A 120 kDa Nuclear Phospholipase Cγ1 Protein Fragment Is Stimulated in Vivo by EGF Signal Phosphorylating Nuclear Membrane EGFR

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ABSTRACT: Intraperitoneal injection of epidermal growth factor (EGF) into mice resulted in the phosphorylation of liver nuclei phospholipase Cγ1 (PLCγ1) at the tyrosine, coincident with the time course of nuclear membrane epidermal growth factor receptor (EGFR) activation. The function of PLCγ1 in mice liver nuclei was attributed to a 120 kDa protein fragment. This 120 kDa protein was immunoprecipitated with the isozyme specific PLCγ1 antibody and was found to be sensitive to a PLCγ1 specific blocking peptide. The 10-partial sequence analysis revealed that the 120 kDa protein contains the PELCQVSLSE sequence at its N-terminal end and the RTRVNGDNRL sequence at its C-terminal end, which reveals that this protein is a major fragment of PLCγ1 devoid of an amino acid portion at the N-terminal end. The tyrosine-phosphorylated 120 kDa protein interacts with activated EGFR, binds phosphatidylinositol-3-OH-kinase enhancer (PIKE), enhances nuclear phosphatidylinositol-3-OH-kinase (PI[3]K) activity, and generates diacylglycerol (DAG) in response to the EGF signal to the nucleus in vivo. The immunoprecipitated 120 kDa protein fragment displayed phosphatidylinositol (PI) hydrolysis activity. These results establish the capacity of EGF-triggered nuclear signaling which is mediated by EGFR itself, located on the inner nuclear membrane. This is the first report identifying a 120 kDa PLCγ1 fragment generated in vivo in the nucleus and capable of discharging the function of nuclear PLCγ1.

Since the discovery that inositol 1,4,5-trisphosphate (IP_3)[1] mobilizes calcium from the nuclear envelope to the nucleoplasm (*1*, *2*) and that the nuclear pools of phosphatidylinositol 4,5-bisphosphate (PIP_2) are metabolized in a manner different from that of the cytoplasm (*3*), one remaining unresolved question is the mechanism by which PLCγ1 is activated in the nucleus. In the cytoplasm, growth factor-dependent (*4*) tyrosine phosphorylation of PLCγ1 generates IP_3 and DAG upon PIP_2 hydrolysis (*5*). Considerable efforts have been made to understand the autonomous phosphoinositide (*6*) and calcium (*7*) signaling in the nucleus. In addition to its action mobilizing calcium from the endoplasmic reticulum (*8*), IP_3 binds to the nuclear inositol trisphosphate receptor (IP_3R) spanning the inner nuclear membrane (*9*, *10*) with its binding domain facing the nucleoplasm. Phospholipase C (*11*) and other components necessary for IP_3 generation are located on the inner nuclear membrane (*6*, *7*). The nucleus also contains other forms of PI-PLC (*6*) and diacylglycerol kinase (*12*). The nuclear localization of PLCγ1 is reinforced by the finding that it stimulates PIKE (*13*), a nuclear GTPase that enhances nuclear PI[3]K activity.

In 1993, Cohen (*14*) showed that within 6 min of the intraperitoneal injection of EGF into mice, STAT proteins were tyrosine phosphorylated in the liver nuclei. However, the specific tyrosine kinase activated in this process remained obscure. Moreover, at that time, the actual nuclear membrane location of EGFR was not so definitive (*15*).

In the study reported here, we have shown that EGF intraperitoneally injected into mice resulted, in liver, in nuclear PLCγ1 phosphorylation at the tyrosine, coincident with the time course of nuclear membrane EGFR tyrosine phosphorylation. This PLCγ1 is a 120 kDa protein derived from the 150 kDa native nuclear enzyme cleaved at the N-terminal end. It can be immunoprecipitated from the EGF-treated mice liver nuclear extract with the isozyme specific PLCγ1 antibody, and it hydrolyzes phosphatidylinositol. The tyrosine-phosphorylated 120 kDa protein fragment interacts with activated nuclear membrane EGFR, binds PIKE, and stimulates nuclear PI[3]K activity in response to the EGF signal to the nucleus in vivo. These data reveal the capacity of EGF nuclear signaling, which is mediated by EGFR located on the inner nuclear membrane. The elusiveness of how the nuclear calcium signal is regulated is dispelled with the knowledge of the mechanism of nuclear PLCγ1 activation.

MATERIALS AND METHODS

EGF Injection and Preparation of Nuclear and Cytoplasmic Extracts. Solutions of EGF (1 mg/mL) in phosphate-

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[1] Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLCγ1, phospholipase Cγ1; EGFR, epidermal growth factor receptor; PIKE, phosphatidylinositol-3-OH-kinase enhancer; PI[3]K, phosphatidylinositol-3-OH-kinase; PI, phosphatidylinositol; RT, room temperature.

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buffered saline (PBS) were injected intraperitoneally into mice at a dose of 10 μg/g of body weight. Mice were sacrificed by cervical dislocation, and the removed liver was placed in ice-chilled PBS. Mouse liver nuclei were isolated employing Pierce nuclear and cytoplasmic extraction reagent as described previously (*16*). Briefly, the liver was cut into small pieces which were immersed in ice-chilled PBS, and rinsed three times. The liver was homogenized using a Dounce homogenizer at a ratio of 500 μL of PBS per 0.6 g wet weight. The homogenate was divided into 500 μL batches in Eppendorf tubes, and the volume in each tube was brought up to 1.5 mL with PBS. Each tube was vortexed for 5 s and centrifuged for 10 min at 500*g*. The floating lipids on the top of the tubes were removed carefully with a Kleenex, and the supernatant was discarded. The loosely packed pellets were aspirated out and placed in 100 μL lots in Eppendorf tubes. Each 100 μL of the pellet was added with 400 μL of cytoplasmic extraction reagent (CER I, Pierce) with a mixture of protease inhibitors (Halt Protease Inhibitor Cocktail, Pierce) at a ratio of 10 μL/mL. The suspension was incubaded on ice for 10 min followed by the addition of 400 μL of a second cytoplasmic extraction reagent (CER II, Pierce), vortexed for 5 s, incubated on ice for 1 min, and centrifuged for 5 min at 16000*g* using an Eppendorf 5415R centrifuge. The resulting supernatant constituted the cytoplasmic extract. The pellet, constituting the crude nuclei, was suspended in an appropriate volume of nuclear extraction reagent (NER, Pierce) with the inhibitors as described above, by being vortexed for 15 s and incubated on ice for 10 min. This procedure was repeated two additional times. The resulting supernatant constituted the mouse liver nuclear extract. The nuclear and cytoplasmic extracts were examined for their NAD pyrophosphorylase, mannose-6-phosphatase, and NADPH cytochrome *c* reductase activities.

