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Epidermal Growth Factor Receptor Phosphorylates Protein Kinase C δ at Tyr332 to form a Trimeric Complex with p66Shc in the H_2O_2-stimulated Cells

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Protein kinase C (PKC) δ is phosphorylated at Tyr311 and Tyr332 and its catalytic activity is enhanced in the H_2O_2-stimulated cells, but the enzymes that recognize these tyrosine residues, especially Tyr332, have been remained to be clarified. The analysis of the endogenous proteins in COS-7 cells revealed that PKCδ binds to p66Shc, an adaptor protein containing two phosphotyrosine-binding domains, in a manner dependent on its tyrosine phosphorylation upon H_2O_2 stimulation. The studies using the mutated PKCδ clarified that PKCδ associates with p66Shc through the phosphorylated Tyr332 residue. Epidermal growth factor (EGF) receptor was detected in the anti-p66Shc immunoprecipitate prepared from the H_2O_2-stimulated cells, and this receptor-type tyrosine kinase phosphorylated PKCδ at Tyr332 *in vitro*. PKCδ was, however, not tyrosine phosphorylated in the EGF-stimulated cells, whereas H_2O_2-induced tyrosine phosphorylation of PKCδ and its association with p66Shc were strongly suppressed by EGF receptor kinase inhibitors such as AG1478 and PD153035. These results indicate that EGF receptor phosphorylates PKCδ at Tyr332 in the H_2O_2-stimulated but not in the growth-factor treated cells, and suggest that PKCδ in the complex with p66Shc and EGF receptor may play a role in the stress-signalling pathway.

Key words: complex formation, EGF receptor, hydrogen peroxide, PKCδ, Shc.

Abbreviations: CH, collagen-homology; EGF, epidermal growth factor; EGFR, EGF receptor; GFP, green fluorescent protein; GST, glutathione S-transferase; PKC, protein kinase C; PTB, phosphotyrosine-binding; SH2, Src homology 2.

The protein phosphorylation reaction is a key process to regulate the cellular functions by transferring a phosphate residue from ATP to the hydroxyl side chain residues in the target proteins. The human genome encodes more than 500 genes of the putative protein kinases, and their products are classified into two categories, protein tyrosine- and serine/threonine-kinases according to their substrate specificity (*1*, *2*). Protein tyrosine-kinases are further divided into two groups, receptor- and non-receptor-type kinases by their domain structures, whereas the protein serine/threonine-kinases show a variety of structural features.

Protein kinase C (PKC) is a typical serine/threonine-kinase family composed of nine genes in mammals, which have the regulatory and catalytic domains in the amino- and carboxyl-terminal halves, respectively (*3*). The catalytic domains are highly homologous among the family, and they are separated into three subfamilies of cPKC, nPKC and aPKC by the structural differences in their regulatory domains. The gene products of the cPKC and nPKC subfamilies are activated by diacylglycerol produced by receptor-mediated hydrolysis of membrane inositol phospholipids and by tumour-promoting phorbol esters, and plays roles in the receptor-coupled membrane signalling as well as in the cell proliferation. On the other hand, the PKC isoforms have three phosphorylation motif sites of serine and threonine that are nearly conserved among the family (*4*, *5*). These motif sites are constitutively phosphorylated in most of the PKC family members, and thus the modification of these residues is regarded to be a prerequisite for maturation of PKC to exhibit a protein kinase activity in the receptor-coupled signalling pathway.

PKCδ, a member of the nPKC subfamily, is expressed ubiquitously among the cells and is activated through multiple mechanisms, in addition to the diacylglycerol- and phorbol ester-dependent manner (*6*). Namely, PKCδ is cleaved by caspase to generate a catalytically active fragment, which inhibits DNA-dependent protein kinase and facilitates apoptosis by binding to the DNA-dependent enzyme. Furthermore, the H_2O_2 treatment induces the protein complex formation of PKCδ through its regulatory domain and the oligomeric PKCδ shows a high catalytic activity (*7*). On the other hand, PKCδ is phosphorylated on tyrosine, in addition to the serine and threonine phosphorylation at the motif sites, in *v-ras*-transformed cells and in the cells stimulated with

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hormones, growth factors, UV radiation, ceramide and H_2O_2 (*6*).

Several tyrosine residues have been identified, thus far, to be the phosphorylation sites in this PKC isoform, and Tyr311 and Tyr332, located in the hinge region between the regulatory and catalytic domains, are phosphorylated predominantly in the H_2O_2-treated cells (*8*). PKCδ recovered from the H_2O_2-treated cells shows a high kinase activity that is independent of diacylglycerol, and the *in vitro* tyrosine phosphorylation at Tyr311 enhances the basal catalytic activity of PKCδ in the absence of the lipid activator. Consistently, the Y311F mutant replacing Tyr311 by phenylalanine was activated less efficiently than the wild type enzyme in the H_2O_2-treated cells. Concerning the tyrosine kinases recognizing Tyr311 of PKCδ, the non-receptor-type kinases such as Src and Lck of the Src family are shown to phosphorylate this residue *in vitro* and *in vivo* (*8*, *9*), whereas Tyr332 is phosphorylated in the cells overproducing Lyn, another Src family kinase (*10*). Lyn is expressed predominantly in haematopoietic cells (*11*), but PKCδ distributes broadly among the cells (*6*), and is phosphorylated at Tyr332 in COS-7 cells (*8*). Therefore, the protein kinase other than Lyn is yet to be identified, that recognizes Tyr332 of PKCδ.

