[CANCER RESEARCH 63, 4692–4697, August 1, 2003]

Loss of Protein Kinase Cα Expression May Enhance the Tumorigenic Potential of Gli1 in Basal Cell Carcinoma[1]

Graham W. Neill,[2] Lucy R Ghali, Judith L. Green,[3] Mohammed S. Ikram, Michael P. Philpott, and Anthony G. Quinn[4]

Centre for Cutaneous Research, Barts and the London, Queen Mary's School of Medicine and Dentistry, University of London, London E1 2AT, United Kingdom

ABSTRACT

Activation of the Sonic hedgehog signaling pathway, primarily through mutational inactivation of the *PTCH1* gene, is associated with the development of basal cell carcinoma (BCC). Gli1, a member of the Gli family of transcription factors, is expressed in BCC and in transgenic mice targeted expression of Gli1 in basal keratinocytes leads to BCC development. In addition to BCC, previous studies have shown that Gli1 is expressed in the outer root sheath (ORS) of the hair follicle but is absent in interfollicular epidermis. In this study, we have characterized the expression pattern of two protein kinase C (PKC) isoforms expressed in BCC and hair follicles. We have then used reporter assays to investigate the effects of these isoforms on Gli1 transcriptional activity. We report that in BCC sections, PKCα but not PKCδ was weakly expressed in the epidermis, whereas in the hair follicle, PKCα was expressed in the ORS and PKCδ in the inner root sheath. In contrast, neither PKCα nor PKCδ was expressed in BCC tumor islands, although both isoforms were often expressed in the surrounding stroma. In mammalian 293T cells, coexpression of constitutively active PKCα reduced the activity of Gli1 in a dose-dependent manner, whereas constitutively active PKCδ increased the activity of Gli1, although this required higher expression levels. Regulation of mutant Gli1 protein localized exclusively to the nucleus was similar to that of the wild-type protein, indicating that nuclear-cytoplasmic shuttling is not a determinant of Gli1 control by either PKC isoform. Furthermore, PKC regulation of Gli1 did not involve activation of mitogen-activated protein kinase signaling. Finally, we show that exogenous Gli1 does not alter the expression of PKCα in human primary keratinocytes, suggesting that loss of this isoform in BCC is not via Hedgehog signaling. As BCCs have been proposed to originate from the ORS, loss of PKCα expression may be relevant to tumor formation; this may, in part, be because of the predicted increase in Gli1 transcriptional activity.

INTRODUCTION

The Hh[5] signaling pathway has been implicated in the regulation of cell patterning and the cell cycle and plays a critical role in proliferation, survival, and growth during embryonic development and in the growth, differentiation, and maintenance of a number of tissues in the mature organism. Much of what is known about the components and regulation of Hh signaling was first elucidated by elegant genetic studies of embryonic segmentation and imaginal disk specification in *Drosophila*, and it is now well established that many of the key elements of the pathway are conserved from invertebrates to vertebrates. In *Drosophila*, in the absence of Hh, signal transduction is repressed by the transmembrane receptor Ptc; this results in the transcription factor Ci being subject to PKA-dependent proteolytic cleavage generating a NH_2-terminal repressor molecule. Conversely, the presence of Hh negates Ptc repression such that Ci is not cleaved and the full-length protein is released to the nucleus where it functions as a transcriptional activator (reviewed in Refs. 1, 2).

Vertebrates differ from *Drosophila* in that there are multiple homologues of a number of components of the pathway. Although this adds to the complexity, the basic principles of the signaling events appear to be maintained, and there is evidence that mammalian homologues can substitute for specific activities when expressed in Drosophila (3). Three mammalian *Hh* genes have been identified: *SHh*, *DHh*, and *IHh* (reviewed in Ref. 4). The mammalian homologues of Ci comprise members of the Gli family of transcription factors that are characterized by five zinc fingers originally described in *Xenopus* transcription factor III A, these are Gli1, Gli2, and Gli3 (5). Several studies have implicated functional redundancy between Gli proteins and, unlike Ci/Gli2/Gli3, Gli1 does not possess a NH_2-terminal repressor domain (reviewed in Ref. 6).

In adult tissues, inappropriate activation of the Hh pathway has been linked to the development of a number of tumor types, including BCC (7). This is primarily because of mutational inactivation of the *PTCH1* tumor suppressor gene, which is proposed to result in increased Gli1 expression (8–11). Also, with regard to medulloblastoma, a recent study reported mutations in *SUFU* resulting in proteins that are unable to export Gli1 from the nucleus (12).

The mechanisms regulating Gli1 in mammalian cells stem mainly by analogy to components identified from studies of Ci regulation in *Drosophila*. PKA and Su(fu) both negatively control Gli1 activity, whereas Fu enhances activity. Su(fu) and Fu regulate Gli1 by influencing its subcellular localization and, in the case of the former, possibly by altering Gli1 transcriptional potential once bound to DNA. The mechanism of PKA regulation has not been determined, but it may influence Gli1 binding to DNA (13–17). The importance of input, however, from other major signal transduction pathways in regulating Hh signaling and Gli1 activity is not known. Recent evidence has shown that Ci is subject to phosphorylation and regulation by CK (18). Analysis of the Gli1 coding sequence reveals multiple CK consensus phosphorylation sites suggesting that it might also be regulated by CK in mammalian cells. Similarly, Gli1 contains multiple PKC consensus phosphorylation sites, indicating that it may also be subject to regulation by this enzyme. In human skin, Gli1 is expressed in the ORS of hair follicles and in BCC but not in interfolicular epidermis (19) Moreover, several PKC isoforms are expressed in human skin (20), and in particular, PKCα is reported in the ORS of murine hair follicles (21). This may be important because the ORS is thought to be a potential source of BCC.

