Recent work has shown that peroxisome proliferator-activated receptor-β (PPARβ) attenuates cell proliferation and skin carcinogenesis, and this is due in part to regulation of ubiquitin C expression. In these studies, the role of PPARβ in modulating ubiquitin-dependent protein kinase Cα (PKCα) levels and phosphorylation signaling pathways was evaluated. Intracellular phosphorylation analysis showed that phosphorylated PKCα and other kinases were lower in wild-type mouse skin treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) as compared with PPARβ-null mouse skin. No differences in expression levels of other PKC isoforms present in skin were observed. Lower ubiquitination of PKCα was found in TPA-treated PPARβ-null skin as compared with wild-type, and inhibition of ubiquitin-dependent proteasome degradation prevented TPA-induced down-regulation of PKCα. The activity of PKCα and downstream signaling kinases is enhanced, and expression of cyclooxygenase-2 (COX-2) is significantly greater, in PPARβ-null mouse skin in response to TPA compared with wild-type mouse skin. Inhibition of PKCα or COX-2 reduced cell proliferation in TPA-treated PPARβ-null keratinocytes in a dose-dependent manner, whereas it only slightly influenced cell proliferation in wild-type keratinocytes. Combined, these studies provide strong evidence that PPARβ attenuates cell proliferation by modulating PKCα/Raf1/MEK/ERK activity that may be due in part to reduced ubiquitin-dependent turnover of PKCα.

Intracellular communication with the external environment, including xenobiotic exposure, can be mediated by receptor/ligand-binding activity of a number of soluble receptors. Receptor activation in response to ligand binding leads to changes in target gene expression that ultimately modulate cellular processes but can also be influenced by intracellular communication facilitated by various signaling networks. Among the pathways that can influence receptor-mediated signaling are protein phosphorylation events that are central to the mechanisms that modulate various signaling pathways leading to changes in cell proliferation, differentiation, and/or apoptosis.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily and are ligand-activated transcription factors (1–3). PPARs modulate target gene expression in response to ligand activation after heterodimerization with retinoid X receptor α and binding to peroxisome proliferator-responsive elements of target genes. There are three different PPAR isoforms, designated PPARα, PPARβ (also referred to as PPARδ), and PPARγ, each with distinct physiological functions (1–3). For example, PPARα mediates the pleiotropic effects of peroxisome proliferators, in particular the regulation of lipid homeostasis and rodent hepatocarcinogenesis (4–7). In contrast to the lipid catabolic function of PPARα, PPARγ mediates adipocyte differentiation and is particularly important for the regulation of glucose homeostasis (8–10). A number of studies have reported that PPARβ is involved in adipocyte proliferation and differentiation (11–13), regulation of lipoprotein metabolism and atherosclerosis (14–16), regulation of skeletal muscle and cardiac fatty acid catabolism (17–19), colon cancer (20–22), and epithelial cell proliferation and differentiation (23–27).

The hyperplastic response to TPA in the epidermis is enhanced in PPARβ-null mice, suggesting that this receptor is critical for attenuating epidermal cell proliferation (24, 28). The exacerbated TPA-induced hyperplasia observed in PPARβ-null mice is likely the result of the lack of increased apoptosis and enhanced replicative DNA synthesis (25). Results from a two-stage carcinogen bioassay show that skin tumor formation, tumor size, and tumor multiplicity are significantly enhanced in PPARβ-null mice compared with wild-type mice (25), demonstrating that PPARβ-dependent attenuation of cell proliferation is functionally significant. However, the mechanisms by which PPARβ mediates attenuation of cell proliferation remain to be fully elucidated. Evidence exists demonstrating that TPA-induced expression of ubiquitin C and the resulting intracellular free ubiquitin pool is transcriptionally regulated by PPARβ and that ubiquitination of proteins induced by TPA is reduced in the absence of PPARβ expression (25). That PPARβ-dependent regulation of ubiquitin C expression contributes to the

*This work supported by National Institutes of Health Grants CA89607 and CA97999 (to J. M. P.) and ES04869 (to G. H. P.). 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.

