PKC-δ and -η, MEKK-1, MEK-6, MEK-3, and p38-δ Are Essential Mediators of the Response of Normal Human Epidermal Keratinocytes to Differentiating Agents

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Previous studies suggest that the novel protein kinase C (PKC) isoforms initiate a mitogen-activated protein kinase (MAPK) signaling cascade that regulates keratinocyte differentiation. However, assigning these functions has relied on treatment with pharmacologic inhibitors and/or manipulating kinase function using over-expression of wild-type or dominant-negative kinases. As these methods are not highly specific, an obligatory regulatory role for individual kinases has not been assigned. In this study, we use small interfering RNA knockdown to study the role of individual PKC isoforms as regulators of keratinocyte differentiation induced by the potent differentiating stimulus, 12-O-tetradecanoylphorbol-13-acetate (TPA). PKC-δ knockdown reduces TPA-activated involucrin promoter activity, nuclear activator protein-1 factor accumulation and binding to DNA, and cell morphology change. Knockdown of PKC downstream targets, including MEKK-1, MEK-6, MEK-3, or p38-δ, indicates that these kinases are required for these responses. Additional studies indicate that knockdown of PKC-η inhibits TPA-dependent involucrin promoter activation. In contrast, knockdown of PKC-α (a classical PKC isoform) or PKC-ε (a novel isoform) does not inhibit these TPA-dependent responses. Further studies indicate that PKC-δ is required for calcium and green tea polyphenol-dependent regulation of end responses. These findings are informative as they suggest an essential role for selected PKC and MAPK cascade enzymes in mediating a range of end responses to a range of differentiation stimuli in keratinocytes.

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INTRODUCTION

The protein kinase C (PKC) kinases comprise a family of enzymes that have a key role in regulating cell growth and differentiation. PKC isoforms are classified into three groups (Newton, 1997). The classical PKC forms (α, β, and γ) are calcium, phospholipid, and diacylglycerol dependent; the novel PKCs (δ, ε, η, and θ) are activated by diacylglycerol and phospholipids, but they do not respond directly to calcium; and the atypical PKCs (ζ and λ) are calcium and diacylglycerol independent but undergo allosteric activation (Nishizuka, 1992; Rosse *et al.*, 2010). Epidermal keratinocytes express the PKC α, βII, δ, ε, η, and ζ isoforms (Osada *et al.*, 1990; Dlugosz *et al.*, 1992; Gherzi *et al.*, 1992; Matsui *et al.*, 1992; Fisher *et al.*, 1993; Shen *et al.*, 2001; Hara *et al.*, 2005). The role of these isoforms has been studied in cultured cells and animal models (Dlugosz and Yuspa, 1994; Acs *et al.*, 2000; Denning *et al.*, 2000; Wheeler *et al.*, 2002; Verma *et al.*, 2006; Aziz *et al.*, 2009; Jerome-Morais *et al.*, 2009). A major ongoing effort is assigning specific functions to individual PKC isoforms in regulating keratinocyte proliferation, differentiation, and apoptosis. This is difficult, as PKC isoforms are activated by common stimuli and share common substrates. For example, the classical (α) and the three novel (δ, ε, and η) PKC isoforms expressed in keratinocytes can be activated by the diacylglycerol analog, 12-O-tetradecanoylphorbol-13-acetate (TPA).

We and others have shown that the novel PKC isoforms stimulate keratinocyte differentiation. This is evidenced by an increase in differentiation-associated responses in the presence of increased nPKC expression (Eckert *et al.*, 2004). These nPKCs, in turn, activate mitogen-activated protein kinase (MAPK) signaling, which results in increased nuclear levels of activator protein-1 (AP-1), C/EBP, and Sp1, and increased binding of these factors to target genes to increase transcription (Efimova and Eckert, 2000; Efimova *et al.*, 2002; Eckert *et al.*, 2004). PKC-δ is the most potent of these activators (Deucher *et al.*, 2002) and its activity can be inhibited by a PKC-δ inhibitor, rottlerin (Efimova and Eckert,

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Abbreviations: AP-1, activator protein-1; EGCG, (-)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; Fra-1, Fos-related antigen-1; hINV, human involucrin; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PKC, protein kinase C; siRNA, small interfering RNA; TPA, 12-O-tetradecanoylphorbol-13-acetate

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2000; Efimova *et al.*, 2004; Zhu *et al.*, 2008). A complicating feature of these studies is that they rely on PKC isoform overexpression, expression of dominant-negative kinases, or the use of isoform-selective chemical inhibitors. Each of these approaches has deficiencies, including the fact that overexpression of a particular PKC isoform or a dominant-negative form may influence the activity of other isoforms, and the fact that most inhibitors are not specific for the target kinase. An example is dominant-negative PKC-δ that inhibits PKC-δ-, PKC-ε- and PKC-η-dependent human involucrin (hINV) promoter activation (Efimova and Eckert, 2000). This may be because dominant-negative PKC-δ may compete for common substrates with other PKC isoforms (Efimova and Eckert, 2000). Thus, these studies, in spite of their utility, do not adequately address the role of individual isoforms.