In this study, we have investigated the pattern of PKCα and PKCδ in BCC sections and associated hair follicles. We have also investigated the effects of both isoforms on Gli1 reporter activity. We report that PKCα is expressed in the epidermis and ORS of human hair

Received 2/20/03; accepted 5/20/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Medical Research Council (to G. W. N., M. S. I., A. G. Q.) and by Cancer Research UK (to L. R. G., J. L. G., A. G. Q.).

[2] To whom requests for reprints should be addressed, at Centre for Cutaneous Research, Barts and the London, Queen Mary's School of Medicine and Dentistry, University of London, 2 Newark Street, Whitechapel, London E1 2AT, United Kingdom. Phone: 44-207-882-7175; Fax: 44-207-882-7171; E-mail: g.w.neill@qmul.ac.uk.

[3] Present address: Division of Parasitology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom.

[4] Present Address: R&D Charnwood, Astrazeneca, Bakewell Road, Loughborough LE11 5RH, United Kingdom.

[5] The abbreviations used are: Hh, hedgehog; Ptc, Patched; Ci, Cubitus interruptus; PKA, protein kinase A; PKC, protein kinase C; SHh, Sonic Hedgehog; DHh, Desert Hedgehog; IHh, Indian Hedgehog; Gli, glioma transcription factor; BCC, basal cell carcinoma; Su(fu), suppressor of fused; Fu, Fused; CK, casein kinase; ORS, outer root sheath; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; mNES, mutant nuclear export sequence.

follicles but is down-regulated in BCC. Furthermore, we show that PKCα is a potent negative regulator of Gli1 transcriptional activity and, as it has been proposed that BCC may originate from the ORS of hair follicles, suggests that loss of PKCα is important to BCC formation.

MATERIALS AND METHODS

Immunohistochemistry. BCC tissue samples were obtained and sections prepared as described previously (19). Primary mouse monoclonal antibodies to detect PKCα and PKCδ were obtained commercially (Transduction Laboratories) and used at a concentration of 1:100. For antigen retrieval, sections were microwave treated under pressure for 4 min at 900 W in preheated citrate buffer (pH 6.0). Sections were then blocked with horse serum to reduce nonspecific binding and subsequently incubated for 90 min at room temperature with primary antibody. Secondary biotinylated antibody (Vectastain Universal Elite ABC kit; Vector Laboratories, Peterborough, United Kingdom) was then applied for 30 min at room temperature, followed by avidin biotin peroxidase complex for 20 min. Visualization was made using 3,3′-diaminobenzidine, then sections were washed in Tris-buffered saline and mounted using Immunomount (Thermo Shandon).

Cell Culture and Retroviral Production. Mammalian fetal kidney epithelial 293T cells were maintained in DMEM supplemented with 10% FCS. Human primary keratinocytes were isolated as described previously (22) and stored in liquid nitrogen before seeding. Primary keratinocytes were cultured in Defined Serum-free Medium in the presence of growth supplement (Invitrogen). Retroviral particles encoding Gli1 and EGFP were produced using the Phoenix packaging line as described previously (23).

Plasmid Construction. Construction of pI2EA-Gli1 (derived from pIRES2-EGFP; Clontech) and SIN-Gli1-EGFP has been described previously (23). To create pI2EA-Gli1mNES, three leucine residues were mutated to valine (L498V, L501V, and L503V) with pI2EA-Gli1 as template using the Site-Directed Mutagenesis kit (Stratagene). The luciferase reporter was created by subcloning 6xGli1 binding sites from the *Bgl*II site of pA10CAT6GBS (24) into the same site of pGL3-Promoter (Promega) creating pGL3P-6GBS.

Transient Transfections and Reporter Analysis. A total of 3×10^5 293T cells were seeded in 6-well plates 16–18 h before transfection using Fugene 6 (Roche). For reporter assays, transfections were performed in duplicate with 0.25 μg of pI2EA-Gli1/pI2EA-Gli1mNES or control pEGFP-C3 (Clontech), 0.25 μg of pGL3P-6GBS, 0.25–1.0 μg of pCO2-PKCα/δ-ca (kindly provided by Peter Parker, London Research Institute, Cancer Research United Kingdom) as indicated, and 0.1 μg of pcDNA3.1-Lac Z (Invitrogen)/well; the total amount of DNA was normalized with empty vector. Six h after transfection, cells were incubated in fresh standard medium (10% FCS); alternatively, for analysis of ERK activation, cells were incubated in low serum medium (0.05% FCS) and in the presence of 1 μM U0126 as indicated. Cells were harvested 24–26 h after transfection in 250 μl of lysis buffer [0.05 M Tris (pH 7.4), 1 mM $MgCl_2$, 0.2% IGEPAL CA-360 (Sigma)], vortexed, and incubated on ice for 15 min. Cell lysates were cleared at 13,000 rpm in a tabletop centrifuge for 1 min, and the supernatant was used for reporter activity and Western blot analysis. Luciferase and β-galactosidase reporter activities were measured with luciferase substrate (Promega) and chlorophenol red-β-D-galactopyranoside (Roche Biochemicals), respectively, using a microtiter plate reader (Victor Multilabel Counter; Wallac, Turku, Finland).

Western Blotting and Antibodies. Protein samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Amersham Pharmacia) according to standard protocols. Protein loading was normalized to Lac z activity or total protein levels. Primary antibodies that were obtained commercially include Gli1 (Santa Cruz Biotechnology), phospho-p38 MAPK (NEB), phospho-ERK (NEB), PKCα (Transduction Laboratories), and phospho-PKCα/βII (NEB). The phospho-serine 657 antibody (PKCα) was a gift from Peter Parker (London Research Institute). Secondary horseradish peroxidase-linked antibodies were obtained commercially (Dako), and immunodetection was performed with enhanced chemiluminescence + reagent (Amersham Pharmacia).

