Protein Kinase Cζ, which Sensitizes Skin to Sun’s UV Radiation–Induced Cutaneous Damage and Development of Squamous Cell Carcinomas, Associates with Stat3

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Abstract

Chronic exposure to UV radiation (UVR) is the major etiologic factor in the development of human skin cancers including squamous cell carcinoma (SCC). We have shown that protein kinase Cζ (PKCζ), a Ca2+-independent, phospholipid-dependent serine/threonine kinase, is an endogenous photosensitizer. PKCζ is among the six isoforms (α, δ, ε, η, μ, and ζ) expressed in both mouse and human skin. PKCζ transgenic mice, which overexpress PKCζ in the basal epidermal cells and cells of the hair follicle, are highly sensitive to UVR-induced cutaneous damage and development of SCC. We now present that PKCζ-overexpressing, but not PKCδ-overexpressing, transgenic mice, when exposed to a single (4 kJ/m²) or repeated (four doses, 2 kJ/m²/dose, thrice weekly) UVR, emitted by Kodak-cell-filtered FS-40 sun lamps, elicit constitutive phosphorylation of signal transducers and activators of transcription 3 (Stat3) at both Tyr705 and Ser727 residues. UVR-induced phosphorylation of Stat3 accompanied increased expression of Stat3-regulated genes (c-myc, cyclin D1, cdc25A, and COX-2). In reciprocal immunoprecipitation/blotting experiments, phosphorylated Stat3 coimmunoprecipitated with PKCζ. As observed in vivo using PKCζ knockout mice and in vitro in an immunocomplex kinase assay, PKCζ phosphorylated Stat3 at Ser727 residue. These results indicate for the first time that (a) PKCζ is a Stat3Ser727 kinase; (b) PKCζ-mediated phosphorylation of StatSer727 may be essential for transcriptional activity of Stat3; and (c) UVR-induced phosphorylation of Ser727 may be a key component of the mechanism by which PKCζ imparts sensitivity to UVR-induced development of SCC. [Cancer Res 2007;67(3):1385–94]

Introduction

Skin cancer is the most common malignancy in the United States with an expected diagnosis of 1.3 million new cases of skin cancer each year (1). Squamous cell carcinoma (SCC) and basal cell carcinoma are the most common nonmelanoma forms of human skin cancer (2). Basal cell carcinoma is rarely life threatening because it is slow-growing and is mostly localized. SCC, unlike basal cell carcinoma, can invade nearby tissues (2). The first site of metastasis usually is a regional lymph node before metastatic growth in distant sites such as the lung and brain. Whereas mortality due to SCC and basal cell carcinoma is low, they still pose a significant societal risk (2). The most important risk factor for nonmelanoma skin cancer is chronic exposure to UV radiation (UVR) in sunlight (3). The UV spectrum, part of the electromagnetic spectrum, which lies between visible light and X-rays, is divided conventionally into three major categories: UVA (315–400 nm), UVB (280–315 nm), and UVC (190–280 nm; ref. 3). Because stratospheric ozone absorbs most of the radiation below 310 nm, UVA and UVB components of sunlight are the most prominent and ubiquitous carcinogenic wavelengths in our natural environment (3, 4).

UVR signal transduction pathways to the development of SCC involve multiple mechanisms. We have reported that targeted overexpression of protein kinase Cζ (PKCζ) in basal epidermal cells of FVB/N mice sensitizes skin to the development of SCC by UVR (4–9). PKCζ is a family of phospholipid-dependent serine/threonine kinases (8). PKCζ is among the six PKC isoforms (α, δ, ε, η, μ, and ζ) expressed both in human and mouse epidermis (7, 8). PKCζ levels in mouse epidermis correlate to the susceptibility of transgenic mice to SCC development by UVR (4, 9). In addition, constitutive activation of Stat3 is observed in UVR-induced development of either human or mouse SCC (10, 11).

STATs comprise a family of seven [Stat1 (α and β splice isoforms), Stat2, Stat3 (α and β isoforms), Stat4, Stat5a, Stat5b, and Stat6] latent transcription factors that reside in the cytoplasm and are encoded by seven distinct genes (12). STATs are activated through tyrosine phosphorylation by a wide variety of growth factors [e.g., epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)] and cytokines [e.g., interleukin (IL)-6], which act through intrinsic receptor tyrosine kinases (12, 13). Tyrosine phosphorylation enables STAT homodimerization or heterodimerization via reciprocal interactivation between the conserved Src homology 2 domain of one monomer and the phosphorylated tyrosine of the other. The dimerized STATs then localize to the nucleus where they bind specific DNA targets and induce the transcription of specific genes (e.g., c-myc, cyclin D1, cyclin E, cdc25A, Bel-2, and Bel-AL; refs. 12, 13).

