein bands, an enhanced chemiluminescence (ECL) Western-blot analysis kit (Amersham) was used according to the manufacturer's instructions.

Measurement of enhancing activity

Enhancing activity was measured as described earlier [1] using A431 cells and ¹²⁵I-EGF. Lyophilized test protein was dissolved in 100 µl of binding buffer and added to the cells with 100 µl pf ¹²⁵I-EGF (counts adjusted to 30000 c.p.m./100 µl, specific activity ≈ 80 µCi/µg). Non-specific binding was determined in the presence of 50 ng of unlabelled EGF. Cells were lysed in lysis buffer and counted in a gamma counter. The enhancing activity was calculated as the percentage of EGF bound above the control level. Non-specific binding was subtracted from all the readings. Each assay was carried out at least thrice, in duplicate.

PLA₂ assay using labelled *Escherichia coli* membranes

PLA₂ assay was carried out using labelled autoclaved *E. coli* as described by Elsbach and Weiss [15]. Briefly, 1 ml of overnight XL1-cell culture was diluted with 75 ml of Luria-Bertani medium containing 175 µl of [³H]oleic acid (10 Ci/mmol; NEN Life Sciences Products) and incubated further for 8 h. After washing the labelled cells with 50 ml of Luria-Bertani medium followed by 50 ml of Tris/BSA [1% BSA (Sigma) in 100 mM Tris (pH 8)] the cells were resuspended in 2 ml of 140 mM NaCl/20 mM Tris (pH 8)/2% BSA. Aliquots (100 µl) of the labelled cells were stored frozen. Incorporation of the label was ≈ 40%. For the assay, labelled *E. coli* cells were washed and resuspended in PLA₂ buffer [100 mM Tris (pH 8)/10 mM CaCl₂/0.1% BSA] such that 100 µl had ≈ 100000 c.p.m. Labelled membranes (100 µl; substrate) were added to serial dilutions of the acid/ethanol-extracted test samples. Biogel P100 purified mouse intestinal EF served as the positive control and wild-type 293 cells as the negative control. The reaction was carried out at room temperature for 1 h after which 250 µl of chilled stop buffer (100 mM EDTA in 1% BSA) was added. Supernatant (400 µl) was collected for counting in the beta counter. The data were plotted as c.p.m. released against concentration of the protein.

Heparin binding and Western-blot analysis

Approx. 10 µg aliquots of HPLC-purified intestinal EF and rEF, and 100 µg of crude acid-soluble 293/SK-3.5m protein, were each mixed with 100 µl of heparin-Sepharose beads (Pharmacia), prewashed and suspended in 500 µl of phosphate buffer and incubated overnight at 4 °C. After incubation, the suspension was centrifuged and the supernatant saved as the unbound protein fraction for Western blotting. The beads were washed three times with phosphate buffer. The heparin-bound proteins were eluted using 2 M NaCl in phosphate buffer, dialysed against

Table 2 Summary of the different clones generated for the study

293 Cells were transfected with different EF cDNA clones as shown in the Table and described in Materials and methods section. Composition of the primers is shown in Table 1. The 63 bp signal sequence is shown separately in the open reading frame (ORF) column.

Clone	Size of the insert	Primers used	5'-UTR	ORF	3'-UTR	Primer-sequence overhangs	Mutation in protein
293/SK-15 (wild type EF)	734 bp	EF(H5'), EF(P6)	7 bp	63 + 378 bp	264 bp	20 bp	Nil
293/SK-8m	801 bp	EF(P5), EF(P6)	87 bp	63 + 378 bp	264 bp	7 bp	Gly ³⁰ → Asp
293/SK-60m	329 bp	EFH5', EF(244-227)	7 bp	63 + 246 bp	Nil	13 bp	Δ83-125
293/SK-14m	598 bp	HEF(229-240), EF(X3')	Nil	0 + 309 bp	264 bp	26 bp	Δ1-37
293/SK-3.5m	461 bp	EF(H5'), EF(FC3)	7 bp	63 + 378 bp	Nil	23 bp	Lys ¹¹⁹ → Glu, Lys ¹²¹ → Glu

MilliQ water, lyophilized and subjected to immunoblotting, along with the unbound fraction, as described earlier.