On the other hand, Shc is an adaptor protein containing multiple protein–protein docking sites such as phosphotyrosine-binding (PTB) domain, collagen-homology (CH) domain and Src homology 2 (SH2) domain (*12*, *13*), and three isoforms, p66, p52 and p46, are generated from the gene by alternative splicing and the use of different initiation codons in mammalian cells. In addition, Shc has three tyrosine phosphorylation sites in the CH domain that also serve as protein–protein docking sites. Upon epidermal growth factor (EGF) stimulation, for example, Shc binds to the tyrosine-phosphorylated EGF receptor (EGFR) via its PTB and SH2 domains, whereas Grb2, another adaptor protein, binds to the tyrosine-phosphorylated Shc through the SH2 domain. Grb2 is constitutively associated with Sos, a Ras guanine nucleotide exchange factor, and the recruitment of the Grb2/Sos complex to the receptor is responsible for the activation of the Ras-dependent Erk/MAP kinase cascade (*12*, *13*). Recently, it has been revealed that PKCδ phosphorylated at Tyr332 binds to the SH2 domain of Shc isoforms in antigen-stimulated mouse bone marrow-derived mast cells (*10*), and that the PKC isoform associates with the SH2 and/or PTB domains of three Shc isoforms in H_2O_2-stimulated COS-7 cells (*14*).

In this study, we investigated the protein kinase that recognizes Tyr332 of PKCδ in H_2O_2-stimulated COS-7 cells, and EGFR was identified as the enzyme that phosphorylates PKCδ at Tyr332. It was then revealed that a novel trimeric complex of EGFR, Shc and PKCδ is generated upon H_2O_2 stimulation of the cells.

MATERIALS AND METHODS

Expression Plasmids—FLAG epitope-tagged expression plasmids of rat PKCδ (amino acids 1–673) and the mutant molecules replacing Tyr311 or Tyr332 by phenylalanine were constructed in pcDNA3 as described previously (*7*, *8*). The protein products were designated as FLAG-PKCδ, FLAG-Y311F and FLAG-Y332F, respectively. The expression plasmid of the glutathione S-transferase (GST) fusion protein of PKCδ (GST–PKCδ) in mammalian cells was generated using pEBG as described previously (*7*). The expression plasmids of green fluorescent protein (GFP)-fusion protein of mouse Shc isoforms, GFP-p66Shc, GFP-p52Shc and GFP-p46Shc, were constructed in pEGFP as reported (*15*).

Antibodies—Anti-FLAG (M2) and anti-α-tubulin antibodies were purchased from Sigma. The anti-GFP antibody was bought from Molecular Probes. Anti-PKCδ and anti-EGFR antibodies were obtained from BD Biosciences Pharmingen, and the monoclonal anti-phosphotyrosine antibody (anti-PY, clone 4G10) and the anti-p66Shc antibody were purchased from Upstate Biotechnology. The anti-GST antibody (Z-5) and antibodies directed against phosphorylated Tyr311 and Tyr332 of PKCδ, anti-pY311 and anti-pY332, respectively, were purchased from Santa Cruz. The antibodies against Erk and phosphorylated Erk were from Cell Signaling. Alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies, horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies and normal rabbit and mouse IgG were purchased from Chemicon.

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator. Gene transfection was performed with Nucleofector kit (Amaxa) according to the manufacturer's protocol, and the cells were cultured in the presence of 10% fetal calf serum for 24 h. The cells were further cultured in the medium containing 0.1% fetal calf serum for 24 h, and then treated with 10 mM H_2O_2 or 100 ng/ml EGF (Calbiochem) for 10 min. Where indicated, the cells were pretreated with either 200 μM of genistein (Wako) for 10 min (*7*), 10 μM of AG1478 (Calbiochem) for 15 min (*16*) or 10 μM of PD153035 (Calbiochem) for 2 h (*17*) before stimulation.