RESULTS

PKC Isoforms Are Differentially Expressed in BCC, Interfollicular Epidermal Cells, and Hair Follicle ORS Keratinocytes. To determine whether PKC may be involved in BCC development, we first investigated the expression of PKCα and PKCδ in BCCs by immunohistochemistry using isoform-specific antibodies. These isoforms are known to be expressed in human primary keratinocytes and

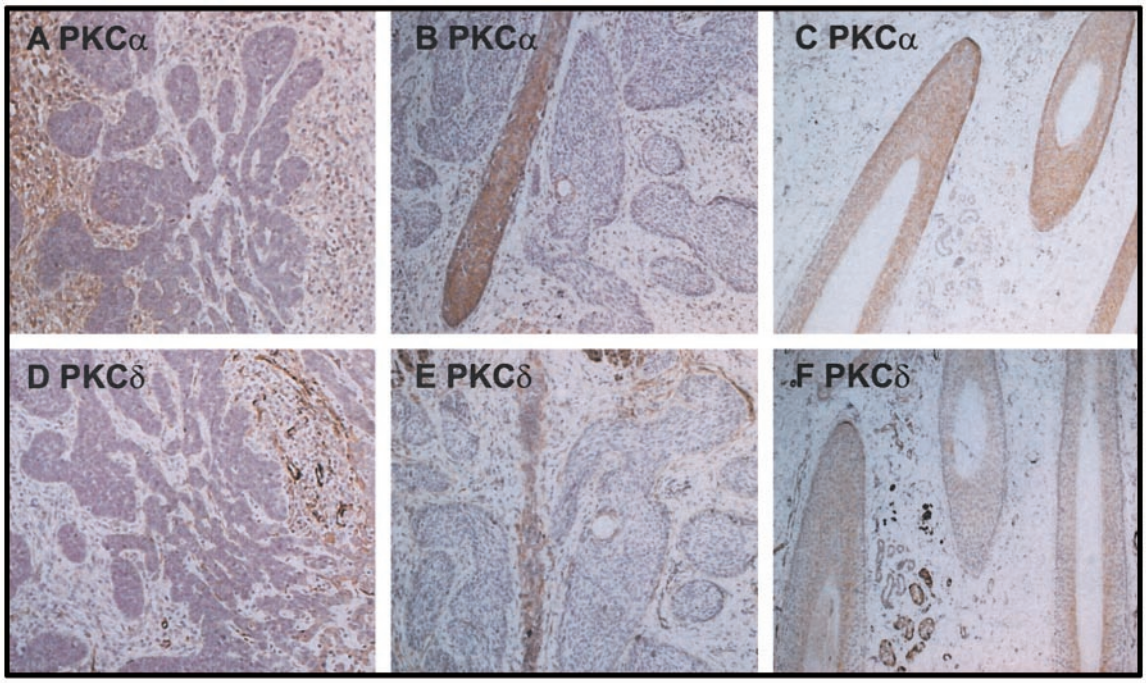

Fig. 1. Immunohistochemical analysis of PKC expression in BCC sections. *A–C*, PKCα was detected in the mesenchyme surrounding tumor islands (*A*) and in the ORS of the hair follicle (*B* and *C*) but not within tumor islands (*A* and *B*). *D–F*, PKCδ was detected in stromal cells and capillaries surrounding tumor islands (*D* and *E*) and in the inner root sheath of the hair follicle (*F*) but not within tumor islands (*D* and *E*). Note that images *A* and *D*, *B* and *E*, and *C* and *F* are of adjacent sections and that images *B*, *C*, *E*, and *F* are from the same section.

represent classical and novel PKC isoforms, respectively (20). Seven different BCC sections were analyzed, including two with hair follicles, which allowed for direct comparison between different structures of the skin. PKCα immunoreactivity was detected in basal keratinocytes of the interfollicular epidermis (data not shown) and strongly in the ORS of hair follicles (Fig. 1, *B* and *C*). In contrast, PKCα was only weakly expressed or absent in BCC tumor islands (Fig. 1, *A* and *B*). PKCα did, however, show heterogeneous expression in the stroma surrounding BCCs (Fig. 1, *A* and *B*), and expression was also observed in the connective tissue sheath surrounding the hair follicle (data not shown). In contrast to strong basal PKCα expression in the ORS of hair follicles, PKCδ expression was mainly restricted to the inner root sheath of the hair follicle, and only occasional, weak immunoreactivity was detected in the ORS (Fig. 1*F*). Similar to PKCα, PKCδ expression was predominantly negative in BCC tumor islands, whereas heterogeneous expression was observed in the stroma (Fig. 1, *D* and *E*).

Gli1 Is Differentially Regulated by PKC Isoforms. In view of the biological differences between Gli1 expressing BCC and ORS keratinocytes and the differences in the expression of the PKC isoforms between BCC and ORS keratinocytes, we investigated the effects of PKCα and PKCδ on Gli1 transcriptional activity. This was performed using PKC-ca (constitutively active) mutants and a luciferase reporter construct containing 6xGli1 binding sites in mammalian 293T cells. PKCα-ca decreased Gli1 activity by over 60% in a dose-dependent manner (Fig. 2*A*), whereas PKCδ-ca increased Gli1 activity in a dose-dependent manner, resulting in a 2-fold stimulation of reporter activity at the highest concentration of plasmid used (Fig. 2*B*). Additional stimulation of Gli1 was achieved using higher concentrations of PKCδ-ca plasmid (data not shown). Neither PKC isoform altered reporter activity when coexpressed with EGFP (data not shown).