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase/ERK kinase; COX-2, cyclooxygenase-2; TBST, Tris-buffered saline with 0.1% Tween-20; LDH, lactate dehydrogenase; MOPS, 4-morpholinoethanesulfonic acid; HBDDE, 2,2′,3,3′,4,4′-hexahydro-1,1′-biphenyl-6,6′-dimethanol dimethyl ether; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; ANOVA, analysis of variance.
mechanisms leading to attenuation of cell proliferation is supported by the observation that inhibition of ubiquitin-dependent proteasome-mediated protein turnover leads to exacerbated hyperplasia in wild-type mouse skin (25). A number of cell signaling components, including kinases such as protein kinase C, are involved in regulating cell cycle progression and apoptosis. Further, the presence of these kinases can be modulated by protein turnover that is mediated by the ubiquitin-proteasome pathway (29, 30). For these reasons, in these studies, the role of PPARβ in the regulation of kinases known to stimulate signaling cascade(s) associated with epithelial cell proliferation was examined.

EXPERIMENTAL PROCEDURES

Mouse Treatments—To examine the role of PPARβ on early events mediated by tumor promotion, skin samples were isolated within 8 h post-TPA application (5 µg/mouse) from both wild-type or PPARβ-null mice (24) and used for isolation of either protein or total RNA as described below. To examine the role of PPARβ after repeated exposure to tumor promotion, wild-type or PPARβ-null mice were treated topically with TPA (5 µg/mouse), followed 48 h later by an additional topical treatment with TPA. Twenty-four hours after the second treatment with TPA, skin samples were obtained from mice and used for isolation of either protein or total RNA as described below.

Protein Isolation—Skin samples from wild-type or PPARβ-null (control and TPA-treated) mice were used to prepare cytosolic proteins. Skin samples were placed in liquid nitrogen and then ground to a fine powder before evaporation of the liquid nitrogen. Ground skin was then homogenized further in buffer containing 20 mM MOPS (pH 7.2), 5 mM EDTA, 2 mM EGTA, and protease inhibitors. Homogenates were centrifuged at 100,000 × g, and the supernatant was used for the cytosolic fraction. The pellet was used for the microsome fraction. The protein concentration of each sample was quantified using the BCA detection system (Pierce).

Phosphoprotein Analysis—Detection of phosphorylated proteins that regulate signaling cascade(s) associated with epithelial cell proliferation was performed by Kinexus using the Kinetworks™ KPSS-1.0 Screen (Kinexus Inc., Cary, Canada) using cytosolic protein samples from wild-type or PPARβ-null (control and TPA treated) mouse skin isolated 8 h post-TPA treatment (5 µg/mouse/application). Proteins of interest were further examined using Western blot analysis to confirm differences detected with the Kinetworks™ KPSS-1.0 Screen. 25–50 µg of cytosolic protein from wild-type or PPARβ-null (control and TPA treated) skin samples or keratinocytes was resolved using SDS-PAGE. The samples were transferred onto a nitrocellulose membrane using an electroblotting method. After blocking in 5% milk in TBST, the membrane was incubated overnight at 4 °C with primary antibody, followed by incubation with a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive proteins were detected after incubation in 125I-labeled streptavidin (Amersham Bioscience). The intensity of the immunoreactive proteins was measured by phosphorimaging analysis. Hybridization signals for specific proteins of interest were normalized to the hybridization signal of the housekeeping protein, lactate dehydrogenase (LDH). The following primary antibodies were used: anti-phospho-PKCα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-MAPK (Cell Signaling Technology, Inc., Beverly, MA), anti-phospho-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA), anti-ubiquitin (Cell Signaling Technology, Inc., Beverly, MA), anti-phospho-PKCα (BD Biosciences) pre-bound to Protein G PLUS-agarose (Santa Cruz Biotechnology). Mouse IgG (Santa Cruz Biotechnology) bound resin was used for control immunoprecipitations. Binding of antibody to resin was performed in phosphate-buffered saline at 4 °C with rocking. After washing, skin cytosol was immunoprecipitated in the presence of buffer for 1.5 h with rocking. After immunoprecipitation, samples were washed five times in buffer containing 1% Nonidet P-40, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was washed with TBST, blocked with 5% milk in TBST, and probed with either a polyclonal anti-ubiquitin antibody (Sigma) or a polyclonal anti-PKCα antibody (Santa Cruz Biotechnology). Immunoreactive protein was detected after incubation in horseradish peroxidase-conjugated anti-rabbit antibody using ECL (Amersham Biosciences). The intensity of the immunoreactive proteins was determined with Image J software (Version 1.30, National Institutes of Health).