RESULTS

Cloning and expression of wild-type full-length EF cDNA in 293 cells

The full-length EF cDNA, comprising 7 bp of 5'-UTR (starting from the Kozak consensus sequence), 63 bp of signal peptide, 378 bp of mature EF protein coding region and 264 bp of the 3'-UTR [10], was cloned in the mammalian expression vector, pRc/CMV and expressed in 293 cells. One of the transfected, G-418-resistant clones, 293/SK-15, was selected based on high expression of EF detected by immunostaining (results not shown). A 14 kDa band was seen on the Western-blot (Figure 1), corresponding to the band in HPLC-purified intestinal EF and which was absent in wild-type 293 cells. This confirmed that the EF gene was expressed in 293/SK-15 cells.

Figure 1 Western-blot analysis of native and recombinant wild-type EFs

HPLC-purified proteins from mouse intestines (lane 1), acid-soluble proteins from 293/SK-15 cells (lane 2) and untransfected 293 cells (lane 3) were extracted as described in the Materials and methods section and separated by SDS/PAGE (17.5% gel). Samples were analysed by immunoblotting using rabbit polyclonal anti-EF antiserum at 1:10 000 dilution and visualized using an ECL kit (Amersham).

Figure 2 Heparin-affinity separation of native and recombinant wild-type EFs

HPLC profile of acid-soluble proteins extracted from mouse intestines (a), 293/SK-15 cells (b) and untransfected 293 cells (c), run on a heparin-affinity column and monitored at 280 nm. The retention time (in min) is marked on every peak.

Characterization of rEF

Earlier studies in our laboratory have shown that EF is a heparin-binding protein [14]. Hence, rEF protein was purified from the crude acid-soluble proteins using a heparin-affinity column. The HPLC profiles of acid-soluble proteins extracted from intestines and 293/SK-15 cells were similar (Figure 2). Each peak was collected individually and tested for enhancing activity. The activity was found to be present in the peak eluting at approx. 18 min in both intestinal proteins as well as 293/SK-15 proteins, but not in non-transfected 293 cells (Table 3). Heparin-affinity-purified rEF showed more than 100 % enhancing activity, which could be abrogated in the presence of heparin (Figure 3). PLA₂ activity could also be abolished by heparin in the blood

Table 3 Radio-receptor assay of the fractions eluting from HPLC heparin-affinity column

Radio-receptor assay was carried out in duplicate wells with each protein peak eluting out of the heparin-affinity HPLC column. Enhancing activity was calculated as percentage EGF binding over control EGF binding (which was taken as 100%). Non-specific binding (76.5 c.p.m.) in the presence of excess unlabelled EGF was subtracted from each reading. The data from one representative experiment are given as means $\pm$ S.E.M.

Sample loaded	Fraction no.	Retention time (min)	Mean c.p.m. ($\pm$ S.E.M.)	Mean specific c.p.m.	EGF binding (%)	Enhancement (%)
Mouse intestinal EF	1	2.79	1196.5 $\pm$ 82.5	1120	109	9
	2	6.60	1122.5 $\pm$ 78.5	1046	101.7	1.7
	3	17.31	1681 $\pm$ 5	1604.5	156	56
	4	18.37	1944 $\pm$ 30	1867.5	181.7	81.7
293/SK-15	1	2.97	1116.5 $\pm$ 20	1040	101.2	1.2
	2	6.30	1125 $\pm$ 10	1048.5	102	2
	3	17.10	1525.5 $\pm$ 80	1449	141	41
	4	18.55	1824.5 $\pm$ 8.5	1748	170	70
293	1	1.22	1114 $\pm$ 23	1037.5	101	1
	2	2.95	1514 $\pm$ 12	1438	140	40
	3	6.76	1416 $\pm$ 5	1339.5	130	30
	4	24.80	1326.5 $\pm$ 90	1250	121.7	21.7
Control			1104 $\pm$ 20	1027	100	—

Figure 3 Enhancing activity of heparin-affinity-purified rEF protein in the presence or absence of heparin

Radio-receptor assay was carried out in duplicate wells using different concentrations of purified rEF in the presence (+) or absence (—) of 10 units of heparin. 125 I-EGF binding in the absence of any additives was considered as a baseline (control). The data from one representative experiment are given as means $\pm$ S.E.M.

agar assay (results not shown). Approx. 1.7 mg of purified EF protein was obtained from 7.8×10^7 293/SK-15 cells.

