Comparison of the activities of wild type and mutant enhancing factor/mouse secretory phospholipase A_2 proteins

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Enhancing factor (EF) protein, an isoform of secretory phospholipase A_2 (PLA_2), was purified as a modulator of epidermal growth factor from the small intestine of the Balb/c mouse. It was for the first time that a growth modulatory property of $sPLA_2$ was demonstrated. Deletion mutation analysis of EF cDNA carried out in our laboratory showed that enhancing activity and phospholipase activity are two separate activities that reside in the same molecule. In order to study the specific amino acids involved in each of these activities, two site-directed mutants of EF were made and expressed *in vitro*. Comparison of enhancing activity as well as phospholipase A_2 activity of these mutant proteins with that of wild type protein helped in identification of some of the residues important for both the activities.

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1. Introduction

Enhancing factor (EF), a mouse homologue of secretory phospholipase A_2 ($sPLA_2$), is a dual function molecule. It shows enhancing activity i.e. it enhances the binding of epidermal growth factor (EGF) to A431 cells in a radio-receptor assay (Deo *et al* 1983) and it also shows phospholipase activity (Mulherkar *et al* 1993). EF is a 146 amino acid protein with a 21 amino acid long signal peptide. A 100 kDa cell membrane receptor for EF has been demonstrated on A431 cells (Mulherkar and Deo 1986). EF along with EGF induces anchorage independent growth of normal rat kidney fibroblasts (Mulherkar and Deo 1986). Studies with heparin and heparitinase in the radio-receptor assay suggested that EF is a heparin binding protein and enhancing activity is inhibited in the presence of heparin (Wagle *et al* 1997). It was, therefore, proposed that EF is a unique molecule that binds itself to the cell membrane, through its own receptor and in turn, provides a binding site for EGF. Immunohistochemical studies have demonstrated that EF is localized in the Paneth cells of the small intestine as well as to the hair follicles of new born mice (Desai *et al* 1991; Mulherkar *et al* 1991).

Secretory phospholipase A_2 ($sPLA_2$) is a highly conserved family of enzymes that has molecular masses of about 14 kDa and requires Ca^{2+} for the catalytic activity (Murakami *et al* 1997). The Ca^{2+} binding loop has a typical glycine-rich sequence and presence of aspartic acid at amino acid 49. The active site of the enzyme shows the presence of highly conserved residues of His^{48}, Tyr^{52}, Tyr^{73} and Asp^{99}. Apart from phospholipid digestion, $sPLA_2$ has the potential to mediate a wide range of biological activities which include mediators of inflammation, ecosanoid generation, antibacterial activity and initiator of cell proliferation. Interestingly, some of these activities of $sPLA_2$ are independent of its catalytic activity. Lys-49, a variant of snake venom type II PLA_2 that is unable to hydrolyze phospholipids, shows cytolytic activity (Francis *et al* 1991). Some of the receptor-mediated functions of $sPLA_2$ are also independent of its catalytic activity. For example $sPLA_2$ receptor mediated

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Abbreviations used: EF, Enhancing factor; EGF, epidermal growth factor; $sPLA_2$, secretory phospholipase A_2.

activation of $cPLA_2$ has been demonstrated in murine bone marrow-derived mast cells and a noncatalytic ligand of this receptor also induced an increase in $cPLA_2$ activity (Fonteh *et al* 2000). $sPLA_2$ induced secretion of *b*-glucuronidase and IL-6 by human macrophages is also thought to be independent of the enzymatic activity (Triggiani *et al* 2000). Our laboratory has reported for the first time the growth modulatory property of mouse $sPLA_2$/EF (Mulherkar and Deo 1986). Deletion mutation studies carried out in our laboratory have shown that enhancing activity and phospholipase activity are two separate activities residing in a single molecule (Kadam and Mulherkar 1999). One of the deletion mutants having only the first 82 amino acids showed enhancing activity but no phospholipase activity. This suggested that enhancing activity does not depend on phospholipase activity. In the present study site-directed mutagenesis of EF/mouse $sPLA_2$ was carried out in order to mutate specific amino acid residues without altering the size of the molecule. The effect of these mutations on both the activities of the molecule was studied by expressing it in 293 cells.