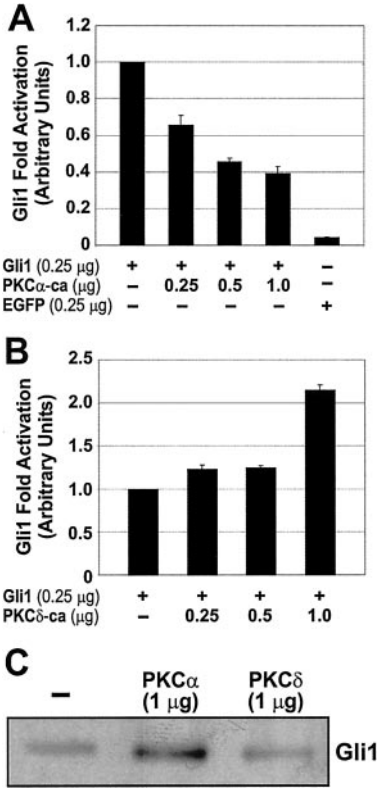

Fig. 2. Effect of PKC isoforms on Gli1 transcriptional activity and protein stability in mammalian 293T cells. *A*, coexpression of Gli1 with increasing amounts of PKCα-ca induced a dose-dependent decrease of Gli1 transcriptional activity. *B*, coexpression of Gli1 with increasing amounts of PKCδ-ca induced a dose-dependent increase of Gli1 transcriptional activity. *C*, Western blot analysis of reporter lysates revealed that coexpression of PKCα-ca induced an increase of Gli1 protein, whereas PKCδ-ca had no effect when compared with expression of Gli1 alone. *Error bars* represent SE of three independent experiments performed in duplicate.

To determine whether changes in Gli1 activity were because of changes in Gli1 protein level, we carried out Western blot analysis of reporter lysates. Fig. 2*C* shows that at the highest concentration of plasmid used (1 μg), PKCα stimulated an increase in Gli1 protein levels when compared with controls, whereas PKCδ had no effect. The paradoxical stimulation of Gli1 expression but down-regulation of reporter activity indicates that PKCα-ca reduction of Gli1 activity would have been more marked had reporter activity been normalized for Gli1 protein.

PKC Regulation of Gli1 Transcriptional Activity Is not Dependent upon Nuclear-cytoplasmic Shuttling. Recent studies have shown that Gli1 is subject to nuclear-cytoplasmic shuttling, which is controlled by nuclear export and nuclear localization motifs (Ref. 15 and unpublished data). Although the subcellular localization of Gli1 is dependent upon cell type, its expression pattern is predominantly cytoplasmic and punctate in 293T cells as shown by an EGFP fusion protein (Fig. 3*A*). Mutation of residues in the nuclear export motif produces a mutant protein that is localized exclusively to the nucleus, Gli1mNES, as shown by an EGFP fusion protein (Fig. 3*A*). Therefore, we sought to determine whether the subcellular location of Gli1 determined its regulation by PKC by analyzing the regulation of Gli1mNES (nonfusion protein). Coexpression of PKCα-ca resulted in a decrease in Gli1mNES activity in a similar manner to that of native Gli1, and coexpression of PKCδ-ca resulted in up-regulation of Gli1mNES activity (Fig. 3*B*). We also confirmed that PKCα-ca did not alter the subcellular location of nuclear Gli1 by coexpression with EGFP-Gli1mNES (data not shown). Combined with the fact that PKCδ regulation is similar between Gli1 and Gli1mNES, this suggests that nuclear-cytoplasmic shuttling is not a determinant of Gli1 regulation by either isoform and indicates that PKC may influence signaling pathways that regulate Gli1 in the nucleus.

PKCα Regulation of Gli1 Is not Dependent upon MAPK Activation. The downstream effects of PKC are mediated through several different pathways, including activation of Raf-MAP kinase kinase-ERK signaling (25, 26). Therefore, we investigated if PKC regulation of Gli1 is mediated via this classical signaling pathway. As PKC-ERK (p42/44 MAPK) activation may be cell specific, we initially determined if either PKC isoform activates ERK in 293T cells. Expression of PKCα-ca, but not PKCδ-ca, induced activation of ERK as shown by the level of phospho-ERK with Western blot analysis (Fig. 4*A*). However, as a recent study has shown that Gli1 induces ERK activation in C3H10T1/2 cells (27), we assessed if Gli1 also induces ERK activation in 293T cells because this could affect our interpretation of PKCα-Gli1 regulation via ERK. We observed no activation of ERK by Gli1 indicating that Gli1 activation of Ras-ERK signaling may be cell specific (Fig. 4*A*).

To determine if PKCα regulation of Gli1 occurs through ERK activation, cells were incubated in the presence of the drug U0126, a specific inhibitor of its upstream activator MAP kinase kinase. The presence of U0126 had no effect on Gli1 activity or protein stability, indicating that PKCα-Gli1 regulation occurs through an ERK-independent mechanism (Fig. 4, *B* and *C*). Western blot analysis confirmed that U0126 (1 μM) inhibited the activation of ERK by PKCα (Fig. 4*C*). The effects of PKC signaling can also be mediated by p38 MAPK (28–31). However, Western blot analysis revealed that neither PKCα nor PKCδ influenced p38 activity as determined by the level of phospho-p38 MAPK, thus excluding this kinase from PKC regulation of Gli1 in 293T cells (data not shown). Our data therefore shows