Immunoprecipitation. Appropriately diluted nuclear extracts were incubated with anti-PLCγ1 antibody and μMACS protein G microbeads, or with anti-EGFR, anti-PIKE, or anti-p110α antibodies and μMACS protein A microbeads, for 30 min on ice. The μMACS columns (Miltenyi Biotech) were placed in the magnetic field of the μMACS separator and equilibrated with a buffer containing 150 mM NaCl, 1% Triton X-100, and 50 mM Tris-HCl (pH 8.0). The incubated nuclear mixture was applied onto the column which was rinsed with 4 $\times$ 200 μL of buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl (pH 8.0), and 100 μL of 20 mM Tris-HCl (pH 7.5). Preheated (95 °C) SDS gel loading buffer (20 μL) was added onto the column and the column incubated for 5 min at room temperature (RT). The eluate, collected as the respective immunoprecipitate, was separated by 10% SDS−PAGE, electroblotted onto nitrocellulose membranes (Bio-Rad), blocked with 5% low-fat milk, incubated with the appropriate antibody, probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody, and developed using the ECL system (Amersham). Details of this procedure are described in ref *16*.

Immunoblotting. Appropriate amounts of nuclear or cytoplasmic extracts were dissolved in sample buffer (95 °C for 10 min), separated by SDS−PAGE (10%), and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% low-fat milk for 1 h at RT, followed by incubation with isozyme specific anti-PLCγ1 or anti-PLCβ (Santa Cruz Biotechnology) or respective anti-EGFR (Santa Cruz Biotechnology or phospho specific anti-EGFR antibodies from Calbiochem) antibodies, overnight, at 4 °C. Immunoblots were treated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and were finally developed using the ECL system. Anti-PLCγ1 antibody was incubated with a 5-fold excess (by weight) of blocking peptide buffer (SFEANQQPFEDFRI, Santa Cruz Biotechnology) in 500 μL of PBS, overnight, at 4 °C for this particular experiment.

Phosphatidylinositol Hydrolysis. Immunoprecipitated with anti-PLCγ1 antibody, the 120 kDa protein fragment was subjected to [^{3}H]phosphatidylinositol ([^{3}H]PI) hydrolysis. The hydrolysis activity (*17*) was quantitated in a 200 μL assay mixture containing 200 μM PI (20 000 cpm [^{3}H]PI), 1 mM EGTA, 10 mM $CaCl_2$, 0.1% sodium deoxycholate, and 50 mM HEPES (pH 7.0). The reaction mixture was incubated at 37 °C for 15 min and the reaction terminated by adding 1 mL of a chloroform/methanol/HCl mixture (100:100:0.6, v/v), followed by addition of 0.3 mL of 1 N HCl containing 5 mM EGTA. After centrifugation, 0.5 mL of the upper aqueous phase was assayed for radioactivity by a liquid scintillation counter.

PI[3]K Activity Assay. Nuclear PI[3]K immunoprecipitate (using anti-p110α polyclonal antibody) from the nuclear extract was washed with the buffer as described previously (*13*) followed by incubation with PI (final concentration of 0.5 mg/mL) in 10 mM HEPES, 1 mM EDTA (pH 7.5), and [γ-^{32}P]ATP (10 μCi/sample). Phosphate incorporation was achieved for 15 min at RT and stopped by addition of a chloroform/methanol/HCl mixture (200:100:0.75, v/v) followed by two washes with a chloroform/methanol/0.6 N HCl mixture (3:48:47, v/v). The lipid-containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica Gel 60) developed in a 2-propanol/acetic acid/H_2O mixture (65:1:34, v/v). The spots were quantified (*18*), after autoradiography, by liquid scintillation spectrometry.

Diacylglycerol Determination. An Amersham reagents system was used, based on a radioenzymatic assay employing diacylglycerol kinase (*19*) quantitatively converting DAG to [^{32}P]phosphatidic acid in the presence of [γ-^{32}P]ATP. [^{32}P]-Phosphatidic acid was separated on Amprep minicolumns and quantified using liquid scintillation spectrometry.

Purification of Nuclear Membranes. Mouse liver nuclei were isolated and purified by centrifugation through 2.2 M sucrose. The suspended nuclear pellet was incubated with 1% (w/v) sodium citrate for 30 min on ice while being stirred gently and centrifuged for 15 min at 500*g*. The resulting supernatant consisted of outer nuclear membranes, which were further purified by centrifugation through a 1.6 M sucrose cushion. The pellet constituting inner nuclear membranes was digested with DNAse I (250 μg/mL) for 14 h at 4 °C and was submitted to centrifugation on a sucrose gradient (*10*) to obtain purified inner membranes.

Partial N-Terminal and C-Terminal Sequence Analysis. Liver nuclear extract from an animal treated for 10 min with EGF was immunoprecipitated with anti-PLCγ1 antibody, separated by SDS−PAGE, and transferred to a nitrocellulose membrane, and the 120 kDa band was cut. For N-terminal analysis, the band was submitted to Edman degradation microsequencing. For C-terminal analysis, it was eluted with 50 mM ammonium bicarbonate overnight at RT and digested

with a mixture of carboxypeptidases P and Y at RT. The digested material was mixed on the target plate with 0.5 μL of saturated α-cyano-4-hydroxycinnamic acid in 70% aqueous acetonitrile containing 0.1% trifluoroacetic acid, dried at RT, and subjected to mass spectrometry (*20*) with an average of 20−50 laser shots recording at 337 nm.

Determination of Protein. Protein contained in the 120 kDa protein fragment immunoprecipitate or anti-p110α antibody immunoprecipitate was estimated by silver nitrate staining of gels and densitometric scanning using BSA as an internal standard (*21*).

Treatment of Isolated Rat Liver Nuclei with EGF and Activation of the 120 kDa PLCγ1 Protein Fragment in Vitro. Isolated rat liver nuclei (*1*) were suspended in a medium containing 0.3 M sucrose, 5 mM $MgCl_2$, 2 mM EDTA, 1.5 mM $CaCl_2$, 1 mM ATP, 100 μM orthovanadate, 5 mM NaF, a mixture of protease inhibitors (Sigma), and 20 mM Tris-HCl (pH 7.5). They were transferred to a 25 mL Erlenmeyer flask and incubated with 100 ng/mL EGF at 37 °C for the indicated time. At the end of the incubation, the Erlenmeyer flasks were chilled on ice for 5 min. Nuclei were sedimented at 1000*g* for 10 min, and the pellets were frozen in liquid nitrogen for 10 min. Nuclei were thawed and resuspended in a minimum volume of the medium described above, and treated with DNAse I (250 μg/mL) for 20 min at 30 °C. Subsequently, they were placed on ice for 5 min, and 1% Triton X-100 was added while the mixture was vortexed for 15 s. They were placed on ice for an additional 10 min followed by centrifugation. The resulting supernatant with appropriate dilution was incubated with anti-PLCγ1 antibody and μMACS protein G microbeads, or with anti-EGFR, anti-PIKE, or anti-p110α antibodies and μMACS protein A microbeads, for 30 min on ice. The remainder of the procedure was followed as described in the previous section.