Co-treatment of TPA and MG-132—Wild-type or PPARβ-null mice were treated topically with MG-132 twice as described previously (25) to inhibit ubiquitin-dependent proteasome degradation, followed by 5 µg of TPA dissolved in 200 µl of acetone 15 min after the last MG-132 application. Skin samples were collected 8 h post-TPA application for Western blot analysis of PKCα as described above.

PPARβ Inhibition of Epithelial Cell Proliferation—PKCα activity was determined by measuring the ability of activated PKC to phosphorylate a substrate in vitro (Upstate Biotechnology Inc.). Raf1 activity and ERK1/2 activity were determined by measuring the activity of immunoprecipitated Raf1 or ERK1/2 to phosphorylate a substrate in vitro (Upstate Biotechnology Inc.). Cytosol from mice treated topically twice with TPA as described above was used for these assays, following the manufacturer’s recommended protocol.

Keratinocyte Culture—A described previously method (31) was used to culture primary keratinocytes obtained from 2-day-old neonates from wild-type or PPARβ-null mice. Briefly, equivalent numbers of keratinocytes were seeded and cultured in low calcium medium (0.05 mM). After 48 h of culture, culture medium was replaced to either control low calcium medium, or low calcium medium containing selective inhibitors of PKCα (HBDDE, 0.4–25.0 µM), or chelerythrine chloride, 0.02–1.0 µM) and COX-2 (NS-398, 5–10 µM), after the 1-h pulse treatment with TPA. After 24, 48, or 96 h of culture, keratinocytes were homogenized, 10 µg of protein was loaded on a 1% agarose gel containing 0.22M formaldehyde, transferred to a nylon membrane, and cross-linked with ultraviolet to fix the RNA. Membranes were hybridized in ULTRAhyb hybridization buffer (Amersham Biosciences) with described previously random primed probes (24), following the manufacturer’s protocol, and washed with salt/detergent solutions using standard procedures.

RESULTS

PPARβ-dependent Regulation of PKCα—To determine if PPARβ influences kinase signaling associated with epidermal cell proliferation and skin carcinogenesis, a Kinetworks™ KPSS-1.0 Screen was performed. Of the proteins examined, significantly higher levels of phosphorylated PKCα, MEK1/2, and p42MAPK/ERK-2 were found in the skin of PPARβ-null mice treated with TPA as compared with TPA-treated wild-type mice (data not shown). Western blot analysis confirmed the presence of PKCα in phosphoprotein bands detected with the Kinetworks™ KPSS-1.0 Screen (Fig. 1A and 2B). Notably, a lower level of phospho-PKCα was detected in wild-type mouse skin 8 h post-TPA treatment, whereas the level of phospho-PKCα was greater in similarly treated samples from PPARβ-null mice (Fig. 1A).

Previous work has demonstrated that PKCα turnover induced by TPA is mediated by ubiquitin-dependent proteasomal degradation (29, 30). To determine if down-regulation of PKCα by TPA treatment in skin is a consequence of reduced ubiquitination, PKCα was immunoprecipitated with an anti-PKCα antibody and probed with either an anti-ubiquitin or anti-PKCα.
antibody. There was a time-dependent increase in the level of PKCα ubiquitination between 1 and 8 h post-TPA in wild-type mouse skin, and this effect was not found in TPA-treated PPARβ-null mouse skin (Fig. 1B). This suggests that PPARβ-null mice are refractory to TPA-induced ubiquitination of PKCα.

The elevated level of PKCα associated with a diminished rate of ubiquitination in PPARβ-null mice suggests that the increased level of PKCα could be the result of reduced ubiquitin-dependent proteasome-mediated turnover. To examine this hypothesis, the effect of proteasomal inhibition by MG-132 on ubiquitination of PKCα expression was evaluated by Western blot analysis. As shown previously (Fig. 1A), TPA treatment caused a reduction in phospho-PKCα in wild-type mice (Fig. 1C). Co-treatment with MG-132, a potent inhibitor of proteasome-mediated protein turnover (using the same protocol that results in exacerbated TPA-induced hyperplasia in wild-type mouse skin (25)), prevented the effect of TPA alone, resulting in a 30% increase in phospho-PKCα, whereas MG-132 alone had no effect of PKCα levels (Fig. 1C). Combined, these results suggest that the lack of TPA-induced expression of ubiquitin C in the absence of PPARβ that is known to lead to lower levels of free ubiquitin and ubiquitinated proteins (25), contributes to reduced turnover of PKCα and subsequent ubiquitin-dependent proteasome-mediated degradation.