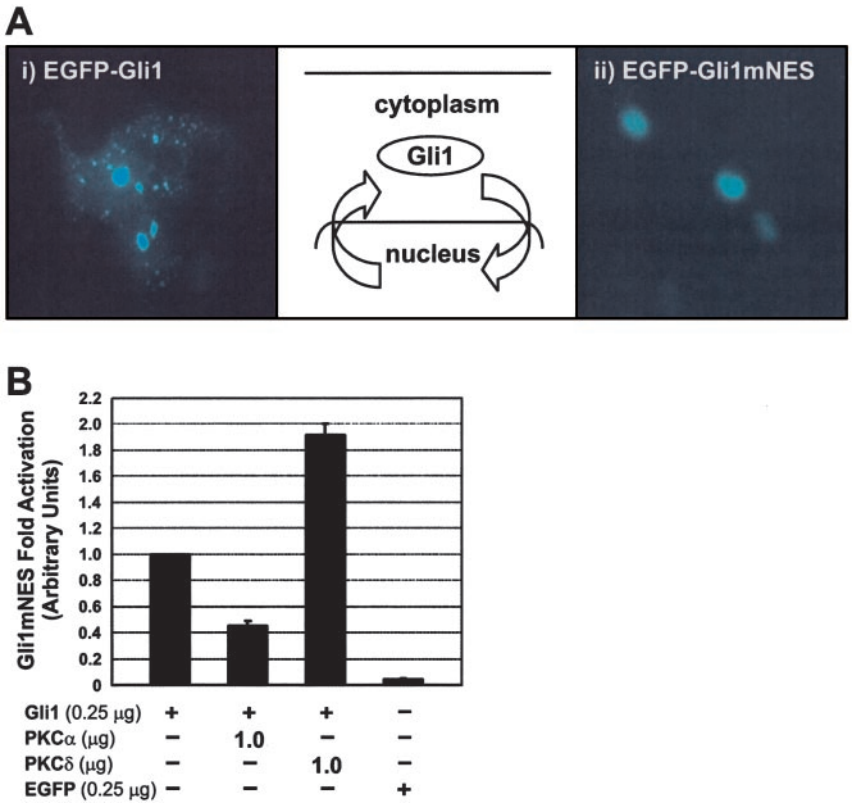

Fig. 3. Nuclear-cytoplasmic shuttling is not a determinant of Gli1 regulation by PKC. *A*, expression of an EGFP-Gli1 fusion protein demonstrates that Gli1 is predominantly cytoplasmic and punctate in 293T cells (*i*); mutation of residues that control nuclear export produces a mutant protein (EGFP-Gli1mNES) that is localized exclusively to the nucleus (*ii*). *B*, coexpression of PKCα-ca reduced Gli1mNES (nonfusion protein) transcriptional activity, whereas PKCδ-ca increased Gli1mNES activity. *Error bars* represent SE of three independent experiments performed in duplicate.

that PKC regulation of Gli1 does not involve p38 MAPK or ERK signaling.

Gli1 Does not Regulate the Expression or Activity of PKCα in Human Primary Keratinocytes. The origin of BCC is uncertain, but proposed sites of tumor initiation include the basal layer of the epidermis and the ORS of the hair follicle. PKCα is expressed in both these regions but is absent in adjacent tumor islands, indicating that its loss of expression may be required for tumor formation or growth. As Gli1 is expressed in the ORS and tumor islands and is fundamental to BCC formation, we investigated if Gli1 affects the expression of PKCα in primary human keratinocytes using retroviral transduction. We have previously shown that retrovirally expressed Gli1 induces endogenous PTCH and Gli2 expression in keratinocytes, indicating protein functionality (23). By Western blot analysis, we found that the total level of PKCα was not altered in Gli1 cells compared with control EGFP cells (Fig. 5). Furthermore, Gli1 did not alter the activation of PKCα as shown with antibodies specific for two of the three phosphorylation sites (threonine 638 and serine 657) that regulate PKCα activity (Fig. 5; Ref. 32, 33). This suggests that loss of PKCα in BCC is not dependent upon Hh signaling.

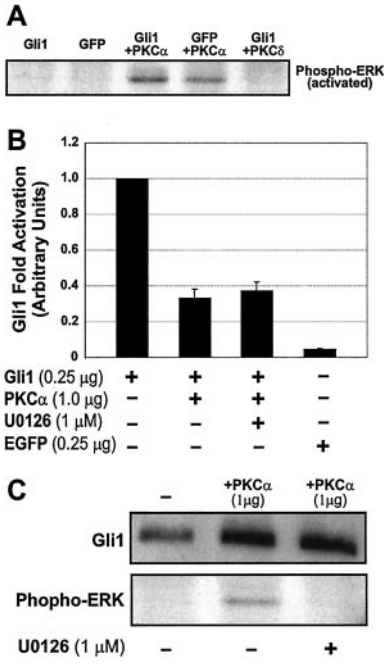

Fig. 4. PKC regulation of Gli1 does not involve MAPK signaling. *A*, PKCα-ca, but not PKCδ-ca or Gli1, induced phosphorylation and activation of ERK in 293T cells as shown by Western blot analysis. *B* and *C*, the presence of the drug U0126 did not inhibit the decrease in Gli1 transcriptional activity or increase in protein stability induced by PKCα-ca; inhibition of ERK activation by U0126 was determined by Western blot analysis of reporter lysates. *Error bars* represent SE of three independent experiments performed in duplicate.

DISCUSSION

Gli1 is expressed extensively in the ORS of human hair follicles but not in interfollicular epidermis (19). The mechanisms regulating Gli1 activity in mammalian cells is still poorly understood with much of the focus on components identified from studies of Ci regulation in *Drosophila.* Although it is well recognized that there are tissue specific differences in the downstream consequences of activation of SHh signaling, the basis for these differences is unclear. In this article, we now present data showing that the transcriptional activity of Gli1 is subject to regulation by PKC and suggest that this may play an important role in tumor development. We have investigated the patterns of expression of PKCα and PKCδ in interfollicular epidermis, hair follicles, and BCC. We found that PKCα is extensively expressed in the ORS of human hair follicles and weakly in basal epidermal keratinocytes, and this is consistent with the expression pattern in murine epidermis (21). A previous study reported that PKCα is not expressed in BCC, and we have confirmed this observation (34).