RESULTS

Tyrosine Phosphorylation of Nuclear PLCγ1 by EGF. The mouse liver nuclear extract, obtained upon injection of EGF at various periods and immunoblotted with isozyme specific anti-PLCγ1 antibody, revealed two protein bands, one at 150 kDa and the other at 120 kDa (Figure 1A). The immunoblot with the anti-phosphotyrosine antibody revealed that the EGF treatment initiated tyrosine phosphorylation of the 120 kDa protein (Figure 1B). This 120 kDa protein was immunoprecipitated with anti-PLCγ1 antibody from the nuclear extract of control and EGF-treated mice, followed by immunoblotting with anti-phosphotyrosine antibody, depicting that, at 10 or 30 min, EGF treatment phosphorylated the 120 kDa protein at the tyrosine (Figure 1C). In the control or after a 60 min EGF treatment, no tyrosine phosphorylation was observed.

Incubating the anti-PLCγ1 antibody with a specific blocking peptide prior to immunoblotting abolished the 150 kDa protein band as well as the tyrosine-phosphorylable 120 kDa protein band (Figure 1D). This suggested that both these protein bands, found in the nuclear extract, were related to PLCγ1. Under similar conditions, in the mouse liver nuclear extract, the isozyme specific PLCβ antibody immunoblotted a single protein band at 155 kDa (Figure 1E), affirming that the 120 kDa protein band was specific to PLCγ1.

The cytoplasmic extracts obtained from control and EGF-treated mice were, likewise, immunoblotted with anti-PLCγ1 antibody, revealing a single 150 kDa band (Figure 1E) which was tyrosine-phophorylated as seen after immunoblotting with anti-phosphotyrosine antibody (Figure 1F). This demonstrated that, in the cytoplasm, it is the 150 kDa protein wihch was tyrosine-phophorylated in vivo in response to EGF treatment.

On the basis of the specific activity of NAD pyrophosphorylase (a specific marker for the nucleus) and mannose-6-phosphatase activity (Table 1), we concluded that the nuclei, as prepared here, were free from cytoplasmic contaminants. The activity of NADPH cytochrome *c* reductase (Table 1), a microsomal marker enzyme, was less than 5% of the total homogenate activity in the isolated nuclei. This shows that the isolated nuclei were devoid of microsomes.

Interaction between Tyrosine-Phosphorylated 120 kDa PLCγ1 Protein Fragment and Nuclear EGFR. Immunoblotting the mouse liver nuclear extract with anti-EGFR antibody revealed a 170 kDa protein band (Figure 2A). The ability of various anti-EGFR antibodies to immunoprecipitate nuclear membrane EGFR was investigated. The EGFR immunoprecipitated with anti-EGFR antibody from the nuclear extract of the control and EGF-treated mice, and immunoblotted with anti-active EGFR antibody, confirmed that in response to a 10 or 30 min EGF treatment, EGFR was phosphorylated on tyrosine (Figure 2B). In parallel, immunoprecipitation either with anti-active EGFR (Tyr^{1173}) or with anti-active EGFR (Tyr^{1068}) antibodies, immunoblotted with the respective antibodies (Figure 2C,D), confirmed that at the 10 or 30 min EGF injection, nuclear membrane EGFR was phosphorylated at the tyrosine and hence activated.

Evidence that the tyrosine-phosphorylated 120 kDa moiety associates with the activated nuclear EGFR, in response to the EGF treatment, was provided by detecting a 120 kDa protein band in the nuclear extract immunoprecipitates obtained with either anti-EGFR antibody (Figure 2E), anti-active EGFR (Tyr^{1173}) antibody (Figure 2F), or anti-active EGFR (Tyr^{1068}) antibody (Figure 2G). Likewise, immunoprecipitation with anti-PLCγ1 antibody revealed a 170 kDa EGFR protein band when it was immunoblotted with anti-active EGFR (Tyr^{1068}) antibody (Figure 2H). In the absence of EGF treatment or with a 60 min EGF treatment, the 120 kDa protein band was not detected in these immunoprecipitates. These data demonstrate that in response to EGF, 120 kDa tyrosine phosphorylation coincides with the activation of nuclear membrane EGFR and triggers the association between the two molecules.

[^{3}H]Phosphatidylinositol Hydrolysis Activity. The 120 kDa protein can be immunoprecipitated from the mouse liver nuclear extract with an isozyme specific PLCγ1 antibody. We have determined the [^{3}H]PI hydrolysis activity present in the immunoprecipitate from the control and EGF-treated mice (Figure 3). Several fold enhanced PI hydrolysis was observed in the immunoprecipitate derived from the nuclear extract of EGF-treated mice, at 10 or 30 min. This was precisely the time course when the 120 kDa protein was tyrosine-phosphorylated in response to EGF.

Nuclear Association of the Tyrosine-Phosphorylated 120 kDa Protein with PIKE, and Nuclear PI[3]K Activity. Since stimulated nuclear PLCγ1 has been shown (*13*) to associate with PIKE, we investigated if such an association exists between the tyrosine-phosphorylated 120 kDa protein frag-