STAT activation is linked to cell proliferation, differentiation, apoptosis, embryogenesis, and immune responses (10, 12). STATs exhibit functional divergence in their roles in oncogenesis. Stat3 and Stat5 promote cell survival whereas Stat1 has been associated with growth inhibitory effects (12, 13). Constitutively activated STATs, in particular Stat3, are found in a number of human cancers (e.g., SCCs, head and neck, breast, ovary, prostate, and lung; refs. 10–12, 14–17). Because naturally occurring mutations of Stat3 have not been observed, constitutive activation of Stat3 seems to be mediated by aberrant growth factor signaling (10, 11, 16). The pioneering work of DiGiovanni and his associates of the role of EGF receptor (EGFR)–mediated Stat3 activation in skin carcinogenesis is noteworthy (10, 17–19). In their findings, activation of STATs (Stat1, Stat3, and Stat5) is an essential component in the
mechanism of mouse skin tumor promotion by diverse tumor promoters. Tumor promoter–induced activation of Stat3 is mediated by EGFR. Furthermore, Stat3 is constitutively activated in both skin papillomas and carcinomas (10, 18). Disruption of Stat3 prevents development of skin tumors elicited by 7,12-dimethylbenz(a)anthracene initiation and 12-O-tetradecanoylphorbol-13-acetate (TPA) promotion (17).

Stat3 has two conserved amino acid (Tyr705 and Ser727) residues that are phosphorylated during Stat3 activation (10, 12). The relative contributions of Stat3 Tyr705 and Stat3Ser727 phosphorylation in UVR-induced Stat3 transcriptional activity and SCC development are unknown. Furthermore, the kinase responsible for Stat3Ser727 phosphorylation in mouse keratinocytes is unknown. We now present in this communication that PKCε associates with Stat3, and PKCε is a Stat3Ser727 kinase. PKCε activation may be an initial signal in UVR-induced constitutive activation of Stat3. PKCε may cross-talk with Stat3 to mediate signals that impart sensitivity to UVR-induced development of SCC.

Materials and Methods

Generation of PKCε transgenic mice. PKCε transgenic mice were generated as previously described (7). Transgenic mice were maintained by mating hemizygous transgenic mice with wild-type FVB/N mice. The mice were housed in groups of two to three in plastic bottom cages in light-, humidity-, and temperature-controlled rooms; food and water were available ad libitum. The animals were kept in a normal rhythm of 12-h light and 12-h dark periods. The transgene was detected by PCR analysis using genomic DNA isolated from 1-cm tail clips (4, 9). The PKCε knockout mice were generated with the LacZNeo cassette by interrupting the coding region of mouse PKCε cDNA. The genetic background used was 129/Ola and C57/BL6 strains. These mice (C57/BL6/129/Ola) were bred for eight generations for mutant transmission to FVB/N for a unified genetic background.

UVR treatment. The UVR source was Kodakel-filtered FS-40 sunlamps (~60% UVB and 40% UVA). Mice were exposed to UVR from a bank of six Kodakel-filtered sunlamps. UVR dose was routinely measured using a UV radiometer. Mice were used for experimentation beginning at 7 to 9 weeks of age. The dorsal skin of the mice was shaved 3 to 4 days before experimentation. Mice were exposed to UVR as indicated in each experiment.

Histology. Mouse skin was excised promptly after euthanasia, placed immediately in 10% neutral buffered formalin, fixed for 1 h in formalin, then transferred to PBS (pH 7.4) and embedded in paraffin. Skin sections of 4-μm thickness were cut for immunohistochemical study.