Immunoprecipitation and Affinity Purification—The following procedures were performed at 0–4°C essentially as described (*8*). Briefly, the cells were washed with phosphate-buffered saline and lysed in 20 mM Tris–HCl at pH 7.5 containing 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10 mM 2-mercaptoethanol, 50 mM NaF, 2 mM Na_3VO_4 and 0.05% protease inhibitor cocktail (Sigma). The lysate was centrifuged for 20 min at 18,000*g*. The resulting supernatant (500–600 μg protein) was incubated for 5 h either with anti-PKCδ, anti-p66Shc, anti-GFP, anti-FLAG or anti-EGFR antibody, and then rProtein G-Sepharose (GE Healthcare) was added. Then, the mixture was further incubated for 1 h. The immunoprecipitate was collected by centrifugation and washed with 20 mM Tris–HCl at pH 8.0 containing 150 mM NaCl, 1% Triton X-100 and 2 mM EDTA for three times. For the affinity purification of GST and GST–PKCδ, the cell extract prepared above was applied to a GSTrap column (GE Healthcare). After washing of the column with the lysis buffer, bounded proteins were eluted with glutathione. The purified proteins were

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measured using the Bradford protein assay reagent (Biorad).

Subcellular Fractionation—The subcellular fractionation of the cells was performed according to (*14*). The washed cells were resuspended in 50 mM HEPES at pH 7.3 containing 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin, and sheared by being passed through a 27-gage needle 10 times. The homogenate was centrifuged at 100,000*g* for 1h, and the supernatant was collected as the cytosol fraction. The pellet was dissolved in the same buffer containing 1% Triton X-100, centrifuged at 15,000*g* for 10 min, and the resulting supernatant was employed as the membrane fraction. The whole cell lysate was prepared by lysing the cells directly in the buffer containing 1% Triton X-100, following the centrifugation at 15,000*g* for 10 min to remove the cell debris.

Immunoblot Analysis—The samples were boiled in SDS-sample buffer, and the proteins were separated by SDS-PAGE and transferred to an Immobilon P membrane (Millipore) (*8*). After incubation with each primary antibody, the alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies were employed as the secondary antibodies, and the colour reaction was carried out using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates for Fig. 1. Alternatively, the horseradish peroxidase-conjugated antibodies were employed as the secondary antibodies, and the chemiluminescence reaction was carried out by using ECL plus chemiluminescence detection kit (GE Healthcare) for Figs 2–5. Pre-stained proteins (broad range, New England Biolabs) were employed as molecular size markers. All results shown in the figures are representative of three independent experiments. Where indicated, immunoblot signals were quantitated by NIH Image J.1.38 software.

In vitro *Phosphorylation*—GST–PKCδ purified as described (1 μg) was incubated with the immunoprecipitate obtained from the H_2O_2-stimulated cells using the anti-EGFR antibody or normal mouse IgG in 5 mM HEPES at pH 7.3 containing 5 mM $MnCl_2$, 2 mM $MgCl_2$, 50 μM Na_3VO_4, 10 μg/ml bovine serum albumin and 40 μM ATP at 30°C for 30 min (*18*). Where indicated, the reaction was carried out in the presence of 1 μM AG1478 (*19*) or 1 μM PD153035 (*20*).

RESULTS

Binding of PKCδ and Shc—It has been revealed that PKCδ is phosphorylated at Tyr332 by Lyn and binds to the Shc isoforms through the tyrosine-phosphorylated residue in antigen-stimulated mouse bone marrow-derived mast cells (*10*). The association of PKCδ and p66Shc was thus studied by the analysis of these two proteins endogenously expressed in COS-7 cells (Fig. 1A), in which PKCδ is phosphorylated at Tyr332 upon H_2O_2 stimulation (*8*). PKCδ was detected in the anti-Shc antibody immunoprecipitate obtained from the H_2O_2-stimulated cells, whereas this enzyme was not found in the immunoprecipitate prepared from the unstimulated cells. Endogenous p66Shc was detected in the immunoprecipitate by the anti-PKCδ antibody from the stimulated but not control cells. Neither PKCδ nor p66Shc was precipitated by the normal rabbit and mouse IgG under the conditions employed. These results indicate that the binding of PKCδ with p66Shc is induced by the H_2O_2 stimulation in COS-7 cells. Next, FLAG-PKCδ was then co-expressed with GFP-p66Shc in COS-7