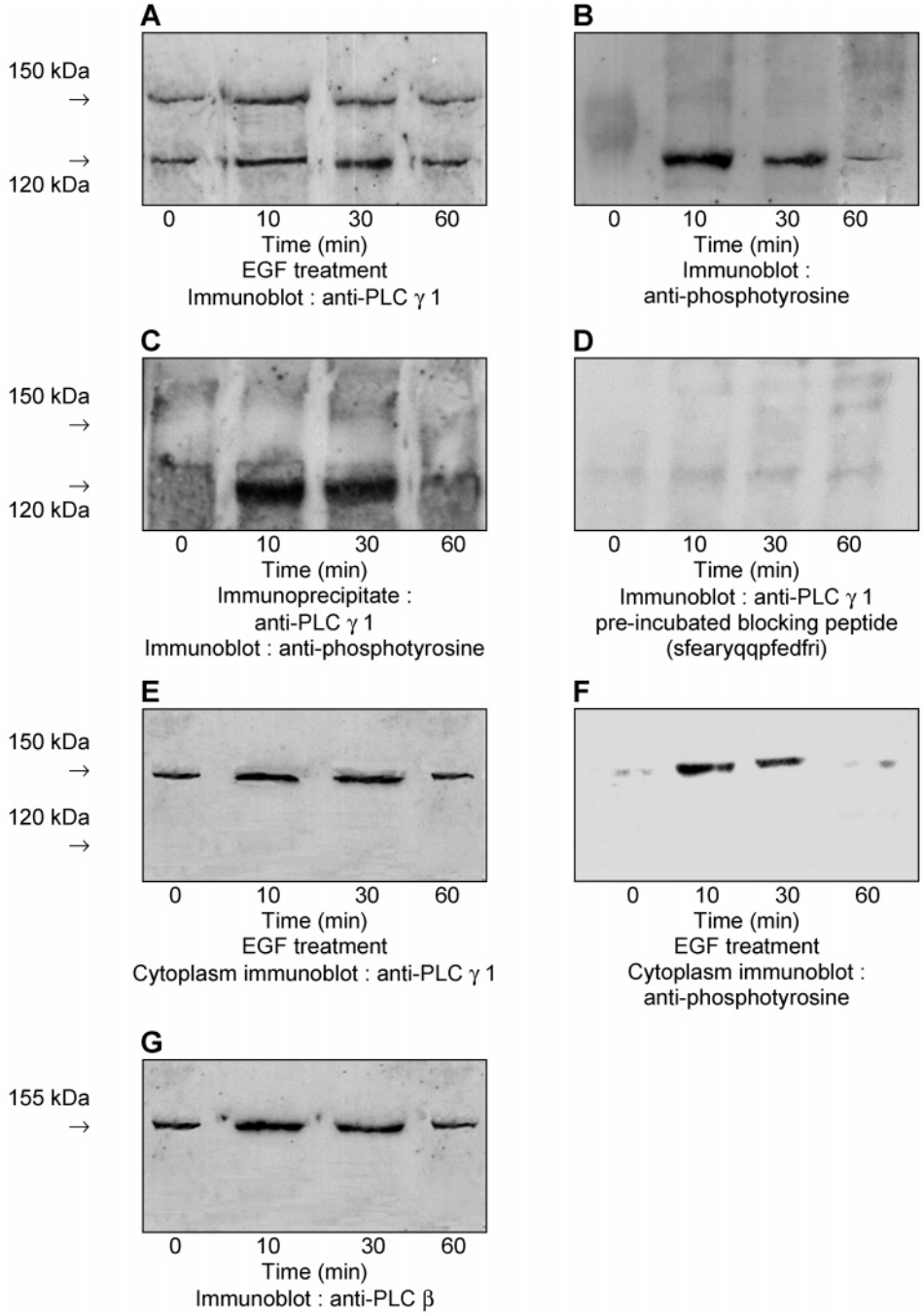

FIGURE 1: Immunoblot with anti-PLC antibodies. (A) Mouse liver nuclear extracts (60 μg of protein), obtained at the indicated time after EGF had been injected intraperitoneally, were immunoblotted with isozyme specific anti-PLCγ1 antibody, depicting two protein bands. (B) Western blotting with anti-phosphotyrosine antibody (as in panel A) revealed a tyrosine-phosphorylated 120 kDa band 10 and at 30 min after EGF injection. (C) Immunoprecipitate obtained with anti-PLCγ1 antibody from nuclear extract (300 μg of protein) immunoblotted with anti-phosphotyrosine antibody confirming that the 120 kDa protein band was tyrosine-phosphorylated after EGF treatment for 10 or 30 min. With or without EGF treatment for 60 min, no tyrosine phosphorylation was seen. (D) Immunoblot as in panel A, except that the anti-PLCγ1 antibody was preincubated with a specific blocking peptide, affirming that the two protein bands were related to PLCγ1. (E) Mouse liver cytoplasmic extracts (70 μg of protein) obtained as in panel A were immunoblotted with anti-PLCγ1 antibody showing a single 150 kDa protein band. (F) Western blotting with anti-phosphotyrosine antibody (as in panel E) revealed a tyrosine-phosphorylated cytoplasmic 150 kDa PLCγ1 in response to EGF treatment. (G) Immunoblot as in panel A, with isozyme specific anti-PLCβ antibody, showed a single 155 kDa band, further confirming that the 120 kDa protein fragment is specific to nuclear PLCγ1.

ment and PIKE. The immunoprecipitates obtained with anti-PIKE antibody (a generous gift from K. Ye) from the control and EGF-treated mice nuclear extracts, immunoblotted with anti-PLCγ1 antibody, revealed the 120 kDa protein band after EGF treatment for 10 or 30 min (Figure 4A). Likewise, in the immunoprecipitate with anti-PLCγ1 antibody, a 98 kDa protein band representing PIKE was immunoblotted with anti-PIKE antibody (Figure 4B). It is known that PLCγ1 interacts with PIKE through its SH3 domain (*13*) spanning from amino acid residue 797 to 841 (*22*). The SH3 domain remains intact in the 120 kDa protein fragment. Interaction between 120 kDa protein and PI[3]K was examined by immunoprecipitating this enzyme with anti-p-110α antobody, in the control and EGF-treated mice nuclear extract, and immunoblotting with anti-PLCγ1 antibody (Figure 4C).

Table 1: Marker Enzyme Activity in Nuclear and Cytoplasmic Extracts[a]

fraction	NAD pyrophosphorylase		mannose-6-phosphatase		NADPH cytochrome *c* reductase	
	total activity	specific activity	total activity	specific activity	total activity	specific activity
liver homogenate	20.5	3.50	435.00	78.70	52.50	14.50
nuclear extract	5.33	17.80	85.60	156.80	2.55	5.80
cytoplasmic extract	1.06	4.20	32.10	94.00	46.40	18.00

[a] NAD pyrophosphorylase activity was determined by monitoring the formation of NADH at 340 nm. Mannose-6-phosphatase activity was quantified by measuring the absorbance at 730 nm. NADPH cytochrome *c* reductase activity was determinated by monitoring the reduction of cytochrome *c* at 550 nm. The total activity is expressed as micromoles per minute, and the specific activity is expressed as the nanomoles per minute per milligram of protein. These data are means of two independent preparations with replicates varying by <10%.