Localization of PKCε and Stat3 by immunofluorescence staining. Paraffin-fixed skin samples from mice were used to determine nuclear cotranslocation of PKCε and Stat3. Sections (4-μm thick) were cut for PKCε and Stat3 staining. After antigen retrieval by incubating samples at 95°C in Tris-urea solution (pH 9.5) for 30 min, the tissue slides were incubated with normal donkey serum (1:10 dilution) for 15 min to block nonspecific binding of the antibodies. Subsequently, the slides were incubated overnight with a mixture of PKCε (goat polyclonal, 1:50 dilution) and Stat3 (rabbit polyclonal, 1:50 dilution) primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) in a humidified chamber. The mixture of antibodies was decanted and the slides were washed thrice in TBS (pH 7.4). The slides were incubated with a mixture of two secondary antibodies, which were raised in two different species and conjugated with two different fluorochromes [donkey anti-goat immunoglobulin (IgG)-FITC for PKCε and donkey anti-rabbit IgG-rhodamine for Stat3; Santa Cruz Biotechnology] for 30 min at room temperature in the dark. The solution of secondary antibodies was decanted and the slides were washed thrice with TBS for 5 min each in the dark. Finally, the slides were mounted with coverslips using a drop of mounting medium and the coverslips were sealed with nail polish to prevent drying and movement under the microscope. All sections were examined with an Olympus Microscope attached with fluorescence detector.

Western blot analysis. Mice were shaved and deplated 24 h before experimentation. The mouse skin was excised and scraped to remove the s.c. fat. The epidermis was removed and homogenized in immunoprecipitation lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl2, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 200 mmol/L Na3VO4, 200 mmol/L NaF, and 1 mmol/L EGTA]. The homogenate was centrifuged at 14,000 × g for 30 min at 4°C. Whole-cell lysate (25–35 μg) was fractionated on 10% or 15% SDS-polyacrylamide gels. The proteins were transferred to Hybond-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham, Piscataway, NJ). The membrane was then incubated with indicated antibodies followed by a horseradish peroxidase–conjugated secondary antibody, and the detection of the signal was developed with Amersham enhanced chemiluminescence reagent and autoradiography using BioMax film (Kodak Co., Rochester, NY). The quantitations of Western blots signals were estimated by densitometric analysis using Totallab Nonlinear Dynamic Image analysis software (Nonlinear USA, Inc., Durham, NC).

PKCε immunocomplex kinase assay. The dorsal skin of the mouse was removed and the epidermis was scraped off on ice with a razor. The epidermis was placed in 0.5 mL of immunoprecipitation lysis buffer, homogenized using a glass Teflon tissue homogenizer, aged for 30 min at 4°C, and centrifuged at 14,000 rpm in a microcentrifuge for 15 min at 4°C. The clear supernatant was used for immunoprecipitation with polyclonal antibody to PKCε or Stat3. Briefly, the lysate was preadsorbed with 5 μL of protein A/G-agarose for 10 min at 4°C. Five micrograms of PKCε or Stat3 antibody and 10 μL of protein A/G-agarose were added to the lysate, and the volume of the lysate was adjusted to 1 mL with lysis buffer. The mixture was incubated for 2 to 4 h at 4°C with agitation. The immunoprecipitate was pelleted at 8,000 rpm in a microcentrifuge, washed, and resuspended in 300 μL of assay buffer [50 mmol/L Tris (pH 7.4), 5 mmol/L EDTA (pH 8.0), 10 mmol/L EGTA (pH 7.9), 0.3% β-mercaptoethanol, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 50 μg/mL PMSF]. Twenty-five microliters of the PKCε immunoprecipitate were assayed in kinase buffer containing 50 mmol/L Tris (pH 7.4), 8 mmol/L MgCl2, 0.136 mmol/L ATP or 0.136 mmol/L [γ-32P]ATP, 3 mmol/L DTT, 34 μg/mL l-α-phosphatidylserine, 3 μg/mL TPA, 1 mmol/L EGTA, and 25 μL immunoprecipitated Stat3. The reaction mixture was incubated at 37°C for 15 min and then immunoprecipitated with Stat3 antibody. The immunoprecipitated samples were then either (a) boiled in SDS sample buffer and separated by 10% SDS-PAGE for the Western blot analysis for the presence of pStat3Ser727 or (b) counted for radioactivity associated with Stat3.

Results

Targeted overexpression of PKCε in mouse epidermis increases sensitivity of skin to UVR treatment for the phosphorylation of Stat3 and the expression of Stat3-regulated genes. A possibility was explored whether PKCε level may be correlated with UVR-induced phosphorylation of Stat3 and expression of Stat3-regulated genes. In these experiments (Figs. 1–3), mice received a single (4 kJ/m2) or repeated (2 kJ/m2, four times, thrice weekly) UVR treatments. Mice were sacrificed at 1, 3, 6, 12, 18, 24, and 96 h post single treatment or 1 and 3 h post multiple UVR treatments. In wild-type mice, both single and multiple UVR treatments elicited rapid and transient increase in the level of phosphorylated Stat3 at both Tyr705 (Fig. 1A and B) and Ser727 (Fig. 1C and D) residues. PKCε overexpression led to UVR-induced constitutive phosphorylation of Stat3 at both Tyr705 and Ser727 residues whereas UVR treatment barely affected the expression of either form of phosphorylated Stat3 in PKCε-overexpressing transgenic mice (Fig. 1A–D).