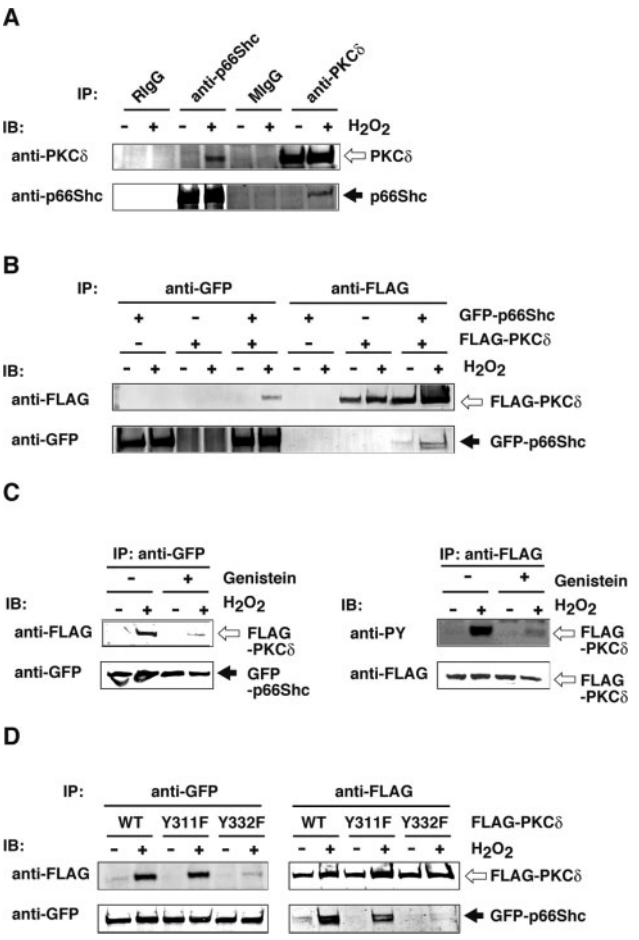

Fig. 1. **The binding of PKCδ and p66Shc in H_2O_2-stimulated cells.** (A) The binding of endogenous PKCδ and p66Shc. The endogenous PKCδ and p66Shc were immunoprecipitated from the H_2O_2-stimulated cells, and immunoblot analysis was carried out using the anti-PKCδ and anti-p66Shc antibodies. The normal rabbit and mouse IgG, RIgG and MIgG, respectively, were employed for immunoprecipitation as controls. (B) The binding of FLAG-PKCδ and GFP-p66Shc. Cells transfected with FLAG-tagged and/or GFP-fused constructs were stimulated by H_2O_2, and the expressed proteins were immunoprecipitated and immunoblot analysis was carried out using the anti-GFP and anti-FLAG antibodies. (C) Effect of tyrosine kinase inhibitor genistein. Cells transfected with FLAG-PKCδ and GFP-p66Shc were pretreated with genistein, and then stimulated by H_2O_2. The expressed proteins were immunoprecipitated by the anti-GFP and anti-FLAG antibodies (right and left panels, respectively), and immunoblot analysis was carried out using the anti-FLAG, anti-GFP and anti-PY antibodies. (D) Binding of PKCδ mutants and p66Shc. Cells transfected with GFP-p66Shc in accompany either of FLAG-PKCδ (WT), FLAG-Y311F or FLAG-Y332F were stimulated by H_2O_2. The expressed proteins were immunoprecipitated and immunoblot analysis was carried out using the anti-FLAG and anti-GFP antibodies.

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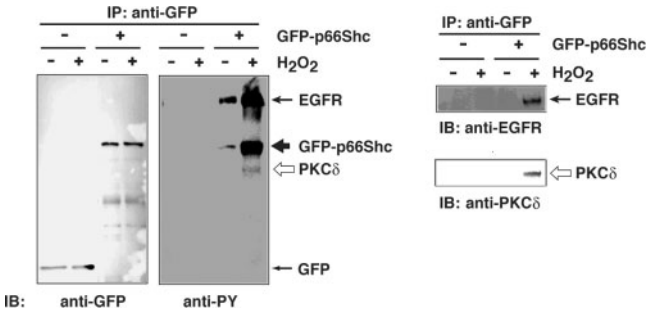

Fig. 2. **The binding of endogenous EGFR and PKCδ with p66Shc in H_2O_2-stimulated cells.** Cells transfected with GFP-p66Shc or GFP were stimulated by H_2O_2. The expressed proteins were immunoprecipitated by the anti-GFP antibody, and immunoblot analysis was carried out using the anti-GFP, anti-PY, anti-EGFR and anti-PKCδ antibodies.

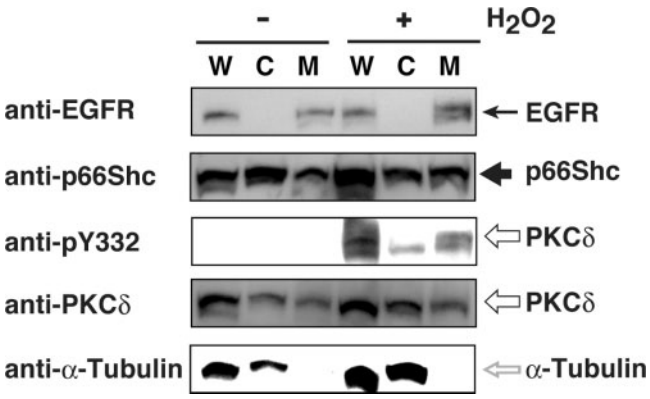

Fig. 3. **Subcellular distribution of PKCδ and p66Shc.** Cells were stimulated by H_2O_2. The whole cell lysate (W) and the cytosol (C) and membrane (M) fractions were prepared, and immunoblot analysis was carried out using anti-EGFR, anti-p66Shc, anti-pY332, anti-PKCδ and anti-α-tubulin antibodies.

