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Cross-talk between epidermal growth factor receptor and protein kinase C during calcium-induced differentiation of keratinocytes

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Abstract: The induction of epidermal differentiation by extracellular Ca^{2+} involves activation of both tyrosine kinase and protein kinase C (PKC) signaling cascades. To determine if the differentiation-dependent activation of tyrosine kinase signaling can influence the PKC pathway, we examined the tyrosine phosphorylation status of PKC isoforms in primary mouse keratinocytes stimulated to terminally differentiate with Ca^{2+}. Elevation of extracellular Ca^{2+} induced tyrosine phosphorylation of PKC-δ, but not the other keratinocyte PKC isoforms (α,ε, η, ζ). We have previously demonstrated that activation of the epidermal growth factor receptor (EGFR) pathway induces PKC-δ tyrosine phosphorylation in basal keratinocytes (Denning M F, Dlugosz A A, Threadgill D W, Magnuson T, Yuspa S H (1996) *J Biol Chem* 271: 5325–5331). When basal keratinocytes were stimulated to differentiate by Ca^{2+}, the level of cell-associated transforming growth factor-α (TGF-α) increased 30-fold, while no increase in secreted TGF-α was detected. Furthermore, Ca^{2+}-induced tyrosine phosphorylation of PKC-δ and phosphotyrosine-association of the receptor adapter protein Shc was diminished in EGFR −/− keratinocytes, suggesting that EGFR activation may occur during keratinocyte differentiation. Tyrosine phosphorylated PKC-δ was also detected in mouse epidermis, suggesting that this differentiation-associated signaling pathway is physiological. These results establish a requirement for the EGFR in Ca^{2+}-induced tyrosine phosphorylation of PKC-δ, and document the production of cell-associated TGF-α in differentiated keratinocytes which may function independent of its usual mitogenic effects.

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Introduction

Several signal transducing systems, including phospholipase C (PLC) (1–3), protein kinase C (PKC) (4–6), and tyrosine kinases (7–10) are activated early in the differentiation of keratinocytes induced by Ca^{2+}. Ca^{2+} rapidly increases phosphatidylinositol turnover resulting in the elevation of cellular diacylglycerol and intracellular Ca^{2+} levels, physiological activators of PKC (2, 3). The activation of PKC during Ca^{2+}-induced differentiation is required for the expression of the granular layer differentiation program (6, 12), and specific PKC isoforms have been implicated in the regulation of keratinocyte differentiation genes (6, 13–16). Keratinocytes express five PKC isoenzymes (α,

δ, ε, ζ and η) which are regulated differentially by lipid and calcium co-factors (6, 17, 18). PKC-α appears to be involved in the up-regulation of loricrin, profilaggrin, SPR-1, involucrin, and transglutaminase expression in keratinocytes (6, 13, 19).

Tyrosine phosphorylation also regulates the activity of PKC isoenzymes (20, 23). For example, transformation of keratinocytes with the v-*ras*^{Ha} oncogene or growth stimulation with epidermal growth factor receptor (EGFR) ligands inactivate PKC-δ by inducing its tyrosine phosphorylation (22, 24). PKC-δ tyrosine phosphorylation by v-*ras*^{Ha} or TGF-α requires a functional EGFR indicating that the EGFR ligands can negatively regulate PKC activity by inducing tyrosine phosphorylation of PKC-δ (24). Tyrosine phosphorylation of PKC-δ in response to other stimuli can activate PKC-δ, indicating both positive and negative regulation of PKC activity can occur by tyrosine phosphorylation (25, 26).

Ca^{2+}-induced keratinocyte differentiation also triggers rapid and sustained activation of several non-receptor protein tyrosine kinases. c-Src activity is increased in human keratinocytes treated with Ca^{2+} plus the Ca^{2+} ionophore A23187 (10). By immunohistochemistry, phosphotyrosine levels are increased in the more suprabasal layers of the epidermis (10). Treatment of keratinocytes with Ca^{2+} also induces the tyrosine phosphorylation of p62, a GAP-associated protein (8). C-fyn activity is specifically induced within 6 h of Ca^{2+}-induced differentiation and increases the phosphorylation of several cellular proteins. Furthermore, c-fyn null mice have a thin epidermis with decreased expression of differentiation markers, and c-fyn null keratinocytes do not differentiate normally in response to Ca^{2+} *in vitro*, indicating that c-fyn is important for the keratinocyte differentiation program (7). While the mechanism of c-fyn activation during keratinocyte differentiation is unknown, activation of the EGFR by TGF-α in keratinocytes stimulates c-fyn activity (24).