The effect of UVR treatment on the Stat3 activation was also determined by immunohistochemical analysis of paraffin-fixed skin specimens. PKCε-overexpressing transgenic mouse lines (TG224
and TG215) elicited a dramatic increase in nuclear staining of Stat3 after UVR treatment (Fig. 2A–C). The extent of Stat3 nuclear staining seems to be proportional to the level of expression of PKCε in transgenic mouse lines (Fig. 2D). On the contrary, deletion of PKCε in mice inhibited UVR-induced nuclear staining of Stat3 (Fig. 2D). In accord with the previous finding (11), intense nuclear staining of Stat3 was observed in both mouse and human SCC specimens (Fig. 2C). Normal human skin scored minimal for nuclear staining for Stat3 (Fig. 2C and D). Inclusion of Stat3 blocking peptide before immunostaining of human SCC specimens completely prevented Stat3 staining, indicating that Stat3 immunostaining was specific (Fig. 2C).

We also determined the level of expression of Stat3-regulated genes after chronic UVR exposures of wild-type and PKCε and PKCδ transgenic mice. The results are illustrated in Fig. 3. The level of expression of cyclooxygenase-2 (COX-2) protein was consistently increased in both PKCε transgenic mouse lines. UVR-induced COX-2 expression seems to be proportional to the level of expression of a Stat3-regulated gene.

**Figure 1.** UVR treatment leads to constitutive phosphorylation of Stat3 at both Tyr705 and Ser727 residues. PKCε transgenic mice (line 215) and their wild-type littermates (four mice per group) were exposed to a single UVR dose (4 kJ/m²). The mice were sacrificed at 1, 3, 6, 12, 18, 24, and 96 h after UVR exposure. In a parallel experiment, PKCε (line 215 and 224), PKCδ transgenic mice (50), and their wild-type littermates (four mice per group) were exposed to chronic UVR exposure [2 kJ/m², four times (Monday, Wednesday, Friday, and Monday)]. The mice were sacrificed at 1 and 3 h after the fourth treatment of UVR. The mouse epidermis was scraped off and total lysates were prepared. The epidermal extract (25 μg protein) was subjected to SDS-PAGE followed by immunoblot analysis with pStat3Tyr705 (A), pStat3Ser727 (C), and whole Stat3 (A) antibodies from Cell Signaling Technology, Inc. (Beverly, MA). Equal loading was confirmed by stripping the blot and reprobing it for β-actin. The quantification of proteins (normalized to β-actin; B and D) was done by densitometry analysis using Total Lab Nonlinear Dynamics Image Analysis Software (Nonlinear USA). Y axis, relative numbers. Columns, mean of Western blots of epidermal extracts from four different mice; bars, SE. Shown is the Western blot of pooled epidermal extracts from four mice. Similar results were obtained in a repeat experiment.
PKC\(\text{q}\) expression in transgenic mice (Fig. 3A and B). However, UVR-induced increases in the expression level of COX-1 were not dramatic in the PKC\(\text{q}\)-overexpressing transgenic mice (Fig. 3A and B). In the high expressing PKC\(\text{q}\) line 215, as compared with wild-type mice, there was a major increase in the levels of cyclin D1, c-myc, and cdc25A after multiple UVR exposures (Fig. 3A and B). Multiple UVR exposures also slightly increased the expression levels of cyclin D1, c-myc, and cdc25A in PKC\(\text{y}\) transgenic mice, implying lack of PKC isoform specificity in the regulation of the expression of these genes (Fig. 3A and B).