cells to further examine the association of these two proteins (Fig. 1B). Analogous to the endogenous proteins, the H_2O_2 stimulation induced the association of FLAG-PKCδ and GFP-p66Shc as detected by the immunoblot analysis following immunoprecipitation using the anti-FLAG and anti-GFP antibodies. These data for the association of PKCδ and Shc are consistent with previous reports in the antigen-stimulated mast cells and H_2O_2-stimulated COS-7 cells (*10*, *14*). When the cells were pre-treated with genistein, a potent tyrosine kinase inhibitor, the H_2O_2-induced association of FLAG-PKCδ to GFP-p66Shc was heavily reduced and genistein naturally suppressed the H_2O_2-induced tyrosine phosphorylation of PKCδ (Fig. 1C). The involvement of tyrosine phosphorylation was further examined by using the PKCδ mutants, FLAG-Y311F and FLAG-Y332F replacing each of the major phosphorylation sites, Tyr311 and Tyr332, respectively, by Phe (Fig. 1D). FLAG-Y332F did not bind to GFP-p66Shc, whereas FLAG-Y311F still bound to the GFP-p66Shc even less significantly as of the wild type PKCδ. Thus, PKCδ was concluded to binds to p66Shc through the phosphorylated Tyr332 in the H_2O_2-treated COS-7 cells. Furthermore, GFP-p52Shc as well as

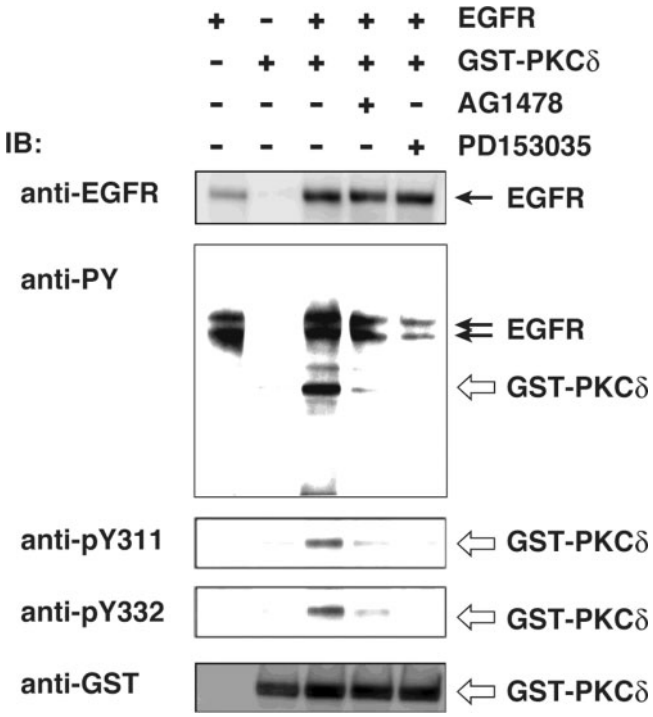

Fig. 4. ***In vitro* tyrosine phosphorylation of PKCδ by EGFR.** The GST–PKCδ protein was incubated in the phosphorylation reaction mixture with EGFR immunoprecipitated from H_2O_2-stimulated cells by the anti-EGFR antibody. The immunoprecipitate obtained using normal mouse IgG was employed for the control lane. Where indicated, the incubation was done in the presence of either AG1478 or PD153035. Immunoblot analysis was carried out using the anti-EGFR, anti-PY, anti-pY311, anti-pY332 and anti-GST antibodies.

GFP-p46Shc showed the properties similar to those of GFP-p66Shc for the binding to PKCδ (data not shown).

Complex Formation of Shc with PKCδ and EGFR—The Shc isoforms have been shown to associate with activated EGFR via its PTB and SH2 domains in the EGF-treated as well as H_2O_2-stimulated cells (*12*, *21–23*). Therefore, the interacting proteins of p66Shc were analysed by using the anti-phosphotyrosine antibody in COS-7 cells to find a clue for the enzyme that phosphorylates Tyr332 of PKCδ (Fig. 2). GFP-p66Shc was weakly recognized by the anti-PY antibody in the control cells, and the H_2O_2 stimulation heavily enhanced tyrosine phosphorylation of GFP-p66Shc. In addition, two tyrosine-phosphorylated proteins with approximate *Mr* of 80 kDa and 180 kDa were found in the immunoprecipitate of GFP-p66Shc from the H_2O_2-stimulated cells (Fig. 2 left panels). The 80 kDa protein, which was absent in the immunoprecipitates from the unstimulated cells and from the cells expressing GFP alone, was normally confirmed to be PKCδ by immunoblot analysis using the anti-PKC δ antibody (Fig. 2 right panels). On the other hand, immunoblot analysis using the antibody against EGFR identified the 180 kDa protein as EGFR, which is expressed endogenously in the cells. A slight association of tyrosine phosphorylated EGFR to exogenously expressed GFP-p66Shc was observed in the unstimulated cells as previously reported (*24*). EGFR was not detected