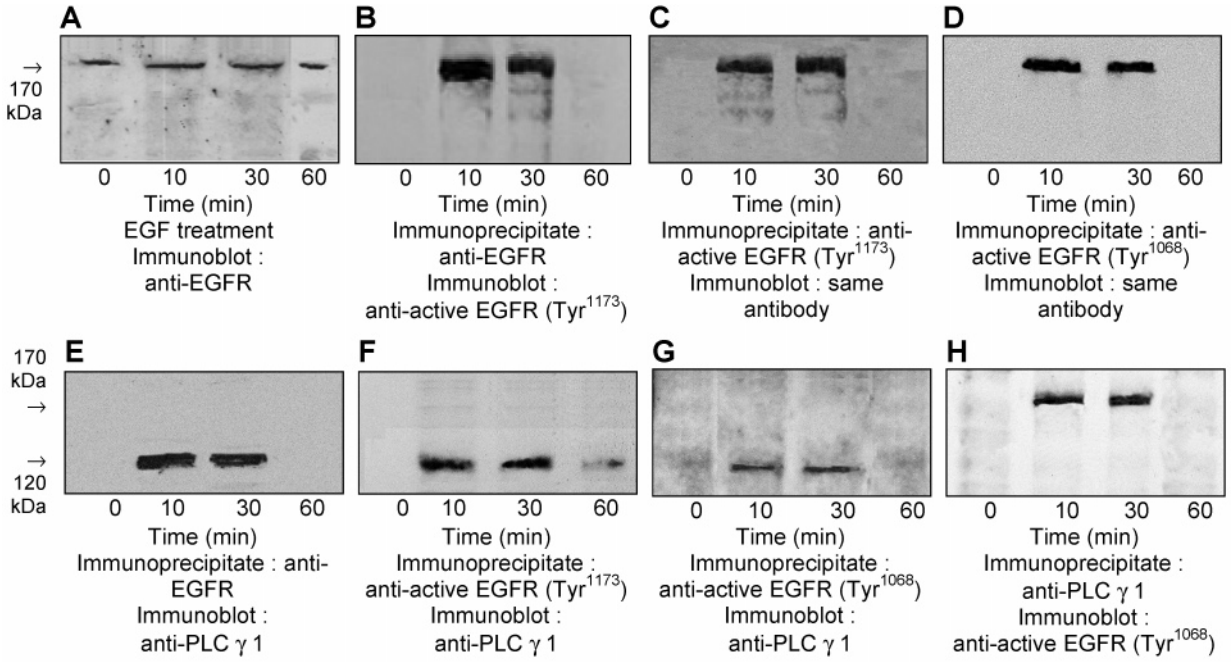

FIGURE 2: EGF-induced activation of nuclear membrane EGFR. (A) Nuclear extract (60 μg of protein) immunoblotted (as in the legend of Figure 1) with anti-EGFR antibodies. (B) Immunoprecipitate obtained with anti-EGFR antibody from nuclear extract (300 μg of protein) immunoblotted with anti-EGFR phospho specific (Tyr^{1173}) antibody depicting the time course of nuclear membrane EGFR tyrosine phosphorylation. (C) Immunoprecipitate with anti-EGFR phospho specific (Tyr^{1173}) antibody immunoblotted with the same antibody confirming EGFR tyrosine phosphorylation as in panel B. (D) Same as panel C with anti-EGFR phospho specific (Tyr^{1068}) antibody. (E) Same as panel B immunoblotted with anti-PLCγ1 antibody. (F) Same as panel C immunoblotted with anti-PLCγ1 antibody. (G) Same as panel D immunoblotted with anti-PLCγ1 antibody. (H) Immunoprecipitate with anti-PLCγ1 antibody immunoblotted with anti-EGFR phospho specific (Tyr^{1068}) antibody.

We have determined the PI[3]K activity contained in the anti-p110α antibody immunoprecipitate (Figure 4D) obtained from the nuclear extracts of control and EGF-treated mice. Augmented PI[3]K activity (45%) was observed after EGF treatment for 10 min, whereas after 30 min, a 30% increase was seen. The increase in nuclear PI[3]K activity in response to EGF was correlated with the time course of association between PLCγ1 and PIKE. The presence of PIKE in purified mouse liver nuclei attests to the fact that PIKE is not found only in nuclei of brain as reported previously (*13*). Studies in progress reveal that some isoforms of PIKE are indeed found in liver (K. Ye, personal communication).

EGF-Mediated Increase in the Nuclear DAG Level. Upon stimulation, PLCγ1 acts on PIP_2, generating IP_3 and DAG. In the liver nuclear extract, a 3-fold increase in the nuclear DAG concentration (Figure 5), after EGF treatment for 10 or 30 min, correlates with the 120 kDa protein moiety tyrosine phosphorylation and, consequently, its stimulation in the nucleus. Unfortunately, until now it has not been possible to quantify the nuclear IP_3 concentration (discussed below).

Localization of EGFR on the Inner Nuclear Membrane. There is a controversy about the localization of EGFR on the nuclear compartments (*23*). The immunofluorescence data (*15*) identifying the nuclear interior as the site for nuclear EGFR localization have been unconvincing (*23*). We have separated the inner and outer membranes from the isolated liver nuclei (*10*). On the basis of the NAD pyrophosphorylase, mannose-6-phosphatase, and NADPH cytochrome *c* reductase activity measurement, we have confirmed, as much as is experimentally feasible, that the inner and the outer membranes were devoid of cross contamination (Table 2). The immunoblotting with anti-EGFR antibody displayed a single 170 kDa protein band located only on the inner nuclear membrane (Figure 6).

N-Terminal and C-Terminal Partial Sequence Analysis of the 120 kDa Moiety. The 10-amino acid sequence at the N-terminal end is Pro-Glu-Leu-Cys-Gln-Val-Ser-Leu-Ser-

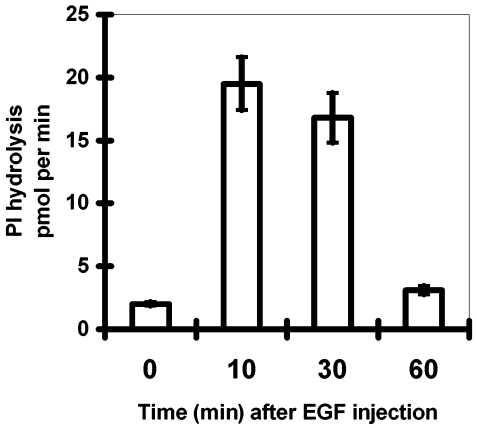

FIGURE 3: [^{3}H]PI hydrolysis. The 120 kDa protein band immunoprecipitated from the nuclear extract of control and EGF-treated mice with anti-PLCγ1 antibody was subjected to [^{3}H]phosphatidylinositol (200 μM, 20 000 cpm) in a 200 μL assay mixture incubated at 37 °C for 15 min. The protein contained in the immunoprecipitate was determined as described previously (*22*).

Glu-. This sequence is identical to that of rat PLCγ1 from Pro^{244} to Glu^{253} (*24*). The mouse PLCγ1 amino acid sequence deduced from cDNA (NCBI entry 41393058) at the same position is similar, but Leu^{246} is replaced with His. This difference comes from a single-nucleotide change. Considering our result and that amino acid 246 is also Leu in rat, bovine, and human, a mistake in the mouse sequence proposed in the NCBI data bank is likely.

The C-terminal sequence that we have determined is -Arg-Thr-Arg-Val-Asn-Gly-Asp-Asn-Arg-Leu. It is identical to the C-terminal sequence of mouse PLCγ1 (NCBI entry 28488251) and to those of rat, bovine, and human. These data reveal that the 120 kDa protein is indeed PLCγ1 which has been deprived of 243 amino acids at its N-terminal end.

Confirmation of the 120 kDa Protein Fragment in an Isolated Rat Liver Nuclei in Vitro Study. Treatment of isolated rat liver nuclei (*1*) with EGF resulted in tyrosine phosphorylation of the 120 kDa protein (Figure 7A) as well as phosphorylation at the tyrosine residue and activation of nuclear membrane EGFR (Figure 7B). Furthermore, association between the 120 kDa protein and EGFR upon EGF treatment (Figure 7C,D) and its interaction with PIKE (Figure 7E) were well founded in the studies in which the isolated nuclear model was used.

DISCUSSION

Despite controversies (*7*, *25*), several lines of overwhelming evidence suggest that the increase in the calcium concentration in the nucleus is regulated in a manner independent of cytosolic calcium signals (*26*). Increases in the nuclear calcium level carry specific biological functions that are distinct from the effects of an increase in the cytosolic calcium level (*27*−*30*). Our knowledge concerning the nuclear generation of IP_3 has been underscored due to the lack of information regarding the upstream signal stimulating nuclear PLCγ1. In this study, we focused on the fact that EGF is capable of delivering signals to the cell nucleus, phosphorylating nuclear membrane EGFR, and triggering nuclear events associated with the nuclear PLCγ1 stimulation.