PKC\(\text{E}\) associates with Stat3. To determine whether there is a direct interaction between PKC\(\text{q}\) and Stat3, reciprocal immunoprecipitation/blotting experiments were done. In these experiments (Fig. 4), the same epidermal protein extracts, prepared from the previous experiments illustrated in Figs. 1–3, were used. The epidermal protein extract was immunoprecipitated with antibodies against PKC\(\text{q}\), PKC\(\text{E}\), Stat3, pStat3Tyr705, or pStat3Ser727. The immunoprecipitates were subjected to immunoblot analysis with antibodies against PKC\(\text{q}\), PKC\(\text{E}\), Stat3, pStat3Tyr705, or pStat3Ser727. As shown in Fig. 4A, PKC\(\text{q}\) coimmunoprecipitated with Stat3, pStat3Tyr705, and pStat3Ser727. In the reciprocal immunoprecipitation/blotting experiments, Stat3, pStat3Tyr705, and pStat3Ser727 coimmunoprecipitated with PKC\(\text{q}\) (Fig. 4A). As shown in Fig. 4B, the inclusion of blocking (neutralizing) peptide in the immunoprecipitation experiments inhibited the coimmunoprecipitation of PKC\(\text{q}\) and Stat3, providing straightforward evidence for the protein-protein interactions of PKC\(\text{q}\) and Stat3. Every immunoprecipitation experiment included a control that contained no primary antibody but preimmune rabbit serum. Neither PKC\(\text{q}\) nor Stat3 was ever pulled down with the preimmune serum. Furthermore, the immunoprecipitation experiments were repeated with both polyclonal and monoclonal antibodies and polyclonal antibody from different commercial suppliers. Irrespective of the source of the antibody, the results were identical.

We further compared PKC\(\text{q}\) and PKC\(\text{E}\) for their ability to associate with Stat3. In this experiment, epidermal extracts prepared from UVR-treated PKC\(\text{q}\) transgenic and wild-type mice were subjected to reciprocal immunoprecipitation/blotting analyses. As shown in Fig. 4C (lanes 1 and 3), the association of PKC\(\text{E}\) with Stat3, pStat3Tyr705, or pStat3Ser727 was barely detectable in the epidermal protein extract prepared from either wild-type or PKC\(\text{q}\) transgenic mice. In contrast, the association of Stat3,
pStat3Tyr705, and pStat3Ser727 with PKCe was strongly increased in epidermal extracts prepared from both wild-type and PKCe transgenic mice (Fig. 4C, lanes 2 and 4).

**Colocalization of PKCe and Stat3.** To confirm the association of PKCe with Stat3, we determined the colocalization of PKCe and Stat3 by double immunofluorescence staining. In this experiment (Fig. 4), 4-μm-thick sections from paraffin-fixed skin samples of UVR-exposed PKCe transgenic mice were used. PKCe and Stat3 localization is indicated by the presence of green and red fluorescence, respectively. The yellow fluorescence indicates colocalization and association of PKCe and Stat3. A few Stat3-positive cells are seen in the untreated skin section. UVR treatment increased nuclear staining (red) of Stat3 (Fig. 4D). PKCe-positive cells (green) are seen in both untreated and UVR-treated skin sections. PKCe staining (green), which is seen in both basal and suprabasal keratinocytes, is predominantly cytoplasmic (Fig. 4D, positive cells pointed by a). A yellow fluorescence indicative of association of PKCe and Stat3 is observed only in UVR-exposed skin sections (Fig. 4, positive cells pointed by b).

PKCe phosphorylates Stat3 at Ser727 residue. PKCe overexpression results in an increase in UVR-induced phosphorylation of Stat3 at the Ser727 residue (Fig. 1) PKCe associates with Stat3 (Fig. 4). These two pieces of evidence prompted us to explore that Stat3 may be a PKCe substrate. To determine that PKCe may directly phosphorylate Stat3, we did both *in vivo* and *in vitro* experiments (Fig. 5A–D). The *in vivo* experiment was done with the PKCe knockout mice. The PKCe knockout mice were generated with the LacZNeo cassette by interrupting the initiating codon. The genetic background used was 129/Ola and C57/BL6 strains. These mice (C57/BL6/129/Ola) were bred for eight generations for mutant transmission to FVB/N for a unified genetic background. Immunoblot analysis of epidermal extract from the dorsal skin indicated a lack of PKCe protein in the PKCe knockout FVB/N mouse epidermis, whereas the heterozygote mice contained less protein than wild-type mice (Fig. 5A). The wild-type, PKCe heterozygous, and PKCe knockout mice were exposed to UVR. The level of expression of pStat3Ser727 was analyzed at 3 h post UVR exposure. Clearly, deletion of PKCe attenuated UVR-induced phosphorylation of Stat3 at the Ser727 residue (Fig. 5A and B). However, the level of expression of total Stat3 remained unaltered in the PKCe knockout mice (Fig. 5A and B).