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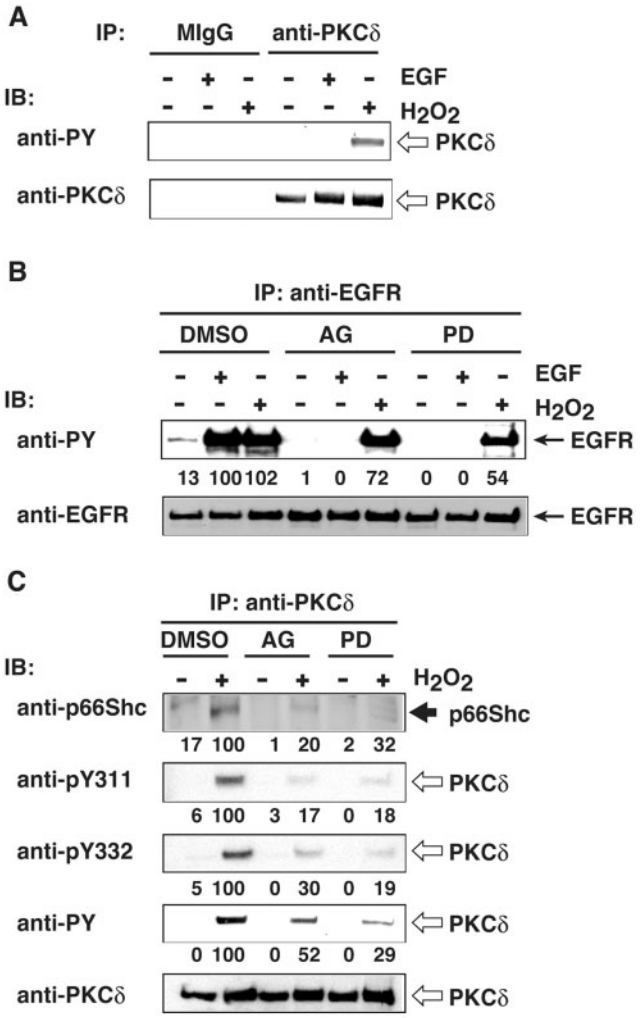

Fig. 5. **Effects of EGFR inhibitors on tyrosine phosphorylation and the binding of PKCδ to p66Shc *in vivo*.** (A) Tyrosine phosphorylation of PKCδ. Cells were stimulated by either H_2O_2 or EGF. The endogenous PKCδ was immunoprecipitated by the anti-PKCδ antibody, and immunoblot analysis was carried out using the anti-PY and anti-PKCδ antibodies. The normal mouse IgG (MIgG) was employed for immunoprecipitation as control. (B) Tyrosine phosphorylation of EGFR. Cells were pretreated with AG1478 (AG), PD153035 (PD) or dimethysulfoxide (DMSO), and then stimulated by either H_2O_2 or EGF. The endogenous EGFR was immunoprecipitated by the anti-EGFR antibody, and immunoblot analysis was carried out using the anti-PY and anti-EGFR antibodies. The specific immunoblot signals of the anti-PY antibody were quantitated, and that in the EGF-stimulated cells is shown as 100% under the panel. (C) Tyrosine phosphorylation of PKCδ and its binding to p66Shc. Cells were pretreated with AG1478 (AG), PD153035 (PD) or dimethysulfoxide (DMSO), and then stimulated with H_2O_2. The endogenous PKCδ was immunoprecipitated by the anti-PKCδ antibody, and immunoblot analysis was carried out using the anti-p66Shc, anti-pY311, anti-pY332, anti-PY, and anti-PKCδ antibodies. The specific immunoblot signals of anti-p66Shc, anti-pY311 anti-pY332 and anti-PY antibodies were quantitated, and those in the H_2O_2-stimulated cells without the inhibitor treatment are shown as 100% under each panel.

in the anti-EGFR immunoblot, presumably because the anti-PY antibody is more sensitive than the anti-EGFR antibody. These results suggest that p66Shc forms a trimeric complex with EGFR and PKCδ. Furthermore, the subcellular distribution of endogenous p66Shc and PKCδ was analysed after separation into the cytosol and membrane fractions comparing with the localization of EGFR (Fig. 3). EGFR was recovered exclusively in the membrane fraction, whereas α-tubulin, a cytosolic marker protein, was reciprocally observed in the cytosol fraction in the control cells. H_2O_2 stimulation did not change the localization of these two proteins. The p66Shc was found predominantly in the cytosol fraction in the unstimulated cells, and this adopter protein was recovered almost equally in the cytosol and membrane fractions in the H_2O_2-stimulated cells. These results indicate that p66Shc translocates from the cytosol to the plasma membrane after H_2O_2 stimulation, presumably through the association with EGFR, in consistent with the previous report (*14*). On the other hand, PKCδ was present both in the cytosol and membrane fractions in the control cells (*6*), and this distribution pattern did not change apparently by the H_2O_2 stimulation. PKCδ phosphorylated at Tyr332, however, appeared in the membrane fraction after the stimulation, indicating that a small but distinct amount of PKCδ, that is phosphorylated at Tyr332 to be able to associate with p66Shc, was detected in the membrane fraction. These results support that the trimeric complex of p66Shc, PKCδ and EGFR was formed in the plasma membrane in the H_2O_2-stimulated cells, and thus EGFR seems to be involved in tyrosine phosphorylation of PKCδ.