2. Materials and methods

2.1 *Site-directed mutagenesis of EF*

Two separate primers were designed to carry out mutations at two separate sites. One of the primers (GE1-5′ P-CTG TGT TAC TCA GAA ATG TTG CTA CAA GA 3′) would change His^{48} and Asp^{49} (amino acid positions according to aligned PLA_2 sequences) to Gln^{48} and Lys^{49} respectively, while another primer (GE2-5′ P-GGT GGG AGT TCT TGT TTT TCA GTA ACT T 3′) was designed to change Tyr^{67} and Tyr^{69} to Asn^{67} and Asn^{69} respectively. 'GeneEditor^{TM} *in vitro* site-directed mutagenesis system' (Promega) was used to carry out mutagenesis of EF cDNA. This system uses antibiotic selection to obtain high frequency of mutants. Plasmid pRcCMV-EF, where full length EF cDNA has been cloned under CMV promoter (Kadam and Mulherkar 1999), was used as template for mutagenesis reaction. The selection oligonucleotide was annealed to template DNA at the same time as a mutagenic oligonucleotide. Subsequent synthesis and ligation of a mutant strand linked the two oligonucleotides. BMH 71-18 *mutS*, which is a DNA repair minus strain of *Escherichia coli*, was used to avoid selection against the desired mutation. Plasmid DNA extracted from this culture was used for second round transformation into *E. coli* JM109, which ensures proper segregation of mutant and wild type plasmids. Mutant plasmid was identified by sequencing.

2.2 *Sequencing*

Double stranded plasmid DNA extracted by the miniprep protocol was quantitated by gel electrophoresis and was used for sequencing. Sequenase version 2·0 double-stranded DNA sequencing kit from USB was used as per the manufacturer's instructions.

2.3 *Transfection of 293 cells with wild type and mutant EF cDNAs*

1×10^5 293 (human embryonic kidney cell line) cells were plated in 60 mm tissue culture plates (Nunc) in DMEM containing 10% bovine calf serum (Hyclone). The cells were transfected with lipofectin (Gibco-BRL) as per the manufacturer's instructions. Transfectants were selected on G-418 (800 μg/ml) for 14 days. The antibiotic resistant clones were picked up and expanded. These clones were screened for expression of EF.

2.4 *Northern blotting*

Expression of EF in positive clones was confirmed by Northern blotting after initial screening by immunostaining. RNA was extracted from untransfected and transfected 293 cells by Chomczynski and Sacchi (1987) method and run on 1·2% agarose gel containing formaldehyde. It was blotted onto a nitrocellulose membrane (Hybond-C extra, Amersham) and probed with [^{32}P]-labelled EF cDNA. One positive clone was selected from each of the three transfections (GE1, GE2 and wild type EF) for extraction of recombinant protein.

2.5 *Extraction of acid-soluble proteins from transfected cells*

Acid-soluble proteins were extracted from untransfected and transfected 293 cells by the standard acid-ethanol procedure used routinely in our laboratory for extraction of EF from mouse intestines (Mulherkar and Deo 1986). Wild type and transfected cells were grown in 10 × 10 cm petri-plates till they attained ~ 80% confluency (~ 1×10^8 cells). Cells were washed thrice with PBS and extracted in HCl and ethanol containing PMSF and Aprotonin as protease inhibitors. The homogenate was extracted overnight at 4°C with constant stirring. After centrifugation at 17,000 *g* for 30 min at 4°C, the pH of the supernatant was adjusted to 5·2, with 25% ammonium hydroxide. The precipitated proteins were discarded after centrifugation at 17,000 *g* for 30 min at 4ºC. The supernatant was then precipitated with two volumes of chilled absolute ethanol and four volumes of chilled ether, and allowed to stand overnight at – 20ºC. The precipitate obtained was

air dried and reconstituted in 1 M acetic acid. The acid-soluble proteins were dialysed against 0·1 M acetic acid at 4ºC. The dialysate consisting of the crude extract of proteins was lyophilized, washed twice with distilled water and aliquots were stored frozen until further use.

2.6 *Immunoblotting*

Acid-soluble proteins were separated on a 17·5% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C extra). ECL Plus Western blot kit (Amersham) was used according to the manufacturer's instructions. Rabbit polyclonal anti-EF antiserum was used at the dilution of 1 : 5000.