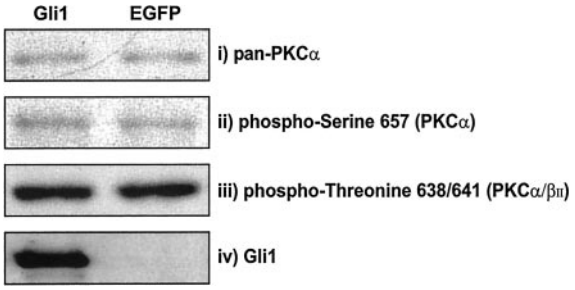

Fig. 5. Gli1 does not regulate the expression or activity of PKCα in keratinocytes. Western blot analysis revealed that the expression of PKCα was not altered in primary cells expressing Gli1 via retroviral transduction (*i*) nor was its activity altered as shown with antibodies specific for residues that control activation (*ii* and *iii*) when compared with control EGFP cells. Confirmation of Gli1 expression was also performed by Western blotting (*iv*).

The combined observations that Gli1 is expressed in the ORS of hair follicles and in BCC but not interfollicular epidermis and, moreover, our observation that PKCα is also expressed in the ORS of the hair follicle but down-regulated in BCC led us to speculate that PKCα might regulate Gli1 activity. We found that coexpression of PKCα reduced the transcriptional activity of Gli1 by $>$60%. Furthermore, we also observed that PKCα increased the level of Gli1 protein and, as such, if the data were normalized for protein content the reduction in Gli1 activity would be even greater. Interestingly, in a previous study we demonstrated by immunostaining that Gli1 is more strongly expressed in the ORS than BCC tumor islands despite an abundance of Gli1 transcript in the tumors (19). Combined with the results of the present study, this suggests that PKCα regulates Gli1 protein levels *in vivo* and that the reduction of Gli1 in BCC compared with the ORS is possibly attributable to the concomitant reduction in PKCα expression.

The data from our reporter assays reveal that Gli1 activity is not completely suppressed by PKCα (Fig. 2*C*). However, it is likely that the transformation to a neoplastic phenotype may only require a slight increase in Gli1 activity to disrupt normal cell homeostasis. Recent evidence has shown that the function of tumor suppressor genes may depend on the level of protein expressed. Haploinsufficiency has been proposed as the genetic basis for neoplasia in PTCH heterozygous mice, and a recent study has demonstrated that after loss of heterozygosity, a 50% reduction in expression of the remaining APC allele may be sufficient to induce colorectal neoplasia (35, 36). By analogy, an increase in the activity of oncogenic transcription factors, either by increased expression or by down-regulation of negative regulatory elements, may be sufficient to promote tumorigenesis.

In BCC, the absence of PKCα in tumor islands may result in Gli1 being transcriptionally more potent, and this may subsequently promote BCC formation and/or growth. Dahmane *et al.* (8) hypothesized that Gli1 expression in BCC could not be accounted for solely by expression of SHh or activation of the pathway via mutation of *PTCH1*. They subsequently proposed that any mutation leading to the induction of SHh signaling would result in Gli1 expression and BCC formation. Alternatively, because SHh signaling is active during hair follicle growth, they also suggested that neoplastic transformation of these cells may induce BCC formation (8). The latter hypothesis correlates with the evidence presented here from which we propose that loss of PKCα expression in the ORS may promote tumor formation. This is also consistent with several immunostaining studies that suggest that BCC originates from the ORS (37–40). As well as the ORS, PKCα is expressed in the interfollicular epidermis and BCC may occur because of ectopic expression of Gli1 in cells of the basal layer. As PKCα is down-regulated in the majority of BCC (Ref. 34 and this study), this indicates that whatever the derivation of the tumor or its mode of Gli1 expression, the activity of Gli1 will be enhanced in the absence of PKCα. It would be interesting to determine the expression profile of PKCα in Gli1-transgenic mice, which develop BCC in the absence of *PTCH1* mutations, and because the profile of PKCα is similar in normal murine epidermis to that presented here (21, 41).

Gli1 is subject to regulation by nuclear import and export sequences (Ref. 15 and unpublished data), and the expression pattern of Gli1 is predominantly cytoplasmic and punctate in 293T cells as it forms part of a protein complex attached to the cytoskeleton. One potential mechanism for PKCα control of Gli1 could be via the regulation of proteins such as Fu or Su(fu) that control the subcellular localization and activity of Gli1 (13–16). We have shown that the regulation of a mutant Gli1 protein localized exclusively to the nucleus is similar to that of the wild-type protein, indicating that PKCα regulation of Gli1 is not dependent upon changes in subcellular location; this suggests that PKCα regulation of Gli1 activity is more direct.

PKC signaling is often mediated through downstream activation of either ERK (p42/44 MAPK) or p38 MAPK. However, using specific drug inhibitors, we have discounted the possibility that PKCα (and PKCδ) regulates Gli1 through MAPK activation. As well as controlling its subcellular localization, Su(fu) also reduces the activity of Gli1 bound to DNA, and it is possible that the negative control of Gli1 by PKA may be through reduced DNA binding (14, 15). Analysis of the Gli1 coding sequence reveals multiple PKC consensus phosphorylation sites throughout the protein, including the zinc fingers, indicating that Gli1 may be a direct target of PKCα. We found no evidence that Gli1 is phosphorylated upon coexpression with PKCα-ca as determined by Western blot analysis (Fig. 2*C*), but this technique may not be sensitive enough to detect phosphorylated Gli1, and more sensitive assays such as two-dimensional gel electrophoresis or *in vitro* labeling may be required. Alternatively, Gli1 could be indirectly regulated by PKCα, and this may or may not involve Gli1 phosphorylation.

Several studies have shown that SHh signaling is crucial for the morphogenesis of skin appendages such as the hair follicle and feather bud (reviewed in Ref. 42), but only little is known about the potential cooperation between SHh and PKC signaling in these structures. The induction of feather bud dermal condensations by SHh-tumor growth factor-β2 signaling is associated with the suppression of PKC expression (43, 44), and exposure of hair follicles to phorbol esthers such as phorbol 12-myristate 13-acetate/12-*O*-tetradecanoylphorbol-13-acetate potently inhibits follicle growth (45–47). Interestingly, the activity of Gli1 is also reduced by phorbol 12-myristate 13-acetate (unpublished data). These studies suggest reciprocal negative regulation between SHh and PKC signaling. How PKCα expression is down-regulated in BCC remains to be determined. We found no evidence that Gli1 regulates PKCα expression or activity in human primary keratinocytes, and this suggests that other factors regulate PKCα in the skin and its appendages.