To determine the role of PPARβ in mediating alterations in PKCα levels and activity in a system that more closely models that which occurs during a two-stage chemical carcinogen bioassay (e.g. repeated treatment with TPA), the effect of TPA after two topical doses was examined. The level of phospho-PKCα was lower than control in wild-type mice after two repeated TPA treatment (Fig. 1D). In contrast, the level of phospho-PKCα in PPARβ-null mice was significantly higher than similarly treated wild-type mice, but lower as compared with control PPARβ-null mice (Fig. 1D). To determine if the difference in PKCα levels detected was specific for PKCα, analy-
ysis of the other PKC isoforms found in skin (32) was performed. Although the level of phosphorylated PKCδ was unchanged in response to TPA treatment in either genotype, the level of phosphorylated PKCe and PKCζ was decreased and increased, respectively, after TPA treatment (Fig. 1D). No difference in the level of phosphorylated PKCe or PKCζ was detected between genotypes. The non-phosphorylated form of PKCζ was also increased in response to TPA in both genotypes (Fig. 1D). Combined, these results show that the change in PKCζ levels in TPA-treated wild-type and PPARβ-null mouse skin are specific for this PKC isoform.

PKC activity was measured with an in vitro kinase assay to determine if the differences detected by Western blot analysis reflected functional differences in enzyme activity. The data revealed a strong correlation between the protein level of PKCα and total PKC activity in both wild-type and PPARβ-null skin cytosol. In wild-type samples, PKC activity decreased after TPA treatment (Fig. 1E), consistent with differences detected by Western blot analysis (Fig. 1D). In contrast, PKC activity was markedly higher in PPARβ-null skin samples (Fig. 1E) as compared with controls, and this was also similar to the level of protein detected.

**Downstream Targets of PKCα Are Elevated in TPA-treated PPARβ-null Mouse Skin—**PKCα can activate a wide range of downstream targets, including the Raf1 and MAPK/ERK pathways (33–35). To determine if the increase in PKC activity observed in PPARβ-null mouse skin leads to activation of these signaling components known to exert positive effects on cell proliferation, enzyme activity was measured and/or Western blot analysis was performed. Raf1 activity measured with an in vitro kinase assay was unchanged by repeated TPA treatment in wild-type mouse skin cytosol (Fig. 2A), whereas Raf1 activity was significantly greater in TPA-treated PPARβ-null mouse skin cytosol compared with control (Fig. 2A). TPA treatment resulted in a decrease in the level of phospho-MEK1/2 and a corresponding decrease in phospho-ERK1/2 in wild-type mice (Fig. 2B). In contrast, TPA treatment resulted in a marked increase in both phospho-MEK1/2 and phospho-ERK1/2 in PPARβ-null mouse skin (Fig. 2B). To determine if the difference in protein levels reflected an increase in enzyme activity, an in vitro kinase assay was performed. Indeed, specific activity of ERK1/2 was significantly higher in cytosol from PPARβ-null mouse skin treated with TPA than similarly treated cytosol from wild-type mouse skin (Fig. 2C). These results suggest that the increase in PKCα activity observed in TPA-treated PPARβ-null mouse skin is transmitted down to the Raf1/MAPK/ERK signal transduction pathway. As such, it would be expected that expression of target genes, known to be regulated by this pathway such as COX-2, would also be increased in PPARβ-null skin treated with TPA. Northern blot analysis was performed on mRNA isolated from wild-type and PPARβ-null mouse skin after TPA treatment. Both genotypes exhibited a marked increase in mRNA-encoding COX-2 in response to TPA as compared with controls (Fig. 3A). However, the increase in mRNA-encoding COX-2 induced by TPA was significantly greater in PPARβ-null samples than in similarly treated wild-type (Fig. 3A). Western blot analyses were also performed to determine if the increase in mRNA-encoding COX-2 was reflected by similar changes in protein levels. Indeed, the expression level of COX-2 was markedly higher in PPARβ-null samples after long-term and repeated treatments with TPA, as compared with similarly treated wild-type mouse skin (Fig. 3, A and B).