2.7 *PLA$_2$ assay by using labelled* Escherichia coli *membranes*

PLA$_2$ activity was also checked using [^{3}H]oleic acid labelled *E. coli* (Elsbach and Weiss 1991). For the assay, labelled *E. coli* cells were washed and resuspended in PLA$_2$ buffer [100 mM Tris (pH8)/10 mM $CaCl_2$/0·1% BSA] such that 100 µl had ~ 100,000 cpm. Labelled membranes (100 µl substrate) were added to serial dilutions of the acid/ethanol extracted test samples. The reaction was carried out at RT for 1 h after which 250 µl of chilled stop buffer (100 mM EDTA in 1% BSA) was added. Supernatant (400 µl) was collected in a Dioxan based scintillation fluid for counting in the beta counter. The data were plotted as cpm released against concentration of the protein.

2.8 *Measurement of enhancing activity*

Enhancing activity was measured as described earlier (Mulherkar and Deo 1986) using A431 cells and [^{125}I]-EGF. Lyophilized test protein was dissolved in 100 µl of binding buffer and added to the cells with 100 µl of [^{125}I]-EGF (counts adjusted to 30,000 cpm/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 sample was tested in duplicate and the assay repeated thrice.

2.9 *Heparin binding and Western blot analysis*

Approximately 75 µg of acid-soluble protein from each of the three clones as well as untransfected 293 cells was mixed with 100 µl of heparin-sepharose beads (Pharmacia), prewashed and suspended in 200 µl of phosphate buffer and incubated overnight at 4°C on a shaker. After incubation, the suspension was centrifuged and the supernatant saved as the unbound protein fraction for Western blotting. The beads were washed with phosphate buffer and the heparin-bound fraction was eluted using 2 M NaCl in the buffer. NaCl-eluted fraction was dialysed using Ultrafree MC tubes (Millipore) and subjected to immunoblotting along with unbound fractions as described earlier.

3. Results

Site-directed mutagenesis reactions were carried out using pRcCMV-EF as template with either GE1 or GE2 as mutant primers. This reaction was used for transformation and the antibiotic resistant colonies were screened for the presence of mutation. In the case of GE1 mutation, colonies were screened by sequencing and one out of six clones sequenced was found to have the GE1 mutation (figure 1a). For GE2 mutation, colonies were screened by restriction digestion as the mutation would result in loss of one of the *ScaI* sites; this was later con-

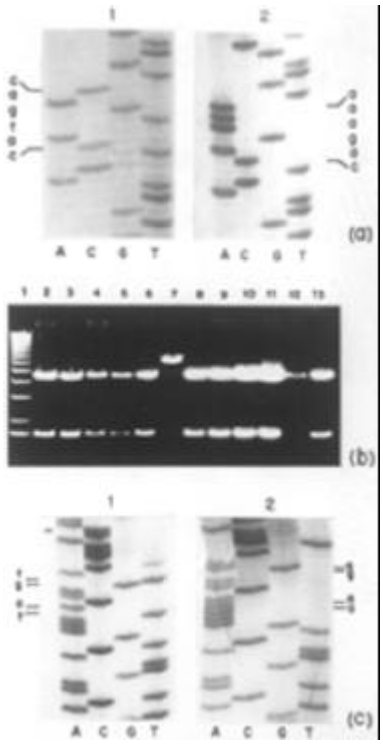

Figure 1. Confirmation of sequence of the mutant EF cDNAs. (**a**) Panel 1 shows wild type EF sequence as compared to panel 2 showing GE1 mutant sequence from one of the clones (ca**t**-g**a**c → ca**g**a**a**a**a**). (**b**) Plasmid minipreps cut with *ScaI* to screen for GE2 mutation. Lane 7 shows the clone with GE2 mutation where one of the *ScaI* sites is lost. (**c**) Panel 1 shows wild type EF sequence compared to panel 2 showing GE2 mutant sequence from one of the clones (**t**a → **a**a and g**t** → g**a**).

firmed by sequencing (figure 1b, c). Plasmid with GE1 mutation was designated as pRcCMV-GE1 and that with GE2 mutation as pRcCMV-GE2. Both these plasmids, along with pRcCMV-EF (wild type) were used for producing recombinant proteins by transfecting into 293 cells.

G418-resistant clones were picked up and expanded. One clone for each of the three transfections was selected on the basis of positive staining in immunohistochemistry (data not shown). Clone 293/GE1, 293/GE2 and 293/wtEF produced mutant GE1, mutant GE2 and wild type EF protein respectively. RNA extracted from these clones as well as untransfected 293 cells were subjected to Northern hybridization using radiolabelled EF cDNA. All three transfectants gave a positive signal (figure 2a), which confirmed the expression of mutant EF cDNAs. Western blot analysis of acid-soluble proteins gave a 14 kDa signal in transfectants and not in the untransfected 293 cells, confirming the production of EF protein by each of these clones (figure 2b). Untransfected cells were used as controls.