Point mutation in the Ca^{2+} -binding loop

During amplification of the full-length EF cDNA, one of the ≈ 800 bp EF cDNAs was found to contain a point mutation at nucleotide position 149 after the first ATG, which resulted in substitution of an A in place of a G, a change from Gly²⁹ (GGC) to Asp²⁹ (GAC) of the mature EF protein (corresponding to position 30 in the aligned PLA₂ sequences). This mutation was found to be in the highly conserved Gly³⁰ residue of the PLA₂ molecule, which is involved in the Ca^{2+} -binding domain [16]. The mutant EF cDNA was cloned into the expression vector pRc/CMV. One of the G-418-resistant clones, designated 293/SK-8m, was selected by immunostaining (results not shown) and cultured for protein extraction.

Figure 4 Enhancing activity of wild-type and mutant rEF proteins

Radio-receptor assay was carried out in duplicate wells as described in the Materials and methods section. Control wells had no protein added to A431 cells. Intestinal EF, purified on HPLC (HPLC-IEF) served as a positive control. Proteins extracted from wild-type 293 cells served as negative control. The experiment was carried out more than three times and data from one representative experiment are given as means $\pm$ S.E.M.

Deletion mutants and point mutations in the C-terminal cationic residues

Deletion mutants of EF cDNAs were obtained by nested PCR strategy, subcloned in pRc/CMV and transfected into 293 cells. 293/SK-60m carried a deletion in the C-terminal end, $\Delta 83$ –125, (Table 2) and 293/SK-14m carried a deletion in the N-terminal region, $\Delta 1$ –37. 293/SK-3.5m carried point mutations in the putative C-terminal heparin-binding residues, Lys¹¹⁹ $\rightarrow$ Glu and Lys¹²¹ $\rightarrow$ Glu (Table 2). Acid-soluble proteins from all the transfected cell lines were extracted and tested for enhancing activity and PLA₂ activity.

293/SK-60m and 293/SK-3.5m showed enhancing activity comparable with 293/SK-15 as well as intestinal EF protein (Figure 4). 293/SK-8m and 293/SK-14m did not show any enhancing activity (Figure 4). Considerable PLA₂ activity was

Figure 5 PLA₂ activity of the acid-soluble proteins

Serial dilutions of acid-soluble proteins extracted from the different transfected cell lines were taken for the enzyme assay. Biogel P100 purified intestinal EF (EF P100) served as the positive control and wild-type (wt) 293 acid-soluble proteins served as a negative control. The c.p.m. released were measured at the end of the assay as described in the Materials and methods section. The data are representative of three independent experiments.

seen in EF P100, 293/SK-15 and 293/SK-3.5m, whereas 293/SK-60m did not show any PLA₂ activity (Figure 5). 293/SK-14m as well as 293/SK-8m were devoid of PLA₂ activity (Figure 5). 293/SK-3.5m exhibited PLA₂ activity comparable with 293/SK-15. A summary of the wild-type and mutant EF protein structures and activities is shown in Figure 6.

Figure 7 Heparin-binding property of SK-3.5m

Acid-soluble proteins from intestinal EF (HPLC-IEF, lanes 1 and 2), 293/SK-15 (lanes 3 and 4) and 293/SK-3.5m (lanes 6 and 7) were incubated with heparin-Sepharose beads as described in the Materials and methods section. The unbound (lanes 1, 3 and 6) and eluted proteins (lanes 2, 4 and 7) were separated by SDS/PAGE (17.5% gel). Samples were analysed by immunoblotting using rabbit polyclonal anti-EF antiserum at 1:10 000 dilution and visualized using an ECL kit (Amersham).