Therefore both tyrosine kinase and PKC pathways are activated during Ca^{2+}-induced keratinocyte differentiation. This prompted us to examine if cross-talk occurs between PKC and tyrosine kinase signaling in differentiating keratinocytes. We found an EGFR-dependent tyrosine phosphorylation of PKC-δ in keratinocytes induced to differentiate with Ca^{2+}. We also detected a significant increase in cell-associated TGF-α in differentiating keratinocytes. The TGF-α production and EGFR-dependent tyrosine phosphorylation of PKC-δ during epidermal differentiation may help orchestrate specialized functions in the epidermis such as keratinocyte migration or acantholysis during the terminal stages of keratinocyte maturation.

Materials and methods

Cell culture

Primary keratinocytes were isolated from newborn BALB/c, or EGFR +/+ and EGFR −/− CD-1 strain mice (27). The keratinocytes were cultured in Eagle's minimal essential medium containing 8% Chelex-treated (Bio-Rad) fetal bovine serum with the final Ca^{2+} concentration adjusted to 0.05 mM as described previously (28). The genotypes of newborn EGFR +/+ and −/− mice were determined as described previously (27), and these keratinocytes were cultured in 0.05 mM Ca^{2+}-containing medium with 1 ng/ml keratinocyte growth factor (KGF) for 2–3 days. After reaching approximately 80% confluence, the cells were cultured without KGF for 3–4 days before treatment. For the induction of differentiation, extracellular Ca^{2+} was elevated by adding an aliquot of concentrated $CaCl_2$ directly to the culture medium to avoid any effects caused by the addition of fresh serum during a medium change. All experiments were performed at least twice with a representative experiment shown here.

Immunoblotting and immunoprecipitation

The cells were washed twice in ice-cold phosphate-buffered saline and scraped into immunoprecipitation lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). For the analysis of differentiation markers, an aliquot of the total lysate was removed and SDS sample buffer (0.15 M Tris, pH 6.8, 5% SDS, 3.5% 2-mercaptoethanol, 35% glycerol) was added. For immunoprecipitations, the lysates were cleared by centrifugation and equal amounts of protein were immunoprecipitated with either 1 μl of antibody to PKC-δ (Calbiochem) in the presence of 50 μl Protein A-Sepharose (Sigma) or 15–20 μl Agarose-conjugated Anti-phosphotyrosine antibody (Upstate Biotechnology Inc.). The immunoprecipitates were washed three times in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and boiled for 5 min in SDS sample buffer.

The samples were run on 8.5% SDS polyacrylamide gels, transferred to nitrocellulose by electrophoresis, and stained with Ponceau S to insure equal amounts of protein were loaded. The membranes were blocked with 5% milk in TBS (10 mM Tris, pH 7.5, 500 mM NaCl), and the total lysate blots were stained with antibodies to keratin 1 (K1), keratin 10 (K10), keratin 14 (K14), loricrin,

and filaggrin as described previously (29). For detection of PKC-δ, an anti-PKC-δ antibody (Calbiochem) was used at 1:5000. Shc was detected with an anti-Shc polyclonal antibody (Upstate Biotechnology Inc.) at 1 μg/ml. EGFR was detected with a sheep antibody at a 1:1000 dilution (30). The specific proteins were detected by staining with anti-rabbit peroxidase-conjugated secondary antibodies (Bio-Rad) at 1:5000 and visualized by chemiluminescence.