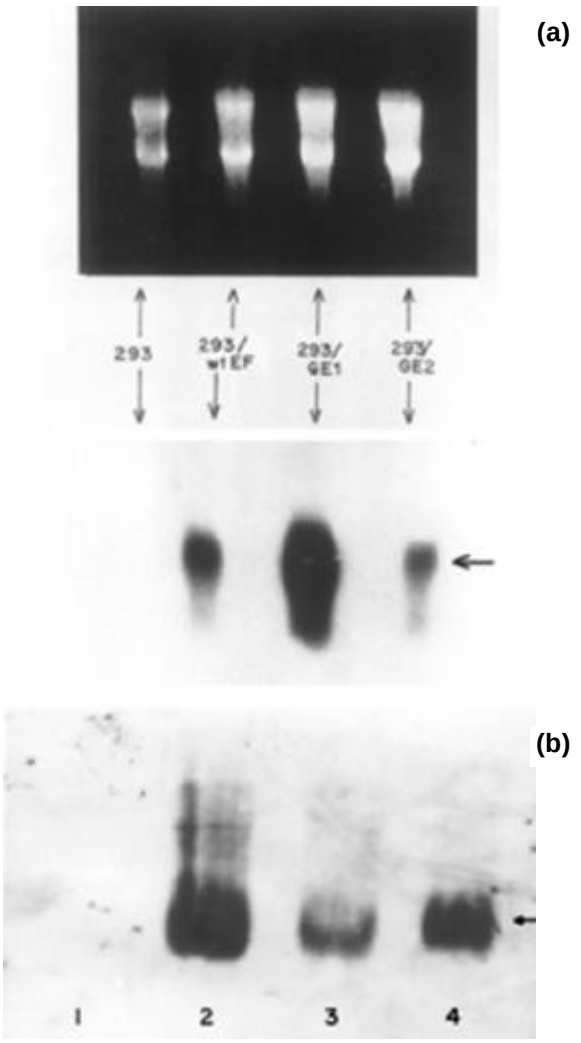

Figure 2. (**a**) Northern blot analysis. RNA extracted from untransfected 293, 293/wtEF, 293/GE1 and 293/GE2 was Northern blotted and probed with [^{32}P]-labelled EFcDNA. ~ 0·8 kb signal was obtained in all the three transfectants (top picture shows ethidium bromide gel and bottom picture is the autoradiogram. Arrow indicates 0·8 kb transcript). (**b**) Western blot analysis. 50 µg acid-soluble proteins from untransfected 293 (lane 1), 293/wtEF (lane 2), 293/GE1 (lane 3), 293/GE2 (lane 4) were separated on 17·5% SDS-PAGE and Western blotted as described in § 2. 14 kDa signal was seen in all the three transfectants (as indicted by arrow). In all cases the positive control (with purified EF) shows a single band.

Acid-soluble proteins from these clones were tested for enhancing and PLA_2 activity. Both the mutants showed loss of enhancing activity (figure 3). Clone 293/GE1 showed complete loss of PLA_2 activity whereas clone 293/GE2 showed partial loss of PLA_2 activity as compared to wild type EF protein (figure 4).

The mutant as well as wild type proteins were also checked for their ability to bind to heparin using heparin-agarose beads. Surprisingly, neither of the mutations resulted in loss of heparin binding (figure 5).

4. Discussion

EF is a 14 kDa molecule isolated from mouse small intestines, identified by its ability to increase the binding of [^{125}I]-EGF to A431 cells in the radio-receptor assay (Mulherkar and Deo 1986). Sequence homology studies have shown that EF belongs to mouse type II $sPLA_2$ family. Hence it is a dual function molecule having enhancing as well as phospholipase activity. Previous studies in our laboratory have shown that both these activities lie in different domains of the EF/$sPLA_2$ molecule (Kadam