Previous studies also suggest that specific MAPK cascade enzymes, including MAPK kinase kinase-1 (MEKK-1), MAPK/extracellular signal-regulated kinase (ERK) kinase 3 (MEK-3), and p38-δ, are required for activation of differentiated gene expression in keratinocytes (Efimova *et al.*, 1998). On the basis of these studies, we proposed that among the three p38 isoforms (α, β, and δ) that are expressed at reasonable levels in keratinocytes (Dashti *et al.*, 2001a, b), p38-δ has the dominant role as a regulator of involucrin gene expression (Efimova *et al.*, 1998, 2003). However, here again, assigning function relied on the use of wild-type and dominant-negative kinase overexpression, and kinase inhibitors (Efimova *et al.*, 1998; Eckert *et al.*, 2003, 2004, 2006).

In this study, as an alternative approach to avoid some of these pitfalls, we use small interfering RNA (siRNA) to reduce the level of individual PKC isoforms and individual MAPK cascade kinases, and then challenge the cells with TPA, a stable analog of diacylglycerol; calcium, a physiologic inducer of keratinocyte differentiation; and (-)-epigallocatechin-3-gallate (EGCG), a chemopreventive agent that stimulates keratinocyte differentiation (Efimova *et al.*, 1998; Efimova and Eckert, 2000; Balasubramanian *et al.*, 2002; Balasubramanian and Eckert, 2004). We examine the effect of reducing individual kinase level on keratinocyte morphology, nuclear transcription factor accumulation, and gene activation. These studies suggest that PKC-δ is a required mediator of the keratinocyte response to several differentiating agents, and that it controls a range of downstream biochemical and morphological end points. PKC-η, and the MAPK cascade enzymes, MEKK-1, MEK-6, and MEK-3, and p38-δ MAPK are also important. In contrast, PKC-α, PKC-ε, p38-α, and p38-β seem not to be the required mediators of these responses.

RESULTS

PKC-δ is required for stimulus-dependent hINV promoter activity

Previous studies show that overexpression of the novel PKC isoforms, δ, ε, or η, leads to increased hINV gene expression (Efimova *et al.*, 2002). To distinguish which of these isoforms is mainly responsible for this regulation, we used siRNA-mediated knockdown. We used TPA as a differentiation stimulus, because TPA has a discrete mechanism of action, as a diacylglycerol mimic (Kazanietz, 2005), and because it is a strong inducer of morphological and biochemical differentiation in keratinocytes (Welter *et al.*, 1995). As shown in Figure 1a and b, knockdown of PKC-δ or PKC-η reduces TPA-dependent hINV promoter activation, but knockdown of PKC-ε is without effect. We also examined the effect of a classical PKC isoform, PKC-α, and show that knockdown has a minimal effect on promoter activity. Thus, among the novel PKC isoforms, only PKC-δ and PKC-η are required as mediators of TPA-dependent hINV promoter activation. In contrast, basal promoter activity (absence of TPA treatment) is not influenced by knockdown of the PKC isoforms.

PKC-δ is required for nuclear accumulation of AP-1 transcription factors and change in cell morphology

PKC-δ was selected for detailed study because its mechanism of action in keratinocytes is heavily studied, it has a central role in controlling keratinocyte processes (Deucher *et al.*, 2002; Wheeler *et al.*, 2002; D'Costa and Denning, 2005; D'Costa *et al.*, 2005; Sitailo *et al.*, 2006; Zhu *et al.*, 2008), and it is an effective regulator of involucrin gene expression (Efimova *et al.*, 2002, 2004). We first examined whether the effect of reduced PKC-δ may be mediated through effects on expression of other PKC isoforms. As shown in Figure 1c, PKC-δ knockdown does not alter the level of other PKC isoforms.

We next examined the effect of reduced PKC-δ level on TPA-dependent downstream responses. Activation of PKC and MAPK kinase signaling has been shown to cause AP-1 factor movement into the nucleus in keratinocytes (Welter *et al.*, 1995). As shown in Figure 2a, TPA-dependent nuclear accumulation of junB, c-fos, junD, and Fos-related antigen-1 (Fra-1) (Welter *et al.*, 1995) is attenuated when PKC-δ level is reduced. A key question is whether this decrease leads to reduced AP-1 factor interaction with binding sites on the hINV promoter. Figure 2b is a gel mobility shift assay that shows increased AP-1 factor DNA binding in response to TPA treatment, and this increase is reduced in extracts from PKC-δ knockdown cells. This is specific binding, as it is competed by radioinert AP-1 site oligonucleotide (Figure 2c).

We next explored whether PKC-δ is required for other end responses. TPA treatment is associated with cell elongation and vesicle accumulation (Efimova and Eckert, 2000; Efimova *et al.*, 2002, 2003). Figure 3a shows that these morphological changes are reduced by PKC-δ knockdown. For example, intracellular accumulation of vacuoles is associated with TPA treatment. Untreated cells lack these vacuoles, but approximately 45–50% of TPA-treated cells are vacuole positive (arrows). In the presence of PKC-δ siRNA, TPA-dependent vacuole formation is reduced to $<7\%$ of cells (Figure 3b). A similar reduction is observed for the formation of elongated spindle-shaped cells. Thus, these findings indicate that PKC-δ is required for TPA-dependent gene activation, nuclear AP-1 factor accumulation, and morphological response. We also compared the role of PKCs α, ε, and η in mediating TPA-dependent morphology change. Figure 3b shows that PKC-η knockdown partially reduces the morphological response, but that PKC-α or PKC-ε knockdown does not restore normal morphology.