Radioimmunoassays

TGF-α levels were assayed in conditioned medium and cell lysates as described previously (31). Briefly, conditioned medium was harvested and debris removed by centrifugation for 5 min at 1000 × ***g***. The attached cells were lysed in 500 μl RIA lysis buffer (25 mM Tris, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM phenylmethylsulfonylfluoride), vortexed, extracted on ice for 45 min, and centrifuged for 10 min at 16,000 r.p.m. TGF-α levels in the cleared supernatants from conditioned medium and cell lysates (cell associated) were determined by radioimmunoassay using a polyclonal sheep antibody to rat TGF-α with rat TGF-α as a standard (32).

Results

Tyrosine phosphorylation of PKC-δ during keratinocyte differentiation

Primary mouse keratinocytes cultured in 0.05 mM Ca^{2+} proliferate and have a basal cell phenotype, but can be induced to differentiate by elevating the extracellular Ca^{2+} above 0.1 mM (28). We examined the tyrosine phosphorylation status of PKC isoforms expressed during Ca^{2+}-induced *in vitro* differentiation of normal, primary mouse keratinocytes. Tyrosine phosphorylated PKC-δ was detected after 48 and 72 h in 0.12 mM Ca^{2+} without any significant change in total PKC-δ levels (Fig. 1A). Tyrosine phosphorylation of the four other PKC isoforms expressed in keratinocytes (α, ε, η, ζ) (6, 18) was not detected (data not shown). In 0.12 mM Ca^{2+}, the induction of tyrosine phosphorylation of PKC-δ occurred after the induction of differentiation markers corresponding to the spinous (K1, K10) and granular layer (filaggrin, loricrin) (36 h) and continued to increase after expression of the differentiation markers had peaked (Fig. 1B).

We also evaluated the effect of 1.4 mM Ca^{2+} on PKC-δ tyrosine phosphorylation; 1.4 mM Ca^{2+} more rapidly induces differentiation markers, morphological differentiation, and is more effective than 0.12 mM Ca^{2+} at inducing keratinocyte transglutaminase (6, 33). Fig. 1C shows that after 24 h, 1.4 mM Ca^{2+} stimulated tyrosine phosphorylation of PKC-δ to a much greater extent