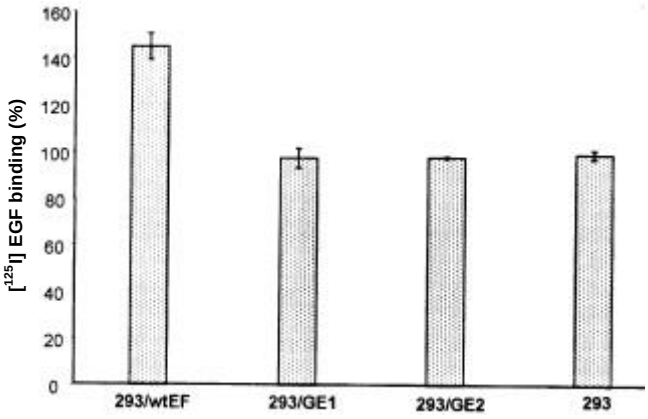

Figure 3. Enhancing activity of wild type and mutant recombinant EF proteins. Radio-receptor assay was carried out in duplicate wells as described in § 2. Control wells had no protein added to A431 cells. Acid-soluble proteins extracted from 293/wtEF served as positive control and those from untransfected 293 cells served as negative control.

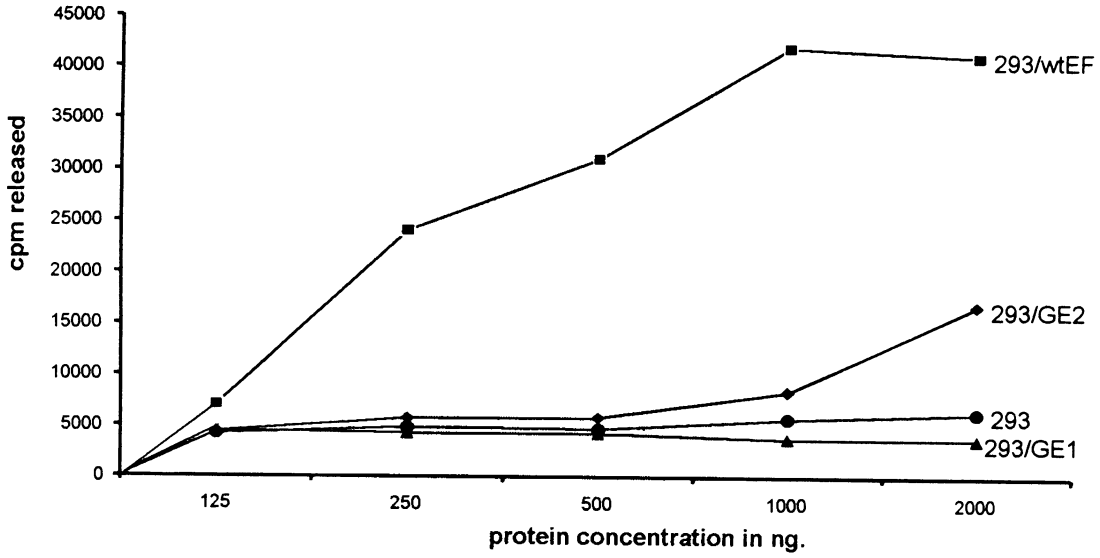

Figure 4. PLA$_2$ activity of the acid-soluble proteins. Serial dilutions of acid-soluble proteins extracted from the transfectants as well as untransfected 293 cells were taken for the enzyme assay. 293/wtEF served as positive control and untransfected 293 served as negative control.

and Mulherkar 1999). In order to identify residues involved in enhancing activity site-directed mutagenesis was carried out.

Most of the amino acids involved in the catalytic reaction of sPLA$_2$ are clearly defined (Murakami *et al* 1997). Asp^{49} and a glycine rich sequence Tyr^{25}-Gly-Cys-X-Cys-Gly-X-Gly-Gly-X-X-X-Pro^{37} form the calcium-binding loop of sPLA$_2$s (Scott *et al* 1990) while His^{48} is the conserved active site residue. A naturally occurring variant of group II sPLA$_2$ isolated from snake venom, Myotoxin II, shows presence of Lys^{49} instead of Asp^{49} and lacks catalytic activity on conventional substrates (van den Bergh *et al* 1989). Nevertheless, it exerts myotoxic and membrane damaging activities comparable to the Asp^{49} sPLA$_2$.

Hence Asp^{49} along with His^{48} were selected for substitution by other residues in order to knock off the PLA$_2$ activity and study the effect on enhancing activity. A specific primer (GE1) was designed to change His^{48} $\rightarrow$ Gln and Asp^{49} $\rightarrow$ Lys. A second mutation was made to change some of the tyrosine residues of EF. This was because previous studies from our laboratory indicated that iodination of EF resulted in loss of enhancing activity which could be due to iodination of some of the tyrosine residues. To study the effect of substitution of some of the tyrosine residues on enhancing activity, a primer (GE2) was designed to change Tyr^{67} and Tyr^{69} to Asn^{67} and Asn^{69} respectively.