To determine whether PKCe was directly involved in the phosphorylation of Stat3 at Ser727, we performed immunocomplex kinase assays. In this *in vitro* kinase assay experiment (Fig. 5C and D), both the enzyme (PKCe) and the substrate (Stat3) were immunopurified from PKCe transgenic mouse (line 215) epidermal protein extracts by using polyclonal PKCe and Stat3 antibodies, respectively. The immunopurified PKCe and Stat3 proteins were reconstituted in the kinase assay buffer and the protein kinase assays were then done. The level of pStat3Ser727 was determined both by immunoblot analysis (Fig. 5C) and by scintillation counting the incorporation of γ-32P into Stat3 (Fig. 5D). The PKCe activation

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**Figure 3.** UVR treatment leads to increased expression of Stat3-regulated genes. As described in the legend to Fig. 1, epidermal extract prepared after chronic UVR exposures was used to analyze cyclin D, c-myc, cdc25A, and COX-2 expression (A). The quantification of proteins (normalized to β-actin) was done by densitometric analysis using TotalLab Nonlinear Dynamics Image Analysis Software (Nonlinear USA). Y-axis, arbitrary numbers. Columns, mean of Western blots of epidermal extracts from four different mice; bars, SE. Shown is the Western blot of pooled epidermal extracts from four mice. Similar results were obtained in a repeat experiment.

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**Figure 4.** Colocalization of PKCe and Stat3 in epidermal extracts. (A) Western blot analysis of PKCe and Stat3 expression in epidermal extracts from wild-type (WT) and PKCe transgenic (PKCe) mice. The quantification of proteins (normalized to β-actin) was done by densitometric analysis using TotalLab Nonlinear Dynamics Image Analysis Software (Nonlinear USA). Y-axis, arbitrary numbers. Columns, mean of Western blots of epidermal extracts from four different mice; bars, SE. Shown is the Western blot of pooled epidermal extracts from four mice. Similar results were obtained in a repeat experiment.
resulted in a dramatic increase in the phosphorylation of Stat3Ser727 (Fig. 5C and D). In contrast, Stat3Ser727 phosphorylation was very low when either L-a-phosphatidyl-L-serine/TPA or Stat3 was omitted from the kinase assay reaction (Fig. 5C and D). Similar results were obtained in two sets of independent experiments. These results clearly indicated that PKCq is a StatSer727 kinase (Fig. 5C and D).

**Discussion**

Chronic exposure to the sun's UVR is linked to the development of human SCC, a metastatic nonmelanoma skin cancer (3). We found, using a novel PKCq transgenic mouse model, that the PKCq level in epidermis dictates the susceptibility of transgenic mice to the induction of SCC by UVR (4, 9). The PKCq transgenic mice, when exposed to UVR (2 kJ/m² thrice weekly), elicited 3-fold increased SCC multiplicity and decreased tumor latency by 12 weeks. PKCq overexpression in mice suppressed UVR-induced sunburn (apoptotic) cell formation and enhanced both UVR-induced levels of specific cytokines and hyperplasia [tumor necrosis factor α (TNFα), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor, and IL-6], implying inhibition of apoptosis and promotion of survival of preneoplastic cells (4, 9, 20). We now present that PKCq may impart sensitivity to UVR carcinogenesis via its association with Stat3, the transcriptional factor that is constitutively activated in both mouse and human SCC (10, 11).
PKCq overexpression, but not PKCq overexpression, in mouse epidermis stimulated UVR-induced phosphorylation of Stat3 at both Tyr705 and Ser727 residues (Figs. 1 and 2). The transcriptional activity of Stat3 involves its dimerization, nuclear translocation, DNA binding and recruitment of transcriptional activators (12, 13). Tyrosine phosphorylation of STATs (Tyr705 in Stat3), as mediated by a wide variety of polypeptides, has been shown to be essential for STAT dimerization and nuclear translocation (12, 13). Stat1, Stat3, and Stat4 share a consensus motif between 720 and 730 in the COOH-terminal transactivation domain in which the serine (Ser727 in Stat3) residue is the target for phosphorylation (21–24). Evidence indicates that cooperation of both tyrosine and serine phosphorylation is necessary for full activation of Stat3 (22). Ser727 phosphorylation of Stat3 is required for transactivation by association with cyclic AMP (cAMP)-responsive element binding protein (CREB)-binding protein/p300 (24). The constitutive phosphorylation of Stat3 at both Tyr705 and Ser727 residues may be essential components of the mechanism by which PKCq mediates sensitivity to UVR carcinogenesis (4, 9). The up-regulation of cell survival genes (e.g., c-myc, cyclinD1, COX-2, and cdc25A) by UVR treatment of PKCq transgenic mice further adds strength to the conclusion that PKCq activation may promote proliferation of UVR-initiated keratinocytes.