Phosphorylation of PKCδ by EGFR in vitro—Then, EGFR was examined whether it phosphorylates PKCδ *in vitro*. GST–PKCδ purified from COS-7 cells was incubated in the kinase reaction buffer with or without EGFR immunoprecipitated from the H_2O_2-stimulated cells (Fig. 4). GST–PKCδ was recognized by the anti-PY antibody after the incubation with EGFR, but the tyrosine phosphorylation did not occur with the control immunoprecipitate. Further, immunoblot analysis using the phosphorylation site-specific antibodies revealed that both of Tyr332 and Tyr311 in the GST–PKCδ are phosphorylated by EGFR. The tyrosine phosphorylation of GST–PKCδ as well as that of EGFR was attenuated when the potent EGFR kinase inhibitors such as AG1478 and PD153035 were added to the reaction mixture. These results indicate that EGFR, but not other protein kinases that may contaminate the immunoprecipitate, recognizes PKCδ *in vitro* at both of these two residues, and suggest that EGFR is responsible for tyrosine phosphorylation of PKCδ at Tyr332 as well as Tyr311 in the H_2O_2-stimulated cells.

Phosphorylation of PKCδ by EGFR in vivo—The involvement of EGFR on tyrosine phosphorylation of PKCδ was studied by monitoring the modification reaction of these two proteins endogenously expressed in COS-7 cells (Fig. 5). The endogenous PKCδ was phosphorylated on tyrosine in the cells after the stimulation by H_2O_2 but not in the cells treated with EGF (Fig. 5A). However, H_2O_2 and EGF induced tyrosine phosphorylation of EGFR efficiently, and the

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EGFR inhibitors, AG1478 and PD153035, reduced H_2O_2-induced tyrosine phosphorylation of EGFR, and abolished its EGF-induced autophosphorylation on the tyrosine residues (Fig. 5B). EGFR inhibitors at a concentration higher than that for *in vitro* analysis (Fig. 4) was employed by the low permeability of these compounds to COS-7 cells (*16*, *17*, *19*, *20*). These results indicate that EGF induces autophosphorylation of EGFR in a manner sensitive to its inhibitors (*25*), whereas H_2O_2 stimulates not only the autophosphorylation but also the tyrosine phosphorylation reaction of EGFR by other kinase(s). These inhibitors decreased the tyrosine phosphorylation at Tyr332 and Tyr311 of PKCδ, and concomitantly attenuated the binding of PKCδ and p66Shc in the H_2O_2-stimulated cells (Fig. 5C). Taken together, it is plausible that EGFR activated by tyrosine phosphorylation by other kinase(s) recognizes Tyr332 of PKCδ to form a complex consisting of EGFR, p66Shc and PKCδ.

DISCUSSION

Tyr311 and Tyr332 have been identified as the predominant tyrosine phosphorylation sites of PKCδ in the H_2O_2-treated cells, and the phosphorylation of Tyr311 enhances its basal catalytic activity in the absence of the lipid activators (*8*), whereas PKCδ phosphorylated at Tyr332 associates with the SH2 domain and/or PTB domain of Shc (*10*). The Src family kinases are reported to phosphorylate PKCδ in various cells (*6*) and Tyr311 is known to be recognized by this family such as Src and Lck *in vitro* and *in vivo* (*8*, *9*). The mutation of Tyr311 by Phe heavily reduces its *in vitro* tyrosine phosphorylation by Lck (*8*), suggesting that Tyr311 is a feasible phosphorylation site by the Src family kinases in PKCδ. On the other hand, Tyr332 is phosphorylated in the cells overproducing Lyn, another Src family kinase (*10*). Lyn is, however, expressed predominantly in haematopoietic cells such as macrophages, mast cells and B lymphocytes (*11*), whereas PKCδ and p66Shc distribute broadly among the cells and tissues (*6*, *13*). Therefore, Lyn is not the sole kinase that phosphorylates Tyr332 of PKCδ.