Enhanced TPA-induced Cell Proliferation in PPARβ-null Keratinocytes Is Dependent on PKCα and COX-2 Activity—The former results demonstrate that, in the absence of PPARβ
expression, enhanced activity of the PKCα/Raf1/MEK/ERK pathway is observed. Increased activity of these pathways is known to lead to increased cell proliferation in a wide range of cell lineages. To determine if these changes specifically contribute to the exacerbated epidermal cell proliferation observed in response to TPA (24, 25), primary keratinocytes were cultured in the presence or absence of specific PKCα or COX2 inhibitors and relative cell proliferation was determined. Interestingly, PPARβ-null keratinocytes proliferate faster (−2×) than wild-type keratinocytes as shown microscopically (Fig. 4A) and by quantification (Fig. 4B). Keratinocytes from both genotypes exhibited a decrease in growth rate after a 1-h pulse treatment with TPA (Fig. 4C). However, after the short lag period observed following the 1-h pulse treatment of TPA, PPARβ-null keratinocytes began to proliferate faster (−5×) than wild-type keratinocytes (Fig. 4C).

Northern blot analysis was performed to determine the expression of mRNAs encoding cell-cycle regulatory proteins, after the pulse treatment with TPA. Constitutive expression of mRNA-encoding cyclin B1 and proliferating cell nuclear antigen was detected in keratinocytes from both genotypes (Fig. 4D). Expression of mRNA-encoding cyclin B1 was gradually decreased, following a significant increase, 9 h after the 1-h pulse treatment of TPA in PPARβ-null keratinocytes, whereas expression of mRNA-encoding cyclin B1 was gradually decreased in wild-type keratinocytes during the same time frame (Fig. 4D). No significant differences in the expression of mRNA-encoding proliferating cell nuclear antigen were observed between genotypes within the first 5 h of culture following TPA treatment. However, a 40% increase in mRNA-encoding proliferating cell nuclear antigen was observed in PPARβ-null keratinocytes after 9 h of culture, and this effect was not found in similarly-treated wild-type keratinocytes (Fig. 4D).

To determine if the increased proliferation rate of PPARβ-null keratinocytes is a consequence of increased PKCα signal transduction, wild-type and PPARβ-null keratinocytes were cultured in the presence of the specific PKCα inhibitors, HB-DDE or chelerythrine chloride. Keratinocytes from PPARβ-null mice exhibited significant dose-dependent inhibition of cell proliferation (Fig. 5, A and B). Both HB-DDE and chelerythrine chloride inhibited cell proliferation in wild-type keratinocytes after TPA treatment; however, this effect was not as marked as compared with PPARβ-null keratinocytes (Fig. 5, A and B). Further, PPARβ-null keratinocytes cultured in the presence of the selective COX-2 inhibitor, NS-398, exhibited significant dose-dependent inhibition of cell proliferation, and this effect was not as marked in wild-type keratinocytes after TPA treatment (Fig. 5C).

DISCUSSION

Results from the present studies, coupled with results from previous studies, provide considerable evidence that PPARβ-dependent up-regulation of ubiquitin C expression and the free intracellular ubiquitin pool, contributes to down-regulation of PKCα and downstream kinases, which in turn leads to attenuation of cell proliferation (Fig. 6). PPARβ-dependent regulation of the ubiquitin C gene could be critical, due to the fact that this gene encodes for ten ubiquitin molecules as compared with ubiquitin B and ubiquitin A that encode for only four or one Ub molecule(s), respectively (36). Data from previous studies demonstrated that, in response to TPA, PPARβ up-regulates ubiquitin C expression in skin, which leads to increased free intracellular ubiquitin levels and increased ubiquitination of proteins, and that these events are absent in PPARβ-null mouse skin (25). Further, the induction of ubiquitin C that is mediated by PPARβ, is linked to the attenuation of epidermal cell proliferation in response to phorbol ester, because co-treatment of TPA with a proteasome inhibitor in wild-type mice resulted in an exacerbated epidermal hyperplastic response, a phenotype similar to that observed in the TPA-treated PPARβ-null mouse epidermis (25). One of the early targets for the ubiquitin-proteasome degradation pathway in response to phorbol ester is PKCα (29, 30). Results from the present studies demonstrate that PKCα levels are significantly lower in TPA-treated skin from wild-type mice, which is coincident with increased levels of ubiquitin C expression, increased intracellular free ubiquitin levels, and ubiquitination of other proteins (25). In contrast, PKCα levels are significantly greater in TPA-treated skin from PPARβ-null mice in the absence of increased expression of ubiquitin C. Specificity for PKCα is shown by the lack of significantly different expression levels of the other PKC isoforms found in skin between genotypes. Further, immunoprecipitation analysis indicates that, although TPA treatment results in increased ubiquitination of PKCα in wild-type mouse skin, ubiquitination of PKCα is lower in TPA-treated PPARβ-null mice. In addition, inhibition of the ubiquitin-dependent proteasomal degradation with MG-132 prevents TPA-induced down-regulation of PKCα. Collectively, these results suggest that one functional role for PPARβ, within the context of TPA-mediated tumor promotion, is to down-regulate PKCα, and that this likely results in attenuation of cell proliferation. This hypothesis is further supported by the observed decrease in
keratinocyte cell proliferation by specific PKCα inhibitors in PPARβ-null cells and the markedly diminished response observed in wild-type cells.