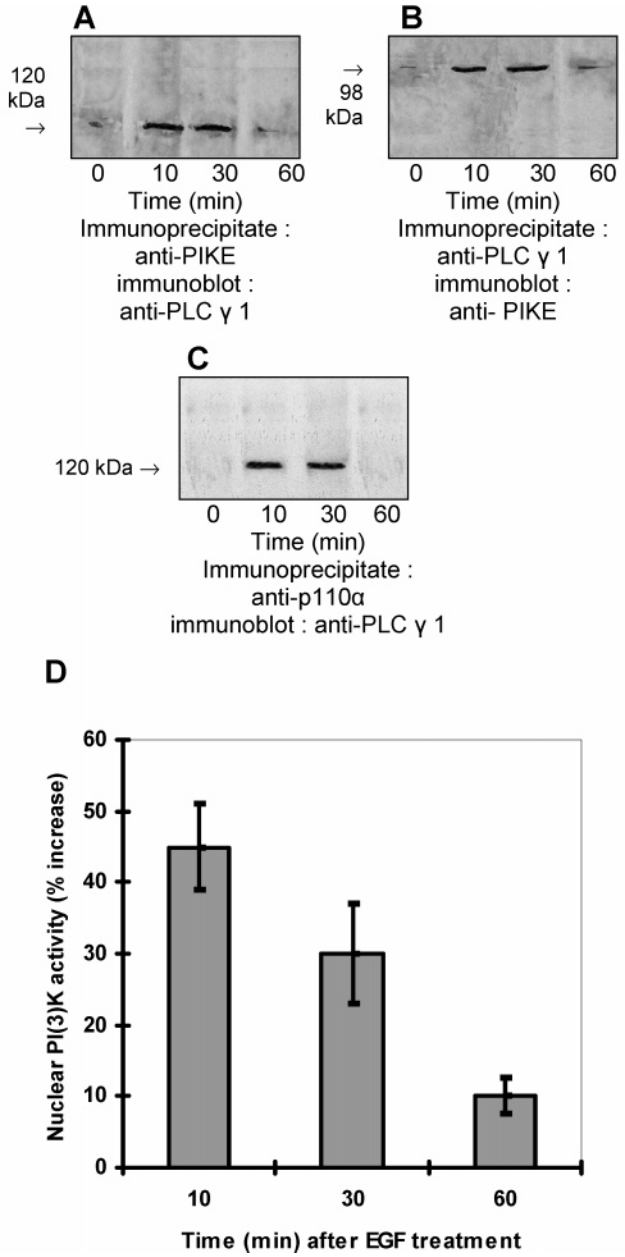

FIGURE 4: EGF mediated association between the 120 kDa protein and PIKE. (A) The immunoprecipitate obtained with anti-PIKE antibody from the nuclear extract (300 μg of protein) of control and treated EGF mice immunoblotted with anti-PLCγ1 antibody revealed the presence of a 120 kDa protein band after EGF treatment for 10 or 30 min. No such association was seen with or without EGF treatment for 60 min. (B) Same as panel A whereby immunoprecipitate with anti-PLCγ1 antibody immunoblotted with anti-PIKE antibody revealing the 98 kDa PIKE band. (C) Immunoprecipitate obtained with anti-p110α antibody immunoblotted with anti-PLCγ1 antibody. (D) Immunoprecipitate obtained with anti-p110α antibody from the nuclear extract (300 μg of protein) of EGF-treated mice depicting the percentage increase in the PI-[3]K activity determined with PI and [γ-^{32}P]ATP (10 μCi/sample).

The injection of EGF into mice induced tyrosine phosphorylation of a 120 kDa protein in the liver nuclei. PLCγ1 has been reported to be a single gene (*24*), encoding a 150 kDa protein. Therefore, we investigated the nature of the 120 kDa protein in the nucleus. Several lines of evidence suggest that the 120 kDa protein is derived from the 150 kDa PLCγ1. First, the 120 kDa protein was immunoprecipitated with isozyme specific anti-PLCγ1 antibody. Second,

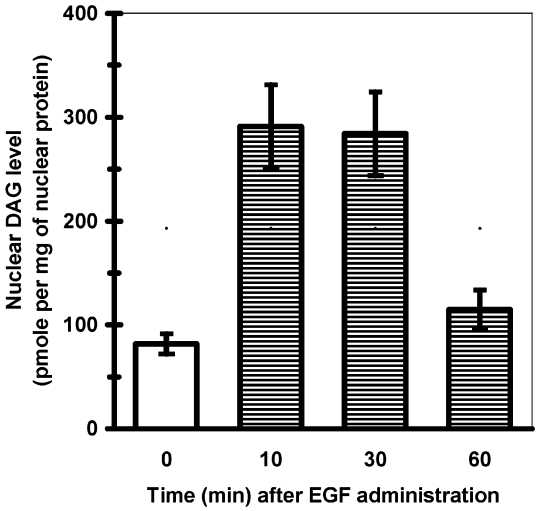

FIGURE 5: DAG level in the nuclear extract. Liver nuclear extracts (300 μg of protein), obtained from the mouse treated with or without EGF at the indicated time, were subjected to DAG determination (*20*) using a radioenzymatic assay employing diacylglycerol kinase quantitatively converting diacylglycerol to [^{32}P]phosphatidic acid in the presence of 10 mM [γ-^{32}P]ATP.

the 120 kDa protein, like the 150 kDa PLCγ1, was sensitive to isozyme specific blocking peptide. Third, the 10 partially sequenced amino acids of the N-terminal end are identical to those of the N-terminal sequence, Pro^{244}−Glu^{253}, found in rat PLCγ1 (*24*) and also identical to that of mouse (NCBI entry 41393058) but with one amino acid difference (at position 246), probably due to a sequencing error. Fourth, the C-terminal amino acid partial sequence was identical to the PLCγ1 C-terminal end. Thus, the 120 kDa protein represents PLCγ1 without N-terminal amino acid residues 1−243. The site of cleavage is located between Arg^{243} and Pro^{244} in PLCγ1. At this stage, we do not known how this cleavage is achieved and regulated in the nucleus in vivo. Although many proteases have been described in the nucleus, the knowledge of their specificity is still in its infancy.

Studies on limited proteolysis (*31*, *32*) and deletion mutation (*22*) activity reported in the literature do indicate that a 120 kDa PLCγ1 (in addition to other fragments) can be generated, but is formed by cleavage at the C-terminal end. Thus, the cleavage giving rise to nuclear 120 kDa PLCγ1 is specific to the nucleus. This is further supported by the data which show that, in the cytoplasm, only 150 kDa PLCγ1 was observed, and this band was indeed tyrosine-phophorylated in response to EGF signaling, confirming previous studies (*17*, *33*−*35*). The observation that another PI-PLC, the nuclear-localized PLCβ, a physiological target of extracellular signal-regulated kinase (ERK) (*36*), was immunoblotted as a single 155 kDa protein under similar conditions confirmed the specificity of PLCγ1 cleavage.