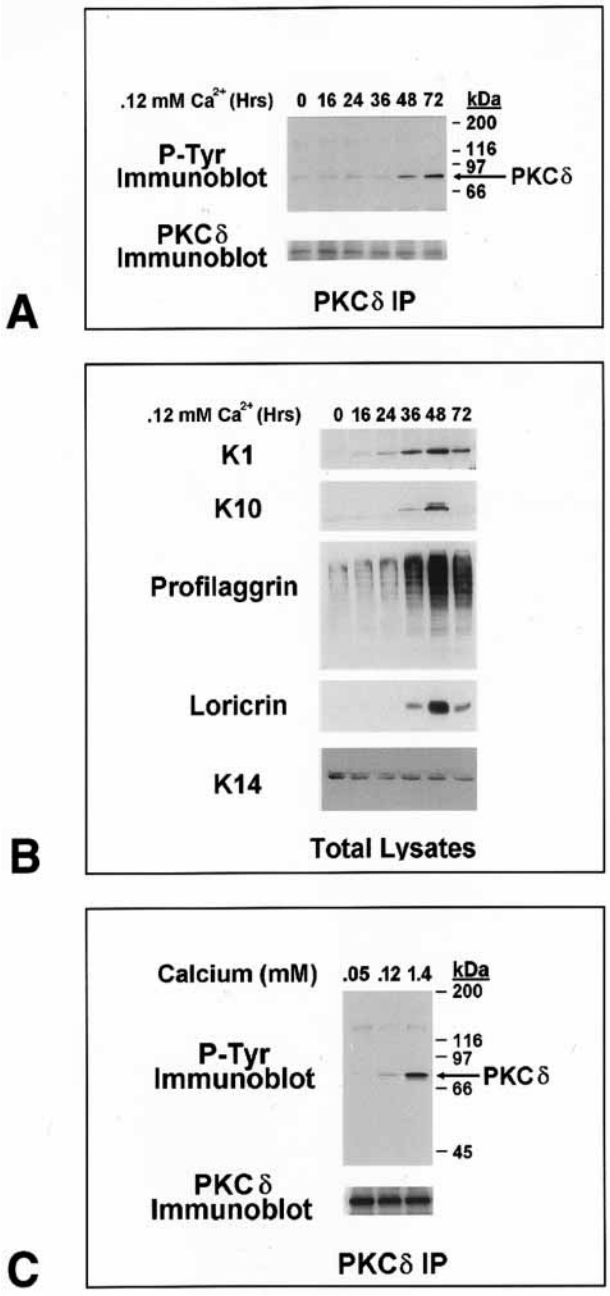

Figure 1. Elevation of extracellular Ca^{2+} stimulates tyrosine phosphorylation of PKC-δ. Primary mouse keratinocytes were switched from media containing 0.05 mM Ca^{2+} to 0.12 mM at the indicated times in panels A and B. In A, PKC-δ was immunoprecipitated and immunoblotted for phosphotyrosine and PKC-δ and in B, the differentiation markers K1, K10, profilaggrin, and loricrin were detected by immunoblotting of total cell lysates. In C, keratinocytes were cultured in 0.05 mM, 0.12 mM, or 1.4 mM Ca^{2+} for 24 h, lysed, and PKC-δ immunoprecipitated from the lysates. The proteins were then immunoblotted for phosphotyrosine and PKC-δ.

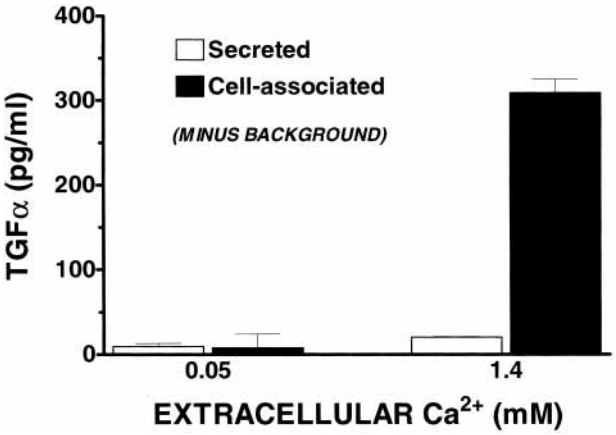

Figure 2. Elevation of extracellular Ca^{2+} induces cell-associated TGF-α in differentiating keratinocytes. Primary mouse keratinocytes were cultured in either 0.05 mM Ca^{2+} or 1.4 mM Ca^{2+} for 24 h, and levels of TGF-α in the conditioned medium (secreted) or cell lysate (cell-associated) were determined by radioimmunoassay.

than 0.12 mM Ca^{2+}. Since 1.4 mM Ca^{2+} induces PKC-δ tyrosine phosphorylation more rapidly and to a greater extent than 0.12 mM Ca^{2+}, 1.4 mM Ca^{2+} was used in the rest of this study.

Production of cell-associated TGF-α in differentiating keratinocytes

TGF-α is synthesized by normal mouse keratinocytes *in vivo* and *in vitro* (34) and can stimulate PKC-δ tyrosine phosphorylation in this cell type (24). To determine if TGF-α production could be responsible for stimulating the tyrosine phosphorylation of PKC-δ, we measured secreted and cell-associated TGF-α levels of cells cultured in basal media (0.05 mM Ca^{2+}) or differentiation media (1.4 mM Ca^{2+}) for 24 h. As shown in Fig. 2, culturing keratinocytes for 24 h in 1.4 mM Ca^{2+} increased the level of cell associated TGF-α approximately 30-fold. The level of secreted TGF-α was very low, and was not induced by Ca^{2+}.