Heparin-binding property of mutant rEF

To check whether the C-terminal cationic residues were involved in heparin binding, acid-soluble proteins from 293/SK-3.5m cells were incubated with heparin-Sepharose beads. On Western-blot analysis of the bound proteins, a 14 kDa band was observed in the extract from 293/SK-3.5m (Figure 7, lane 7) comparable with that in intestinal EF and 293/SK-15 (Figure 7, lanes 2 and 4, respectively). No protein was detected in the unbound fractions in intestinal EF (Figure 7, lane 1) nor in the 293/SK-15 (Figure 7, lane 3). Although a faint 14 kDa band was seen in the unbound fraction in 293/SK-3.5m (Figure 7, lane 6) the majority of the protein was found to be bound to the heparin-Sepharose

Figure 6 Wild-type and mutant EF protein structures and their properties

The structures of the proteins used in the study and their enhancing and PLA₂ activities are shown. SP, signal peptide. The numbers in the line diagram denote the amino acid residues in the mature EF protein.

beads (Figure 7, lane 7). Mutation in the C-terminal cationic residues Lys¹¹⁹ and Lys¹²¹ did not appear to interfere with the heparin-binding property of EF.

DISCUSSION

EF is a 14 kDa molecule isolated from mouse small intestines, identified by its ability to increase the binding of ¹²⁵I-EGF to A431 cells in the radio-receptor assay [1,2]. In the presence of EGF, EF stimulates DNA synthesis *in vitro* in NR6 cells (cells devoid of functional EGF receptors) as well as inducing anchorage-independent growth in normal rat fibroblasts [2]. Therefore, we have proposed that EF gives the cell a growth advantage by making EGF available to it. To explain the increased binding, we had proposed that EF binds to its own cell-surface receptor and in turn offers a binding site for EGF [2].

On sequencing the EF protein as well as the cDNA, EF was found to be a sPLA₂, belonging to group II [8,9]. Hence there was a possibility that the enhancing activity was brought about by hydrolysing phospholipids on the cell surface and exposing cryptic EGF receptors, thereby increasing the EGF binding. Besides having structural similarity to rat and human sPLA₂, EF also shared some functional characteristics with PLA₂. EF was found to require Ca²⁺ for its enhancing activity (S. Kadam and R. Mulherkar, unpublished observations) and the enhancing activity could be blocked with heparin [14]. Whether enhancing activity was dependent on phospholipase activity or not had to be investigated. Modifying the PLA₂ active sites in the EF molecule would help in understanding whether the enzyme action is essential for enhancing activity or whether the two activities are independent of each other.

rEF is secreted but is tightly bound to the membrane

The full-length EF cDNA, 293/SK-15, expressed in 293 cells, was found to be identical to the native, intestinal EF. Although rEF was secreted out, it was found to be tightly associated with the membranes and could be dissociated with 1 M NaCl (results not shown). Phylogenetic analysis of the mammalian PLA₂ family using 16 aligned PLA₂ sequences has shown previously that mouse EF belongs to the membrane-associated PLA₂ family with a bootstrap value of 98 % [10]. There have been reports that the membrane-associated PLA₂s are essentially identical to sPLA₂ but, unlike sPLA₂, tightly associated with the membrane [17]. Ishizaki et al. [18] have reported that PLA₂ from rat spleen is tightly associated with the membrane and that sorting machinery within the cell plays a crucial role in anchoring the molecule to the cell surface, whereas Ono et al. [17] have reported that the association of the PLA₂ from rat spleen with the membrane fraction could be due to the cluster of hydrophobic amino acid residues on the molecular surface.

EF does not bind heparin via C-terminal cationic residues

PLA₂s have been shown to bind to heparin-like cell-surface molecules [19–23]. EF also binds heparin, and heparin inhibits EF activity by blocking its binding site on the cell membrane [14]. Cationic residues involved in heparin binding are highly conserved in the rat, human and mouse PLA₂ sequences. Murakami et al. [13] demonstrated by site-directed mutagenesis that the C-terminal residues Lys¹¹⁹ and Lys¹²¹ are important for association of the mouse sPLA₂ with heparin and cell surfaces, although they are not essential for the catalytic activity. Their results showed that mutations in the two residues resulted in a reduced capacity of the molecule to bind to heparin and cell-surface heparan sulphate proteoglycans compared with wild-type sPLA₂ [13]. In