Disruption of the ubiquitin-proteasome pathway can significantly alter molecular and biochemical events due to imbalanced activity of proteins, including cell cycle regulatory proteins and tumor suppressor proteins, resulting in cell proliferation and cancer (37). Indeed, there is compelling evidence demonstrating dysregulation of the ubiquitin-proteasome pathway in a variety of cancers (37). However, both increased and decreased degradation of specific regulatory proteins have been linked to enhanced cell proliferation, inhibition of apoptosis, or increased tumor growth. For example, increased degradation of the cyclin-dependent kinase (CDK) inhibitor p27 mediated by the ubiquitin-proteasome pathways is associated with progression of tumor growth at both early and late stages of breast and colorectal carcinogenesis (38, 39). In contrast, decreased ubiquitin-proteasome-dependent degradation of cyclins is associated with increased levels of specific cyclins and enhanced cell proliferation in ovarian cancer cell lines (cyclin E) as well as squamous cell carcinomas (cyclin D1) (40–42) and may be associated with other human cancers (37, 43). Recent evidence has also demonstrated that a number of proto-oncogenes are regulated by ubiquitin-dependent protein turnover, including c-myc and c-fos (44–47), although the direct role of this type of regulatory pathway in cancer has not been critically evaluated to date. Together, this suggests that ubiquitin-dependent regulation of the cell cycle is likely very complex and cell-type specific. Therefore, in addition to polymorphisms or altered expression of specific proto-oncogenes and tumor suppressors, alterations in their turnover mediated by the ubiquitin-proteasome pathway also contribute to the molecular mechanisms underlying cancer. There is more than one mechanism by which ubiquitin-dependent protein turnover could be affected, including mutations/polymorphisms in substrate proteins for specific E2/E3 that lead to altered ubiquitination, mutations/polymorphisms in specific E2s or E3s

**FIG. 4.** PPARβ regulates keratinocyte proliferation. A, qualitatively enhanced proliferation of PPARβ-null (−/−) keratinocytes as compared with wild-type (+/+) Representative photomicrographs of primary keratinocytes cultured in low calcium medium were obtained after days 2–5 of culture. B, quantitatively enhanced proliferation of PPARβ-null (−/−) keratinocytes as compared with wild-type (+/−). Cell number was quantified with a Coulter counter over the 9-day culture period. *, significantly different than control (p ≤ 0.05) as determined by ANOVA. C, increased cell proliferation in TPA-treated (−/−) keratinocytes. Equivalent numbers of keratinocytes were seeded on 12-well plates, and treated with TPA (25 ng/ml) for 1 h, 3 days after plating. Cell number was quantified with a Coulter counter over the 9-day culture period. *, significantly different than control (p ≤ 0.05) as determined by ANOVA. D, enhanced expression of mRNA-encoding cyclin B1 and proliferating cell nuclear antigen in TPA-treated (−/−) keratinocytes. Primary keratinocytes from (+/+) and (−/−) mice were treated with TPA (25 ng/ml) as described, and RNA was isolated and used for Northern blot analysis using the indicated cDNA probes. Hybridization signals were quantified and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as -fold increase to respective control.
that lead to altered ubiquitination, and alterations in expression of the different Ub genes, as suggested from the present studies. Although there is a limited amount of information defining the role of the specific ubiquitin genes in the regulation of protein turnover, it has been shown that glucocorticoids can significantly regulate the level of free ubiquitin and subsequent ubiquitination of proteins in skeletal muscle through transcriptional modulation of ubiquitin C (48). This may be important clinically, because muscle catabolism occurring with diabetes through a similar mechanism may contribute to the symptoms associated with disease. Therefore, it will be of great interest to determine if other proto-oncogenes similar to PKCα, which are known to be regulated by the ubiquitin-proteasome degradation pathway, are also dysregulated in the absence of PPARβ expression and to determine if specific E3 ligases are associated with this turnover. Elucidation of this regulation may provide new molecular targets for the treatment and prevention of skin carcinogenesis.