EGFR is required for the induction of PKC-δ tyrosine phosphorylation

Since previous studies indicated that EGFR triggers PKC-δ tyrosine phosphorylation in keratinocytes (24) and TGF-α production increased in differentiating keratinocytes (Fig. 2), we hypothesized that the EGFR would be required to transduce the downstream signals leading to tyrosine phosphorylation of PKC-δ during keratinocyte differentiation. To test the requirement for the EGFR in stimulating PKC-δ tyrosine phosphorylation, we isolated keratinocytes from mice harboring wild type and genetically disrupted EGFR alleles (27). Fig. 3A shows that a 24-h exposure of keratinocytes to 1.4 mM Ca^{2+} stimulated tyrosine phosphorylation of PKC-δ in the EGFR wild type (+/+) cells, but not in the EGFR-deficient (−/−) cells. The level of total PKC-δ was not different between EGFR +/+ and −/− keratinocytes while the EGFR was not detected in EGFR −/− cells (Fig. 3A). In addition to PKC-δ, we detected an increase in the growth factor receptor adapter protein Shc in phosphotyrosine immunoprecipitates from differentiating EGFR +/+ keratinocytes, but not from differentiating EGFR −/− keratinocytes

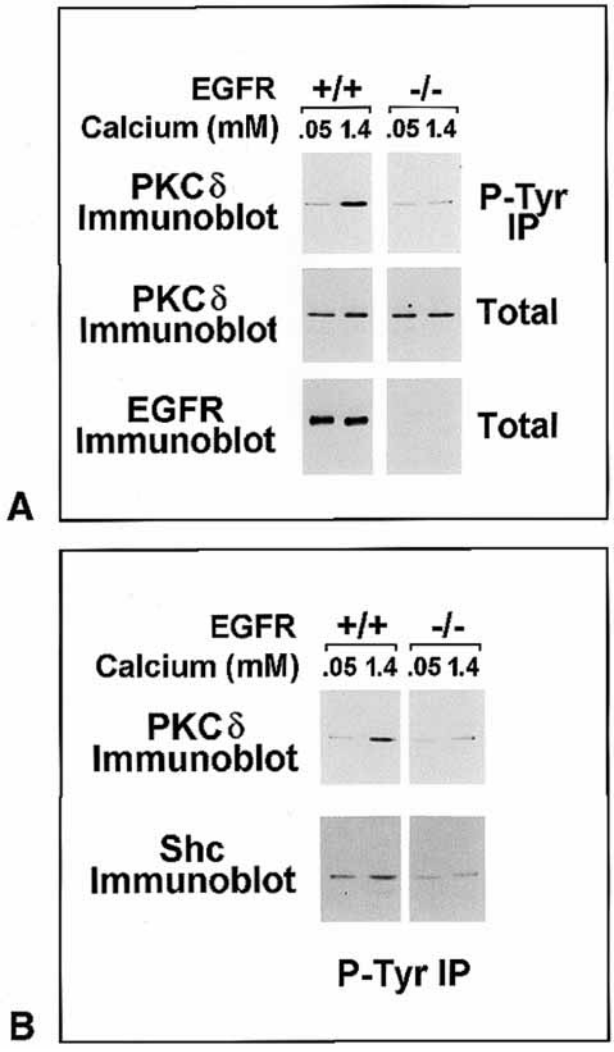

Figure 3. Calcium-induced tyrosine phosphorylation of PKC-δ and Shc requires a functional EGFR. Keratinocytes isolated from mice having wild type EGFR alleles (EGFR +/+) or harboring disrupted EGFR alleles (EGFR −/−) were cultured in 0.05 mM Ca^{2+} or 1.4 mM Ca^{2+} for 24 h. Phosphotyrosine containing proteins were immunoprecipitated and immunoblotted for PKC-δ in panels A and B and for Shc in panel B. In panel A, total cell lysates were immunoblotted for PKC-δ and EGFR.