The mechanism by which PKCq may enhance UVR-induced phosphorylation of Stat3 at the tyrosine residue is not yet defined. STATs are activated through tyrosine phosphorylation by a wide variety of growth factors (e.g., EGF and PDGF) and cytokines (e.g., IL-6), which act through intrinsic receptor tyrosine kinases (6, 12, 13). It remains to be determined whether PKCq-mediated induction of growth factors (e.g., TNFα and EGF; refs. 4, 9, 21), via either an autocrine or a paracrine mechanism, influences the phosphorylation of tyrosine residue of Stat3 (Fig. 6).

Depending on the cellular context, Stat3 has been shown to be a substrate for several protein kinases (25–27). Members of the mitogen-activated protein kinase and e-jun NH2-terminal kinase family of serine kinases are shown to mediate Stat3Ser727 phosphorylation (27). Our results indicate that PKCq, which interacts with Stat3 (Fig. 4), is a Stat3Ser727 kinase in mouse keratinocytes (Fig. 5). Our conclusion is further strengthened by the fact that (a) PKCq-deficient mutant mice failed to elicit UVR-induced phosphorylation of Stat3Ser727 and, (b) in immunocomplex kinase assays, PKCq directly phosphorylated Stat3Ser727 (Fig. 5).

The mechanism by which PKCq may associate and mediate the phosphorylation of Stat3Ser727 is unclear. A few motifs in the signal-transducing proteins are known to activate Stat3. For example, extracellular signal–regulated kinase has been reported to be involved in Stat3Ser727 phosphorylation through the YSTV docking sites for Stat3 (25). IFN-γ receptor was shown to cause phosphorylation on Ser727 of Stat3 through its YDKP docking motif. The YXXQ motif in gp130 is also important for serine phosphorylation of Stat3 (25). It is notable that mouse PKCq has three repeats of the YXXQ motif (regions 176–179, 199–202, and 468–471). Two of the motifs occur in the TPA-binding region and the third in the ATP-binding domain. Any one of these YXXQ motifs present in PKCq may bind and facilitate the serine phosphorylation of Stat3.

Figure 5. PKCq is a Stat3Ser727 kinase. PKCq depletion inhibits phosphorylation of Stat3Ser727 (A). PKCq knockout (eKO), PKCq heterozygous (eHet), and wild-type mice were exposed to UVR four times (2 kJ/m2 per exposure on Monday, Wednesday, Friday, and Monday). Mice were sacrificed 3 h post last UVR exposure and the epidermal extracts were prepared as described in Materials and Methods. The protein extracts were analyzed for the expression levels of pStat3Ser727 and whole Stat3 by immunoblot analysis (A and B). A, lanes 1, 3, and 5, unexposed mice; lanes 2, 4, and 6, UVR-exposed mice. C and D, immunocomplex kinase assay. The dorsal skin of the PKCq transgenic mice was shaved and depilated 24 h before experimentation. The mice were euthanized, the dorsal skin was removed, and the epidermis was scrapped off on ice with a razor. PKCq assay was done using a kit (Protein Kinase C enzyme blotassay system) from Amersham Biosciences following the manufacturer’s protocol. The epidermis was placed in 0.5 mL of immunoprecipitation lysis buffer for immunoprecipitation with either PKCq or Stat3 antibodies. The immunoprecipitate was pelleted at 8,000 rpm in a microcentrifuge, washed, and resuspended in 300 μL of assay buffer [50 mmol/L Tris (pH 7.4), 5 mmol/L EDTA (pH 8.0), 10 mmol/L EGTA (pH 7.9), 0.3% i-mercaptoethanol, 5 μg/mL aprotenin, 5 μg/mL leupeptin, and 50 μg/mL phenylmethylsulfonyl fluoride]. Twenty-five microliters of the immunoprecipitate were assayed in kinase buffer containing 50 mmol/L Tris (pH 7.4), 8 mmol/L MgCl2, 0.136 mmol/L ATP with or without [γ-32P]ATP, 3 mmol/L DTT, with or without 34 μmol/L of l-ω-phosphatidyl-L-serine, 3 μg/mL TPA, and 1 mmol/L EGTA, and with or without 25 μL of Stat3. The PKCq assays were run in the absence of added l-ω-phosphatidyl-L-serine (PS)/TPA, Stat3, PKCq, and ATP. The reaction was incubated at 37°C for 15 min. The reaction mixture was immunoprecipitated with Stat3Ser727 antibody. The phosphorylated level of Stat3Ser727 was determined either by Western blot analysis (C) or by scintillation of radioactivity associated with 32P-Stat3Ser727 (D). No PKCq activity was detected in the absence of added ATP.
The results indicate that PKC\(\gamma\)-mediated Stat3Ser727 phosphorylation may be an important component of the mechanism by which PKC\(\gamma\) imparts sensitivity to UVR-induced development of SCC. The role of Stat3Ser727 phosphorylation in UVR-induced activation of Stat3 transcriptional activity can be explored using Stat3Ser727Ala knock-in mice. There are two reports explaining the generation of genetically engineered Stat (Stat1 and Stat3) serine-mutant knock-in mice (28, 29). Both strains of mice with knock-in mutations are viable, normal, and fertile (28, 29). Varinou et al. (28) showed, using a Stat1Ser727 to alanine knock-in mouse, that phosphorylation of the Stat1 transactivation domain is required for Stat1 regulated transcriptional activity. Similarly, Shen et al. (29) have shown, using knock-in mouse models, that Stat3Ser727 plays an essential role in postnatal survival and growth.