Increased PKCα activity in response to TPA treatment is known to be associated with epidermal hyperplasia and tumor promotion (49). Overexpression of epidermal PKCα increases TPA-induced inflammation and expression of COX-2 and leads to enhanced epidermal hyperplasia (50). The phenotype of the PPARβ-null mouse in response to TPA treatment is consistent with these observations, because exacerbated inflammation and hyperplasia are noted from the present and previous studies (24, 25). Increased tumor multiplicity as a result of PKCα overexpression is not observed (50), suggesting that the increase in PKCα activity in PPARβ-null mouse skin is not the sole mechanism underlying increased tumor multiplicity previously observed (25). However, epidermal TPA treatment in the presence of overexpressed PKCα can enhance activation of the MAPK/ERK cascade (51). Additionally, PKCα can directly phosphorylate Raf1, which also feeds into the MAPK/ERK pathway, and this type of activation has been shown to cause
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transformation of NIH3T3 cells (33). Consistent with these reports, results from the current studies demonstrate that in the presence of increasingly phosphorylated PKCα, higher levels of activated MEK1, MAPK/ERK, and enhanced cell proliferation can be found. Because it is well known that COX-2 expression can be regulated by both changes in mRNA stability and transcription, and that these events are mediated by the MAPK/ERK pathway (55–56), it is of great interest to note that the induction of COX-2 resulting from TPA treatment, as measured at both the mRNA and protein level, was substantially higher in PPARβ-null mouse skin. This could be due to increased phosphorylation of nuclear transcription factors that leads to increased expression of target genes such as COX-2 (Fig. 6). Similar to the inhibition of cell proliferation observed with PKCα inhibition, COX-2 inhibition leads to marked inhibition of keratinocyte proliferation in TPA-treated PPARβ-null cells, an effect that is attenuated in wild-type cells that express significantly lower levels of COX-2. Results from the present, and previous studies (25), suggest that PPARβ attenuates epithelial cell proliferation and tumor formation by up-regulation of ubiquitin C and free intracellular ubiquitin pools. This leads to increased turnover of target proteins such as PKCα, thus preventing further kinase activity of RaI and MAPK/ERK that prolongs signaling events that lead to stimulation of cell proliferation (Fig. 6).

It is of interest to note that the exacerbated hyperplastic response to TPA observed in PPARβ-null mouse skin in vivo also occurs in cultured keratinocytes that do not express PPARβ. This demonstrates the usefulness of this model system for future studies to elucidate specific PPARβ-dependent mechanisms that regulate cell proliferation. The role of PPARβ in cell proliferation is somewhat controversial, as there is evidence suggesting that activation of PPARβ can promote cell growth in some models (55, 56) and inhibit cell proliferation in other models (25–27, 28). Some of these differences could be cell/tissue-specific. For example, in skin cells, deletion of PPARβ exacerbates cell proliferation in response to tumor promotion by TPA, in two distinctly different null mouse models (24, 25, 28). Additionally, potent PPARβ ligands can inhibit keratinocyte cell proliferation (27) and lead to reduced expression of cyclin A expression in cultured keratinocytes (23). This is consistent with the induction of keratinocyte differentiation that occurs in response to ligand activation of PPARβ (23, 26, 27), because chemicals that induce differentiation are more likely to cause slower cell growth, due in part to a diminished population of precursor cells. This is of interest because there are a number of reports that differentiation of other cell types can be induced by ligand activation of PPARβ, including F9 teratocarcinoma cells (57) and THP-1 monocytes (58). Therefore, results from the present studies provide further support for the hypothesis that ligand activation of PPARβ in skin will effectively inhibit cell proliferation and could be a useful molecular target for the treatment and prevention of skin cancer. This hypothesis is currently under examination in this laboratory.

Acknowledgment—The authors gratefully acknowledge Debra Wolgemuth for providing cDNA probes.

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J. Biol. Chem. 2005, 280:9519-9527.
doi: 10.1074/jbc.M413808200 originally published online January 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413808200

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