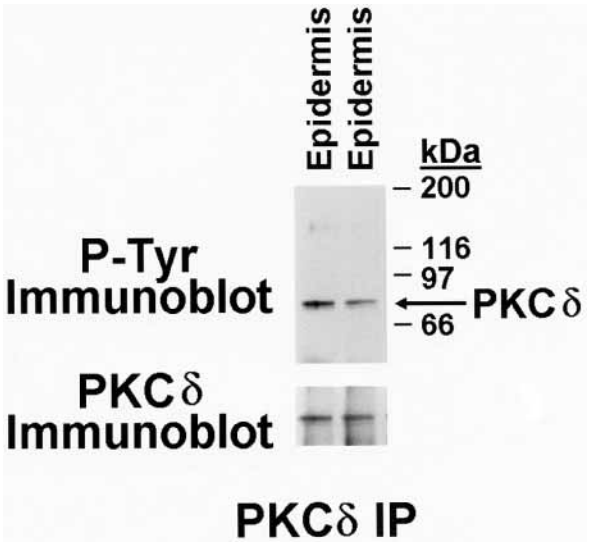

Figure 4. PKC-δ is tyrosine phosphorylated in adult mouse epidermis. The epidermis from 2 adult mice were scraped from the dermis after a brief heat treatment and homogenized individually in immunoprecipitation lysis buffer. PKC-δ was immunoprecipitated from each lysate and phosphotyrosine and PKC-δ detected by immunoblotting. Each lane represents an independent sample.

(Fig. 3B). These results establish a requirement for the EGFR in the induction of tyrosine phosphorylation of PKC-δ and Shc during keratinocyte differentiation.

In vivo *tyrosine phosphorylation of PKC-δ*

We examined the tyrosine phosphorylation status of PKC-δ from mouse epidermis to determine if PKC-δ tyrosine phosphorylation occurs *in vivo*. Fig. 4 shows tyrosine phosphorylated PKC-δ in extracts from the epidermis of 2 mice. The majority of cells in the epidermis are differentiated, and these are a likely source for the tyrosine phosphorylated PKC-δ. This is the first demonstration of PKC-δ tyrosine phosphorylation *in vivo*.

Discussion

During the terminal differentiation of epidermal keratinocytes, activation of PKC is required for the expression of granular differentiation markers, including profilaggrin, loricrin, transglutaminase, and SPR-1 (6, 12, 19, 33). PKC activation also results in the down-regulation of the spinous keratins K1 and K10, making it a central regulator of the keratinocyte differentiation program (12). PKC can be regulated by tyrosine phosphorylation in keratinocytes (22, 24), and tyrosine kinases become activated during keratinocyte differentiation (7–10). In this study, we demonstrate that TGF-α is produced by differentiating keratinocytes, and that tyrosine kinase and PKC signaling pathways are integrated during keratinocyte differentiation.

The maximum tyrosine phosphorylation of PKC-δ induced by 0.12 mM Ca^{2+} occurred at 48–72 h, which is after the expression of differentiation markers at 36 h (Figs 1A and 1B). This suggests that PKC-δ tyrosine phosphorylation was not an early triggering event for marker expression. Given that PKC activation induces differentiation marker expression and that tyrosine phosphorylation of PKC-δ can inhibit its enzymatic activity, the PKC-δ tyrosine phosphorylation may be a signal to turn off the differentiation program (12, 22, 24). The magnitude and kinetics of PKC-δ tyrosine phosphorylation were graded to the Ca^{2+} concentration, with 1.4 mM Ca^{2+} more rapidly inducing tyrosine phosphorylation than 0.12 mM Ca^{2+} (Fig. 1C). The higher induction of PKC-δ tyrosine phosphorylation by 1.4 mM Ca^{2+} is consistent with the tyrosine phosphorylation occurring late in the differentiation program since 1.4 mM Ca^{2+} is more effective than 0.12 mM at inducing late differentiation events such as transglutaminase activity and cornification, as opposed to early differentiation marker gene expression (6, 35). High concentrations of Ca^{2+} (>1.0 mM) are not effective inducers of structural differentiation marker expression in mouse keratinocytes, further supporting a link between PKC-δ tyrosine phosphorylation and a decrease in differentiation markers (6, 36).

We observed an induction of cell-associated TGF-α in differentiating keratinocytes, but no increase in soluble TGF-α (Fig. 3). TGF-α mRNA and protein are also found throughout the normal mouse epidermis (34), however no induction of TGF-α mRNA was detected in cultured keratinocytes induced to differentiate (37). Therefore, the increase in cell-associated TGF-α during keratinocyte differentiation may result from post-transcriptional mechanisms such as increased translational efficiency or protein stabilization. TGF-α is synthesized initially as a membrane anchored proform which is released upon proteolysis into the extracellular environment (38). Both the membrane pro-form of TGF-α and secreted form of TGF-α are active signaling ligands capable of binding to and activating the EGFR (39). Several lines of evidence are consistent with the TGF-α we detected in differentiating keratinocytes functioning as a signaling ligand. Two downstream targets of the EGFR signaling pathway, PKC-δ and Shc, were detected in phosphotyrosine immunoprecipitates after keratinocytes were cultured for 24 h in 1.4 mM Ca^{2+} (Figs 3A and 3B), conditions where TGF-α was also detected. In addition, the