It has been shown that PKCδ is tyrosine phosphorylated in the EGF-stimulated cells (*26*). EGFR is, however, not regarded as the direct enzyme phosphorylating PKCδ, and the Src family kinases are indicated to recognize this PKC isoform in the downstream of EGFR (*26*). Supporting this supposition, the purified EGFR phosphorylates PKCδ much less significantly than the Src family kinases *in vitro* (*26*). In this study, however, inhibitors specific to the EGFR kinase such as AG1478 and PD153035 were shown to reduce the tyrosine phosphorylation of Tyr332 as well as that of Tyr311 in the H_2O_2-stimulated cells. Furthermore, EGFR purified from the H_2O_2-stimulated cells phosphorylated these two residues *in vitro*. The tyrosine kinases contaminated the EGFR preparation, if any, are not responsible for the *in vitro* tyrosine phosphorylation of PKCδ, because this *in vitro* phosphorylation was sensitive to the EGFR inhibitors. Accordingly, it is reasonable to conclude that Tyr332 as well as Tyr311 are phosphorylated directly by EGFR, although the Src family kinases efficiently recognize Tyr311 of PKCδ. Importantly, EGFR, expressed in various cells, was revealed to phosphorylate Tyr332 of PKCδ in addition to Lyn rich in the blood cells.

EGFR forms a homodimer on the ligand binding, and the dimerization activates its intrinsic tyrosine kinase activity to autophosphorylate on different residues to recruit the proteins including SH2 or PTB domain and triggers the downstream signalling events (*27*, *28*). EGFR is also tyrosine phosphorylated and activated in the H_2O_2-stimulated cells, but this phosphorylation is proposed to be catalysed by Src rather than by the receptor kinase itself (*23*, *24*, *29*, *30*). Namely, it has been shown that EGFR is phosphorylated by Src at Tyr845 and Tyr1101, located to the activation loop of the catalytic domain and in carboxyl-terminal region, respectively, in the H_2O_2-stimulated cells (*24*, *29*, *30*). Especially, the phosphorylation of the former residue is regarded to stabilize the activation loop of EGFR. Consistently, EGF-induced tyrosine phosphorylation of EGFR was blocked by the inhibitors of AG1478 and PD153035 as mentioned earlier, that compete with ATP at the active site of the enzyme; however, these inhibitors suppressed tyrosine phosphorylation of EGFR just slightly in the H_2O_2-stimulated cells. Therefore, EGFR was confirmed to be phosphorylated by other kinase(s), presumably by the Src family members, under the oxidative stress in a manner independent of the intrinsic protein kinase activity of EGFR. In contrast to EGFR, the tyrosine phosphorylation of PKCδ was not detected significantly after the EGF treatment, whereas the modification was induced in the H_2O_2-stimulated cells as previously reported (*31*, *32*). The tyrosine phosphorylation level of PKCδ in the ligand-treated cells may be much lower than that in the H_2O_2-stimulated cells under the conditions employed. Apparently, EGFR activated in the ligand-treated cells and in the H_2O_2-stimulated cells show that the individual catalytic properties against PKCδ. EGFR molecules are modified at different tyrosine residues in the cells treated with EGF and H_2O_2, respectively (*23*, *24*, *29*, *30*), and thus they may show distinct substrate specificities. Alternatively, PKCδ structurally changed to form a protein complex in the H_2O_2-stimulated cells (*7*) may be a suitable substrate of EGFR.

In this study, the trimeric complex of EGFR, p66Shc and PKCδ was found in the H_2O_2-stimulated cells, but not in the EGF-treated cells, in a manner dependent on the EGFR-mediated Tyr332 phosphorylation of PKCδ. There are two possibilities for the complex formation in activated cells: p66Shc first binds to the tyrosine phosphorylated EGFR, and then PKCδ is phosphorylated to associate with the complex; PKCδ initially associates with p66Shc after tyrosine phosphorylation by EGFR, and then they bind to EGFR through p66Shc. It is thus interesting to assume that PKCδ activated by the phosphorylation at Tyr311 plays a role in the trimeric complex through phosphorylating its substrate proteins. It is known that the association of Shc with EGFR induces activation of the MAP-kinase cascade through recruiting the Grb2/Sos complex to contribute cell survival (*13*), whereas PKCδ is, in contrast, involved in apoptosis, cell cycle arrest and senescence (*33–35*), suggesting that PKCδ may attenuate cell growth by

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inhibiting the MAP-kinase activity. Therefore, we overexpressed the wild type PKCδ as well as the Y311F and Y332F mutants to examine the effect of activated PKCδ on the MAP-kinase cascade upon H_2O_2 stimulation, but no obvious change of the Erk activity was observed in the transfected cells (data not shown). On the other hand, PKC is shown to phosphorylate Ser138 in p66Shc (*36*), and the phosphorylation at this serine residue is proposed to be essential for the adaptor protein for the binding to PTP-PEST, a tyrosine phosphatase, to terminate the MAP-kinase activation. It is interesting to assume that PKCδ in the protein complex recognizes Ser138 of p66Shc. Further studies are required to elucidate the roles of the active PKCδ in the trimeric complex for the regulation of cell survival and death in the stress signaling.

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