Finally, in addition to PKCα, we have also investigated the expression pattern of PKCδ and its effect on Gli1 reporter activity. In contrast to PKCα, PKCδ stimulated Gli1 reporter activity. However, as we did not detect PKCδ expression in BCC tumor islands, it is most likely that PKCδ up-regulation of Gli1 activity does not play an important role in BCC tumor formation *per se*. Despite this, PKCδ was expressed in stromal cells surrounding tumor islands, and we cannot exclude the possibility that PKCδ mediates epithelial-mesenchymal cross-talk and may influence tumor development in this way.

In summary, we have determined that PKCα is normally expressed in the basal layer of the epidermis, as well as the ORS of the hair follicle. Both of these compartments have been proposed as the sites

of tumor formation, and although our data are unable as yet to shed light on which of these may be the origin of tumors, we have shown that PKCα negatively regulates Gli1 activity. This is important because PKCα is down-regulated in BCC, whereas Gli1 is expressed in BCC. We therefore propose that loss of PKCα expression in BCC may play a key role in tumor development.

ACKNOWLEDGMENTS

We thank Peter Parker for providing the PKC plasmids and PKCα antibody and Philip Whitehead for technical advice (London Research Institute). We also thank to Dr. Muy-Teck Teh (Queen Mary University of London) for his considerable help in preparation of the illustrations.