Mutant proteins were tested for enhancing activity as well as PLA$_2$ activity. Mutation GE1 [H^{48} $\rightarrow$ Q and D^{49} $\rightarrow$ K] which changes amino acids required for Ca^{2+} binding, resulted in loss of PLA$_2$ activity as expected but

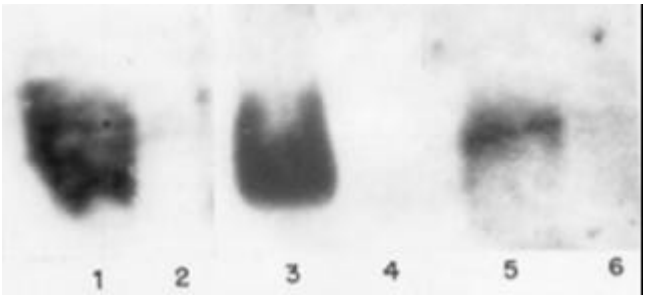

Figure 5. Heparin binding property of mutant proteins. Acid-soluble proteins from 293/wtEF (lanes 1 and 2), 293/GE1 (lanes 3 and 4) and 293/GE2 (lanes 5 and 6) were incubated with heparin-Sepharose beads as described in § 2. The high-salt eluted (lanes 1, 3, 5) and unbound (lanes 2, 4, 6) proteins were separated by SDS-PAGE (17·5%) and Western blotted. 14 kDa signal was seen in the eluted factions of all the proteins and not the unbound fraction.

it also caused loss of enhancing activity. This confirmed that Ca^{2+} binding is required for both the activities. Hence, though both the activities are independent of each other, Ca^{2+} binding is essential for both of them.

Mutation GE2 [Y^{67} $\rightarrow$ Q and Y^{69} $\rightarrow$ Q] resulted in loss of enhancing activity suggesting that these tyrosine residues are important for enhancing activity. Heparin binding analysis showed that neither of the mutations resulted in loss of heparin binding. This indicates that these amino acids do not form part of heparin binding domain. However they could be involved either in binding of EF to cell membrane or to EGF. We have shown before that mouse

$sPLA_2$/EF has a cell surface receptor that is probably a heparan sulfate proteoglycan (Wagle *et al* 1997). C-terminal lysine residues of type II sPLA2 have been demonstrated to be involved in binding to heparin as well as cell surface heparan sulfate proteoglycan (Kadam and Mulherkar 1999). Replacement of Lys^{119} and Lys^{120} reduced the affinity of mouse and rat PLA_2s for heparin and discontinued its binding to the cell surface. But not all the residues for heparin binding and cell surface binding are common (Murakami *et al* 1996). Replacement of Lys^{103} and Lys^{104} did not reduce the affinity of $sPLA_2$ for heparin but did reduce its cell surface binding significantly. Replacement of Tyr^{67} and Tyr^{69} (GE2 mutation) in EF does not affect the heparin binding but it abolishes enhancing activity. Using this mutant molecule it would be possible to study if these tyrosine residues could affect the cell surface binding of EF/mouse $sPLA_2$. However, whether their substitution caused a conformational change in the molecule remains to be elucidated. We did not measure binding or activity when cells are transfected with the vector alone but do not expect there to be any difference.

GE2 mutation also caused partial loss of PLA_2 activity. Apart from mouse $sPLA_2$, human and rat type II $sPLA_2$ show presence of tyrosine at position 67 (Murakami *et al* 1997). Our results demonstrate that Tyr^{69} along with Tyr^{67} is also important for catalytic activity in mouse $sPLA_2$. Unfortunately we were unable to measure the binding of mutant proteins to A431 cells. Given that neither mutant lost heparin binding activity our data would have been stronger if we could have shown that mutant proteins could bind cells but were inactive. But for reasons that remain unclear, we are unable to label EF protein and retain binding activity.

From these results we can conclude that though enhancing activity and phospholipase activity are two independent activities of the same molecule, there are some amino acids that appear to be common for both activities. His^{48}, Asp^{49}, Tyr^{67} and Tyr^{69} are the residues, which are important for both these activities.

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