Having defined the relationship of the 120 kDa protein with PLCγ1, we addressed the function of this protein in the nucleus. Immunoblotting with anti-active EGFR antibody (Figure 2B) revealed that the 170 kDa protein band, depicting EGFR, was tyrosine-phosphorylated after injection of EGF into mice for 10 or 30 min. Immunoprecipitation with anti-active EGFR antibodies and subsequent immunoblotting with the corresponding antibody (Figure 2C,D) further confirmed that in response to the EGF signal the EGFR was activated in the nucleus. Since the time course of EGF activation coincides with the time course of the 120 kDa protein tyrosine phosphorylation, it is reasonable to propose that the 120 kDa protein undergoes tyrosine phosphorylation in the nucleus by the activation of the nuclear membrane EGFR itself, in response to EGF. It is intriguing that the 150 kDa moiety is not a target for activated nuclear resident EGFR. This could be related to the composition and architecture of the inner nuclear membrane, the resident site for nuclear EGFR, which is distinct from that of the plasma membrane. Thus, we can postulate that the conformation of the activated EGFR in the nuclear membrane is different from that in the plasma membrane in such a way that its target is the 120 kDa moiety.

To explore further the mechanism of activation of the nuclear PLCγ1, interaction between the EGFR and the 120 kDa moiety in the nucleus was investigated. The immunoprecipitates obtained with anti-EGFR antibodies from the control and EGF-treated mice nuclear extract, when immunoblotted with anti-PLCγ1 antibody, revealed a 120 kDa protein in each case that was studied (Figure 2E−G). Conversely, after a similar treatment, the immunoprecipitate obtained with anti-PLCγ1 antibody immunoblotted with anti-EGFR antibody revealed a 170 kDa protein band. The association between the two proteins was seen after EGF treatment for 10 and 30 min but not after 60 min.

Having established the capacity of EGF to trigger tyrosine phosphorylation of the nuclear EGFR as well as of the 120 kDa protein moiety, and having defined their interaction, we investigated the [^{3}H]PI hydrolysis activity contained in the 120 kDa protein immunoprecipitate from the control and EGF-treated mice nuclear extract. Indeed, the ability of EGF to stimulate PI hydrolysis, as catalyzed by the 120 kDa protein, was well-founded (Figure 3). The intrinsic hydrolysis activity further confirmed that we are dealing with a protein moiety with intact PLCγ1 catalytic properties. The preferred and differential physiological significance of PI hydrolysis by PLCγ1 has been reported (*33*).

PLCγ1 is a crucial mediator of a variety of growth factor receptor mitogenic actions (*34*, *37*). The mitogenic activity of PLCγ1 is monitored by its SH3 domains, and not by its phospholipase activity (*38*). One of the mechanisms of PLCγ1 mitogenic activity involves its interaction with nuclear PIKE, a nuclear GTPase, and subsequent activation of nuclear PI[3]K activity (*13*). This provided the basis for exploring the downstream events in the nucleus, monitored by nuclear PLCγ1 stimulation. EGF's ability to trigger the interaction between the 120 kDa protein and PIKE (Figure 4A,B) as well as with PI[3]K (Figure 4C) was established. This association was further supported by augmented nuclear PI[3]K activity (Figure 4D) in the immunoprecipitate from the nuclear extract, employing anti-p110α antibody. The increased PI[3]K activity was dependent on the time of EGF treatment and corresponded to the conditions under which the EGF-mediated complex was formed among nuclear PIKE, the 120 kDa moiety, and PI[3]K.

Another downstream target that was studied was DAG formation in the nucleus. Upon EGF stimulation, a more than 3-fold increase in DAG levels was observed at 10 and 30 min. This time course once again affirms the authenticity of the 120 kDa protein serving as nuclear PLCγ1, although there are other nuclear sources of DAG generation (*39*).

Table 2: Marker Enzyme Activity[a]

fraction	NAD pyrophosphorylase		mannose-6-phosphatase		NADPH cytochrome *c* reductase		5′-nucleotidase	
	total activity	specific activity	total activity	specific activity	total activity	specific activity	total activity	specific activity
liver homogenate	28.50	3.95	630.00	97.65	61.25	8.46	21.32	3.90
nuclei	8.50	23.90	128.60	229.00	1.21	4.60	0.08	0.25
outer nuclear membrane	0.03	3.28	4.20	765.00	0.04	1.00	ND[b]	ND[b]
inner nuclear membrane	1.50	65.95	0.51	21.25	0.03	1.05	ND[b]	ND[b]

[a] Activities of NAD pyrophosphorylase, mannose-6-phosphatase, and NADPH cytochrome *c* reductase were determined as described in Table 1. 5′-Nucleotidase activity was determinated as described in ref *10*. The total activity is expressed as micromoles per min, and the specific activity is expressed as nanomoles per minute per milligram of protein. These data are means of two independent preparations with replicates varying by <10%. [b] Not detected.

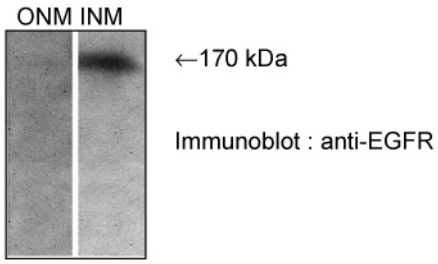

FIGURE 6: EGFR is located on the inner nuclear membrane. Immunoblot with anti-EGFR antibody employing purified inner nuclear membrane (INM) and outer nuclear membrane (ONM), 5 μg of protein in each case, showing the 170 kDa EGFR band located on the inner membrane.

One would very much like to determine IP_3 levels in the nuclei. This has not been possible up to now. That notwithstanding, overwhelming experimental evidence from a number of laboratories indicates that it is the IP_3 generated in the nucleus which is responsible for mobilizing nuclear envelope calcium. For instance, IP_3 in the nucleus, microinjected (*40*) or released from a cage (*41*), mobilizes nuclear envelope calcium. Functional IP_3R is located on the inner nuclear membrane (*10*), and is phosphorylated by nuclear protein kinase C (*9*).

In this context, an analogy can be put forward with the ryanodine receptors (RyR) which are also located on the inner nuclear membrane (*42*, *43*) with their agonist-binding sites facing toward the nucleoplasm. A functionally active CD38/ADP-ribosyl cyclase (*44*) is present on the inner nuclear membrane generating cyclic ADP-ribose (c-ADPr). c-ADPr and IP_3 are agonists for RyR and IP_3R, respectively. This analogy between c-ADPr and IP_3 makes it most likely that IP_3 is generated within the nuclei like c-ADPr. It is possible that nuclear-generated IP_3 remains long enough to mobilize nuclear envelope calcium but not long enough to be detected with available techniques.

Colocalization of EGFR with nuclear lamin and its binding directly to the promoter region of cyclin D1 in vivo have been advanced as evidence for nuclear localization of EGFR (*15*). However, immunofluorescence data (*15*) were not convincing (*23*). Precise EGFR localization is important for any future studies directed toward signal transduction-mediated regulation of nuclear events. We have now resolved this question by showing that the 170 kDa EGFR band is detected only on the inner nuclear membrane (Figure 6).

In vivo results were confirmed by in vitro studies using the well-tested isolated rat liver nuclear model system which we have used in many previous publications (refs *1*, *7*, *9*, *10*, *16*, *21*, and *29* and others uncited). When isolated nuclei were treated with EGF, the 120 kDa protein was phosphorylated at tyrosine in a time-dependent manner (Figure 7A), which was analogous to the in vivo observation. Similarly, nuclear membrane EGFR was activated (Figure 7B) and associated with tyrosine-phosphorylated 120 kDa moiety (Figure 7C,D). An interaction between the 120 kDa moiety and PIKE (Figure 7E) was also well-founded in the isolated nuclei.