our studies, mutant 293/SK-3.5m, carrying similar mutations in the C-terminal Lys residues (Lys¹¹⁹ → Glu and Lys¹²¹ → Glu), could bind heparin (although the affinity for heparin was not checked), and demonstrated both enhancing and PLA₂ activities. This suggests that the ability of the mutant EF to bind to the cell surface and bring about an increase in the binding of EGF was not affected. Diccianni et al. [23] have shown that a peptide corresponding to 26 residues at the N-terminal end of porcine pancreatic PLA₂ can rescue PLA₂ from heparin inhibition. Dua and Cho [24] have also reported that the cationic residues at the N-terminal region of the human sPLA₂ are responsible for interaction with the heparin. Based on the present data and our earlier studies [14], we propose that the N-terminal basic residues, Arg¹⁰, Lys¹², Lys¹⁵ and Arg¹⁶, could be involved in heparin binding.

Enhancing activity is Ca²⁺-dependent

sPLA₂s have been shown to be dependent on Ca²⁺ for their enzymic activity [25]. All catalytically active sPLA₂s are known to have a Ca²⁺-binding loop with a highly conserved consensus motif, Xaa-Cys-Gly-Xaa-Gly [26]. Gly³⁰ and Asp⁴⁹ have been found to be essential for the enzymic activity and are highly conserved in all sPLA₂s [16,25,27–29]. This motif is present in the EF molecule as well [9]. A full-length EF cDNA obtained by PCR strategy had a point mutation at the nucleotide position 149 from the ATG, resulting in an amino acid change from Gly to Asp at position 29 (position 30 when aligned with other sPLA₂s). This point mutation was found to be in the Ca²⁺-binding loop. In studies from our laboratory, EF activity was found to be lost if Ca²⁺ was depleted from the binding medium, thus indicating that enhancing activity is Ca²⁺-dependent (results not shown). The mutant protein confirmed this observation. Recombinant protein from 293/SK-8m showed neither enhancing activity nor PLA₂ activity. It is possible that the Ca²⁺ ion stabilizes the EF molecule and plays an important role in EGF binding. SK/293-60m has the Ca²⁺-binding loop and displays enhancing activity, whereas in SK/293-14m this region is deleted and the molecule is devoid of enhancing activity.

Enhancing activity in deletion mutants

Sites and patterns detection analysis [30], and comparison of the EF sequence with known PLA₂ molecules, suggested the existence of two PLA₂ active sites centred on His⁴⁸ and Asp¹¹³ [31]. A deletion mutant, 293/SK-60m, which had a 43 amino acid deletion at the C-terminal end, retained its enhancing activity but showed no PLA₂ activity. This demonstrates that enhancing activity is independent of PLA₂ activity. Another mutant, 293/SK-14m, which had an N-terminal deletion of 37 amino acid and an intact C-terminal end, with a molecular mass of 10 kDa, shows no enhancing activity and no PLA₂ activity. This was expected, as the Ca²⁺-binding loop was deleted from the molecule. The ability of the molecule to bind the cell surface as well as EGF appears to reside in the N-terminal end.

Mechanism of action of EF

To explain the mechanism of action of enhancing activity based on the present data, we once again propose that EF binds to the cell surface, presumably via heparan sulphate proteoglycans, and in turn offers a binding site for EGF. EF by itself may not be able to bind EGF but when bound to its cell-surface receptor could be creating a binding site for EGF. The mutation-analysis studies indicate that the enhancing activity and the phospholipase activity are independent of each other and that the N-terminal

end of the EF molecule is perhaps involved in the enhancing activity. Whether the EF-EGF complex is responsible for transducing signals into the cell or whether it only sequesters EGF molecules on the cell surface is not yet clear.

EF appears to be a molecule with dual function; growth modulation via increased EGF binding, and phospholipase A₂ activity. There are other reports of such bifunctional molecules. Tissue inhibitor of metalloproteinase is a molecule that has both anti-protease activity and growth-factor activity [32]. PLA₂ from bovine seminal plasma has been shown to be a platelet-activating factor acetylhydrolase [33]. Rosenberg et al. [34] have reported that in the neurotoxic snake venom PLA₂, the N-terminal region and basic residues away from the enzymic active-site region are important in neurotoxicity and may occur independently of phospholipid hydrolysis. Based on our studies, we propose that the N-terminal region of the EF molecule is important in the EGF-binding activity and that one of the functions of the mammalian sPLA₂ molecules could be growth modulation.

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