The results (Fig. 4) of the physical interaction of PKC\(\gamma\) with Stat3 also raise a possibility that PKC\(\gamma\) may be a transcriptional coactivator of Stat3. The molecular basis of gene activation of DNA binding transcriptional factors involves the recruitment of different coactivator complexes. Activation of transcription requires the recruitment of phosphorylated coactivators to facilitate access of the transcriptional machinery to the DNA template. Thus far, many reports are available showing that transcriptional activation of Stat3 depends on its interaction with different coactivators (such as CREB-binding protein/p300, the nuclear receptor binding protein, and NcoA/SRC1; refs. 30–32). Stat3 can regulate different genes such as \(p21^{\mathrm{waf1}}\), \(c\)-myc, cyclic D1, and Bcl-2 (33–35). It remains to be determined whether PKC\(\gamma\) is a novel coactivator of Stat3.

In summary, PKC\(\gamma\) is linked to the development of SCC by UVR in PKC\(\gamma\) transgenic mice (4, 9). PKC\(\gamma\) signals the induction of certain cytokines (e.g., TNF\(_{\alpha}\)), which may play an important role in the development of UVR-induced SCC (4, 9). The PKC\(\gamma\)-overexpressing transgenic mice, when exposed to either a single or...
repeated UV dose, elicited constitutive phosphorylation of Stat3 at both Tyr705 and Ser727 (Fig. 1). UVR-induced constitutive phosphorylation of Stat3 accompanied an increased expression of a number of Stat3-regulated genes such as c-myc, cyclin D1, cdc25A, and COX-2 (Fig. 3). Mouse epidermal Stat3 common immunoprecipitated with PKCδ and PKCε phosphorylated Stat3Ser727 (Figs. 4 and 5). These observations have led us to suggest that in intact skin in vivo, (a) the phosphorylation of Ser727 is essential for maximum transcriptional activity of Stat3; (b) UVR-induced phosphorylation of Ser727 is a key component by which PKCε imparts sensitivity to UVR-induced development of SCC; (c) Stat3 is a substrate for PKCδ; and (d) PKCε is a transcriptional coactivator. The PKCε transgenic mice provide a unique model to investigate human SCC (4, 9, 21, 36–38). Histologically, SCC in PKCε transgenic mice, like human SCC, is poorly differentiated and is metastatic (37). PKCε, a novel calcium-independent PKC isoform (37, 39–42), has been shown to be a transforming oncogene (43–45), a predictive biomarker of breast cancer (46) and prostate cancer (15, 45). Stat3 is linked to the development of wide variety of human cancers (14–16, 47–49). Our findings are the first report to show the association of PKCε with Stat3 and its activation on UV treatment. In conclusion, the available evidence, including the results presented here, indicates that PKCε and Stat3 may be potential molecular targets for human cancer prevention and treatment.

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Protein Kinase Cε, which Sensitizes Skin to Sun's UV Radiation–Induced Cutaneous Damage and Development of Squamous Cell Carcinomas, Associates with Stat3

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