In this study, we report that most of the functional domains of nuclear 120 kDa PLCγ1, including sites for tyrosine phosphorylation, remain completely intact. It may be recalled that PLCγ1 contains two SH2 domains and one SH3 domain, located between the X and Y domains (*35*). The SH2 domains are involved in the phosphotyrosine-dependent association of PLCγ1 with activated EGFR or other growth factor receptors. This association is followed by the tyrosine phosphorylation of PLCγ1 which activates its enzymatic action. It is through the SH3 domain that PLCγ1 interacts with PIKE (*13*) which in turn enhances nuclear PI[3]K activity in a manner independent of the phospholipase enzymatic activity.

PLCγ1 also contains two PH (pleckstrin homology) domains; the first one is located in the region of residues 27−142 and is present in all the PLCs, while the second one, split by the two SH2 and SH3 domains, is found only in PLCγs. It is this split PH domain that binds EF-1α and PIP_2. EF-1α activates the hydrolysis activity of PLCγ1 that produces IP_3 from PIP_2 (*45*). Another domain that was identified is composed of four EF hands which are supposed to bind calcium, one of which is located in the fragment of residues 165−177 (according to PROSITE). Thus, in the 120 kDa protein fragment, the first PH domain and the first EF hand are missing. The PH-EF domain is also missing in higher plants (*46*) and in two spliced variants of PLCβ4, from mammalian retina (*47*), suggesting that it is not absolutely necessary for biological activity.

It has been shown that the PH domain of PLCγ1 is required for the association of PLCγ1 with the human insulin receptor (IR) β-subunit while its SH2 domains are necessary for association with EGFR (*48*) or PDGFR (*49*). In addition, a PLCγ1 mutant lacking the PH-EF domain (301 amino acids at the N-terminal end), which is almost similar to the nuclear 120 kDa moiety (lacking 243 amino acids at the N-terminal end), is still able to interact with EGFR, but it is incapable of interacting with IR (*48*).

Differences in the interaction of 120 and 150 kDa PLCγ1 with some other tyrosine kinases are likely since PLCγ1 is involved in the transduction of at least 100 different tyrosine kinase receptors and various nonreceptor tyrosine kinases,

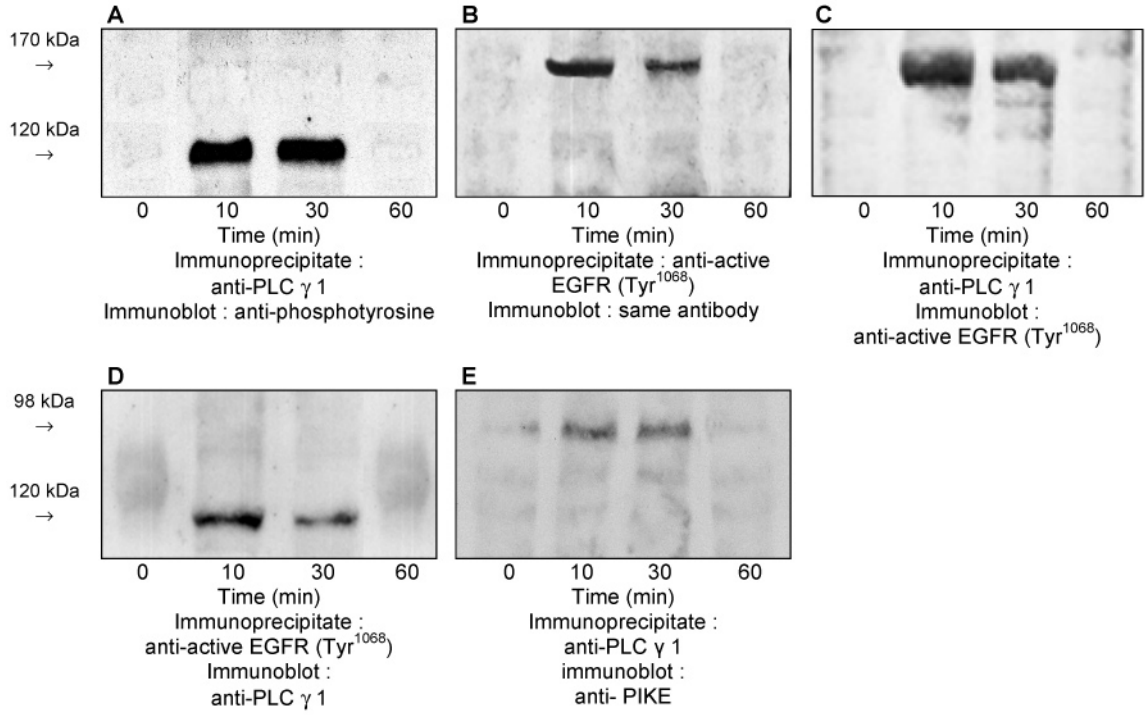

FIGURE 7: Protein fragment (120 kDa) and nuclear EGFR activation by EGF in isolated nuclear model. (A) Rat liver nuclear extracts (300 μg of protein), obtained at the indicated time after EGF treatment, were immunoprecipitated with anti-PLCγ1 antibody and immunoblotted with anti-phosphotyrosine antibody depicting that EGF treatment for 10 or 30 min tyrosine-phosphorylated the 120 kDa protein fragment. With or without EGF treatment for 60 min, such a phosphorylation did not occur. (B) Immunoprecipitate with anti-active EGFR phospho specific (Tyr^{1068}) antibody immunoblotted with the same antibody, with a similar EGF treatment, showing a 170 kDa EGFR protein. (C) Immunoprecipitated with anti-PLCγ1 antibody like in panel A immunoblotted with anti-active EGFR phospho specific (Tyr^{1068}) antibody. (D) Immunoprecipitate with anti-active EGFR phospho specific (Tyr^{1068}) antibody immunoblotted with anti-PLCγ1 antibody. Panels C and D confirm the association between EGFR and 120 kDa. (E) Immunoprecipitated with anti-PLCγ1 antibody immunoblotted with anti-PIKE antibody revealing a 98 kDa protein, confirming interaction between the two moieties upon EGF mediation.

many of these being known to be located in the nucleus. Differences in their interaction with the nuclear membrane are also expected since the PH domain mediates binding of 150 kDa PLCγ1 to phosphatidylinositol 3,4,5-trisphosphate (*50*).

In conclusion, we have discovered a 120 kDa protein, a cleaved 150 kDa PLCγ1 moiety in the liver nuclei in vivo, activated by EGF with a time course similar to that of nuclear membrane EGFR phosphorylation. EGF also promotes interaction between the 120 kDa protein and PIKE augmenting nuclear PI[3]K activity. To the best of our knowledge, such a PLCγ1 proteolytic product in vivo, with intact functions, has not been described previously.

Future research will be required to address the mechanism of PLCγ1 cleavage in vivo in the nucleus. The field of partial proteolysis (*51*, *52*) endowed with the promises for advancing the understanding of signal transduction pathways will, we hope, encompass nuclear events in future. The results documented in this paper warrant a need for filling this gap.

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