association of both PKC-δ and Shc with phosphotyrosine depended on the presence of the EGFR (Fig. 3B). These results suggest that the cell-associated TGF-α we detected or some other EGFR ligand is functional in signaling.

Although the EGFR is required for calcium-induced PKC-δ tyrosine phosphorylation (Fig. 3), the EGFR doesn't directly phosphorylate PKC-δ *in vitro* (24). The Src and Fyn tyrosine kinases are activated in response to EGFR activation and do phosphorylate PKC-δ *in vitro* (24, 40). However, the activation of Src by Ca^{2+} and ionophore treatment of human keratinocytes is not specific for keratinocyte differentiation since it also occurs in kidney tubular cells and fibroblasts (10, 41). Fyn is the only known Src family kinase activated during Ca^{2+}-induced differentiation of mouse keratinocytes, and Src family kinases are involved in the regulation of both differentiation and cell–cell adhesion in the epidermis (7, 42). The kinetics of PKC-δ tyrosine phosphorylation in Fig. 1 are consistent with the kinetics of Fyn activation during keratinocyte differentiation (7) suggesting that Fyn is the proximal kinase phosphorylating PKC-δ.

Several signaling molecules downstream from the exogenously stimulated EGFR are not activated or tyrosine phosphorylated during calcium-induced keratinocyte differentiation. These including PLC-γ_1, phosphatidylinositol 3-kinase (8), ras (43), and MAP kinase (44). In fact, the Ca^{2+} signal for differentiation blocks EGF-induced activation of ras/MAP kinase pathway at the level of ras activation (44). Extracellular Ca^{2+} also blocks EGF-induced DNA synthesis in keratinocytes, thus supporting a role for activation of the ras/MAP kinase branch of the EGFR signaling cascade in cell proliferation (45). Taken together, extracellular Ca^{2+} induces the accumulation of cell-associated TGF-α and activates a subset of EGFR signaling components, while simultaneously inhibiting others.

The EGFR tyrosine kinase is critical for maintenance of skin homeostasis. Genetic disruption of the EGFR in mice resulted in multiple defects in skin organization, including aberrant hair follicle development/morphogenesis, thin epidermis, and reduced numbers of cornified cell layers in certain mouse strains (27, 46, 47). In the EGFR −/− mice used in this study (CD-1 genetic background), the primary defect in the epidermis was a generalized disorganization and premature hair follicle maturation (27, 46). If PKC-δ is normally tyrosine phosphorylated in EGFR +/+ differentiating hair follicles, then in EGFR −/− follicles, the lack of PKC-δ tyrosine phosphorylation may accelerate the differentiation of the follicular keratinocytes. The EGFR has been localized to the basal and immediate suprabasal layers of the epidermis by immunohistochemistry (48) and to the basal layer by ^{125}I-EGF binding (49, 50). ^{125}I-EGF binding also decreases in keratinocytes induced to differentiate with Ca^{2+} (51). Decreased levels of EGFR in the differentiating layers of the epidermis may be due to ligand-induced down-regulation (52) or occupancy of the receptor (Fig. 2)

Thus, multiple kinase systems are integrated into the regulation of keratinocyte differentiation. We have demonstrated a novel cross-talk between PKC and tyrosine kinases, two signaling components required for proper keratinocyte differentiation. This integration of signaling cascades may be required for the tight control of normal epidermal stratification and differentiation. In light of our findings that PKC-δ is tyrosine phosphorylated *in vivo*, it will be of interest to assess whether alterations in this signaling pathway occur and play a functional role in skin diseases such as ichthyosis, psoriasis, and cancer where the balance between growth and differentiation in the skin is disrupted.

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