REFERENCES

1. Currie, P. D. Hedgehog's escape from Pandora's box. J. Mol. Med., *76:* 421–433, 1998.
2. Ingham, P. W. Transducing Hedgehog: the story so far. EMBO J., *17:* 3505–3511, 1998.
3. von Mering, C., and Basler, K. Distinct and regulated activities of human Gli proteins in *Drosophila*. Curr. Biol., *9:* 1319–1322, 1999.
4. Zardoya, R., Abouheif, E., and Meyer, A. Evolution and orthology of hedgehog genes. Trends Genet., *12:* 496–497, 1996.
5. Kinzler, K. W., Ruppert, J. M., Bigner, S. H., and Vogelstein, B. The *GLI* gene is a member of the Kruppel family of zinc finger proteins. Nature (Lond.), *332:* 371–374, 1988.
6. Matise, M. P., and Joyner, A. L. Gli genes in development and cancer. Oncogene, *18:* 7852–7859, 1999.
7. Toftgard, R. Hedgehog signalling in cancer. Cell Mol. Life Sci., *57:* 1720–1731, 2000.
8. Dahmane, N., Lee, J., Robins, P., Heller, P., and Ruiz i Altaba, A. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. Nature (Lond.), *389:* 876–881, 1997.
9. Green, J., Leigh, I. M., Poulsom, R., and Quinn, A. G. Basal cell carcinoma development is associated with induction of the expression of the transcription factor Gli-1. Br. J. Dermatol., *139:* 911–915, 1998.
10. Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., and Bale, A. E. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. Cell, *85:* 841–851, 1996.
11. Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., and Scott, M. P. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science (Wash. DC), *272:* 1668–1671, 1996.
12. Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., Hao, A., Goldstein, A. M., Stavrou, T., Scherer, S. W., Dura, W. T., Wainwright, B., Squire, J. A., Rutka, J. T., and Hogg, D. Mutations in SUFU predispose to medulloblastoma, Nat. Genet., *31:* 306–310, 2002.
13. Ding, Q., Fukami, S., Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., Dlugosz, A., Nakafuku, M., and Hui, C. Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1. Curr. Biol., *9:* 1119–1122, 1999.
14. Kaesler, S., Luscher, B., and Ruther, U. Transcriptional activity of GLI1 is negatively regulated by protein kinase A. Biol. Chem., *381:* 545–551, 2000.
15. Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Unden, A. B., Sandstedt, B., Toftgard, R., and Zaphiropoulos, P. G. Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat. Cell Biol., *1:* 312–319, 1999.
16. Murone, M., Luoh, S. M., Stone, D., Li, W., Gurney, A., Armanini, M., Grey, C., Rosenthal, A., and de Sauvage, F. J. Gli regulation by the opposing activities of fused and suppressor of fused. Nat. Cell Biol., *2:* 310–312, 2000.
17. Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J., and Rosenthal, A. Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. J. Cell Sci., *112:* 4437–4448, 1999.
18. Price, M. A., and Kalderon, D. Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by glycogen synthase kinase 3 and casein kinase 1. Cell, *108:* 823–835, 2002.
19. Ghali, L., Wong, S. T., Green, J., Tidman, N., and Quinn, A. G. Gli1 protein is expressed in basal cell carcinomas, outer root sheath keratinocytes and a subpopulation of mesenchymal cells in normal human skin. J. Investig. Dermatol., *113:* 595–599, 1999.
20. Reynolds, N. J., Baldassare, J. J., Henderson, P. A., Shuler, J. L., Ballas, L. M., Burns, D. J., Moomaw, C. R., and Fisher, G. J. Translocation and down-regulation of protein kinase C isoenzymes-α and -ϵ by phorbol ester and bryostatin-1 in human keratinocytes and fibroblasts. J. Investig. Dermatol., *103:* 364–369, 1994.
21. Wang, H. Q., and Smart, R. C. Overexpression of protein kinase C-α in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF-α expression but not tumor promotion. J. Cell Sci., *112:* 3497–3506, 1999.
22. Rheinwald, J. G., and Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell, *6:* 331–343, 1975.
23. Regl, G., Neill, G. W., Eichberger, T., Kasper, M., Ikram, M. S., Koller, J., Hintner, H., Quinn, A. G., Frischauf, A. M., and Aberger, F. Human GLI2 and GLI1 are part of a positive feedback mechanism in basal cell carcinoma. Oncogene, *21:* 5529–5539, 2002.
24. Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J. Biol. Chem., *274:* 8143–8152, 1999.
25. Schonwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, P. J. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol. Cell. Biol., *18:* 790–798, 1998.
26. Soh, J. W., Lee, E. H., Prywes, R., and Weinstein, I. B. Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element, Mol. Cell. Biol., *19:* 1313–1324, 1999.
27. Xie, J., Aszterbaum, M., Zhang, X., Bonifas, J. M., Zachary, C., Epstein, E., and McCormick, F. A role of PDGFR-α in basal cell carcinoma proliferation. Proc. Natl. Acad. Sci. USA, *98:* 9255–9259, 2001.
28. Chen, N. Y., Ma, W. Y., Huang, C., Ding, M., and Dong, Z. Activation of PKC is required for arsenite-induced signal transduction. J. Environ. Pathol. Toxicol. Oncol., *19:* 297–305, 2000.
29. Osborn, M. T., Berry, A., Ruberu, M. S., Ning, B., Bell, L. M., and Chambers, T. C. Phorbol ester induced MDR1 expression in K562 cells occurs independently of mitogen-activated protein kinase signaling pathways. Oncogene, *18:* 5756–5764, 1999.
30. Rahman, A., Anwar, K. N., Uddin, S., Xu, N., Ye, R. D., Platanias, L. C., and Malik, A. B. Protein kinase Cδ regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. Mol. Cell. Biol., *21:* 5554–5565, 2001.
31. Shimizu, T., Kato, T., Jr., Tachibana, A., and Sasaki, M. S. Coordinated regulation of radioadaptive response by protein kinase C and p38 mitogen-activated protein kinase. Exp. Cell Res., *251:* 424–432, 1999.
32. Bornancin, F., and Parker, P. J. Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase C-α. Curr. Biol., *6:* 1114–1123, 1996.
33. Bornancin, F., and Parker, P. J. Phosphorylation of protein kinase C-α on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. J. Biol. Chem., *272:* 3544–3549, 1997.
34. Reynolds, N. J., Todd, C., and Angus, B. Overexpression of protein kinase C-α and -β isozymes by stromal dendritic cells in basal and squamous cell carcinoma. Br. J. Dermatol., *136:* 666–673, 1997.
35. Yan, H., Dobbie, Z., Gruber, S. B., Markowitz, S., Romans, K., Giardiello, F. M., Kinzler, K. W., and Vogelstein, B. Small changes in expression affect predisposition to tumorigenesis. Nat. Genet., *30:* 25–26, 2002.
36. Zellner, A., Fetell, M. R., Bruce, J. N., De Vivo, D. C., and O'Driscoll, K. R. Disparity in expression of protein kinase C α in human glioma *versus* glioma-derived primary cell lines: therapeutic implications. Clin Cancer Res., *4:* 1797–1802, 1998.
37. Asada, M., Schaart, F. M., de Almeida, H. L., Jr., Korge, B., Kurokawa, I., Asada, Y., and Orfanos, C. E. Solid basal cell epithelioma (BCE) possibly originates from the outer root sheath of the hair follicle. Acta Derm. Venereol., *73:* 286–292, 1993.
38. Kruger, K., Blume-Peytavi, U., and Orfanos, C. E. Basal cell carcinoma possibly originates from the outer root sheath and/or the bulge region of the vellus hair follicle. Arch. Dermatol. Res., *291:* 253–259, 1999.
39. Kurzen, H., Esposito, L., Langbein, L., and Hartschuh, W. Cytokeratins as markers of follicular differentiation: an immunohistochemical study of trichoblastoma and basal cell carcinoma. Am. J. Dermatopathol., *23:* 501–509, 2001.
40. Markey, A. C., Lane, E. B., Macdonald, D. M., and Leigh, I. M. Keratin expression in basal cell carcinomas. Br. J. Dermatol., *126:* 154–160, 1992.
41. Nilsson, M., Unden, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G., and Toftgard, R. Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. Proc. Natl. Acad. Sci. USA, *97:* 3438–3443, 2000.
42. Chuong, C. M., Patel, N., Lin, J., Jung, H. S., and Widelitz, R. B. Sonic hedgehog signaling pathway in vertebrate epithelial appendage morphogenesis: perspectives in development and evolution. Cell Mol. Life Sci., *57:* 1672–1681, 2000.
43. Ting-Berreth, S. A., and Chuong, C. M. Sonic Hedgehog in feather morphogenesis: induction of mesenchymal condensation and association with cell death. Dev. Dyn., *207:* 157–170, 1996.
44. Ting-Berreth, S. A., and Chuong, C. M. Local delivery of TGF β2 can substitute for placode epithelium to induce mesenchymal condensation during skin appendage morphogenesis, Dev. Biol., *179:* 347–359, 1996.
45. Harmon, C. S., Nevins, T. D., and Bollag, W. B. Protein kinase C inhibits human hair follicle growth and hair fibre production in organ culture. Br. J. Dermatol., *133:* 686–693, 1995.
46. Harmon, C. S., Nevins, T. D., Ducote, J., and Lutz, D. Bisindolylmaleimide protein-kinase-C inhibitors delay the decline in DNA synthesis in mouse hair follicle organ cultures. Skin Pharmacol., *10:* 71–78, 1997.
47. Xiong, Y., and Harmon, C. S. Interleukin-1β is differentially expressed by human dermal papilla cells in response to PKC activation and is a potent inhibitor of human hair follicle growth in organ culture. J. Interferon Cytokine Res., *17:* 151–157, 1997.