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Protein Kinase C Is Involved in the Regulation of *hairless* mRNA Expression during Mouse Keratinocyte Differentiation

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The *hairless* (*hr*) gene is a putative transcriptional factor whose mutations lead to hair loss in animals and humans. As a step toward understanding the role of the *hr* gene, we investigated the expression of *hr* mRNA in mouse keratinocyte differentiation. Treatment of mouse primary keratinocyte cultures with phorbol-12-myristate-13-acetate (PMA) reduced DNA synthesis and sequentially induced an up-regulation of p21Cip1/WAF1 (p21), *hr* and involucrin (inv) mRNAs in a time-dependent fashion, suggesting that an increase in *hr* gene expression is associated with keratinocyte differentiation. This up-regulation was blocked by the RNA synthesis inhibitor actinomycin D. However, an increase in *hr* mRNA, but not in inv mRNA, was seen in cells treated with the protein synthesis inhibitor cycloheximide, suggesting that new protein synthesis is involved in the suppression of *hr* transcription or in the degradation of *hr* mRNA in the steady state. The up-regulation of *hr* mRNA expression by PMA was blocked by the protein kinase C (PKC) inhibitor, GF109203X. These data indicate that PKC activation is involved in the up-regulation of *hr* mRNA expression during mouse keratinocyte differentiation. © 2001 Academic Press

***Key Words: hairless* gene; involucrin; protein kinase C; phorbol ester; GF109203X; keratinocytes.**

The *hairless* (*hr*) gene is a putative transcriptional factor that contains a single zinc-finger motif with potential DNA binding capacity. The *hr* gene was first isolated from the *hairless* (*hr/hr*) mice that carry a spontaneous proviral integration in the *hr* gene (1, 2). Subsequently, the highly conserved human and rat homologues were cloned (3, 4). Mutations at various locations of the *hr* gene have been identified in different *hairless* mouse strains (1, 5–7) as well as in humans with congenital papular artrichia (3, 8–12). At present, the molecular mechanism underlying the pathophysiology of hairlessness caused by the *hr* mutations remains unclear.

The expression of the *hr* gene has been detected in multiple tissues, with the highest level seen in the skin and brain (2, 10, 12). The *hr* gene is a thyroid hormone-responsive gene in developing rat brains, but the same hormone response was not observed in the skin (4). In the skin, *hr* expression was mainly detected in hair follicles and interfollicular epidermis (2), in consistent with the notion that *hr* plays an important role in the biology of hair follicles and keratinocytes. However, the regulation of *hr* expression in keratinocytes is not understood.

The epidermis is composed of four layers of keratinocytes each expressing a unique pattern of differentiation markers, with the basal cells maintaining the proliferative capacity and the capacity to differentiate as they move upward to the skin surface (13). Based on the observation that *hr* is highly expressed in the epidermis and hair follicles (2), we hypothesize that the *hr* gene may be involved in keratinocyte differentiation. To test this hypothesis, in the present study, we used mouse primary keratinocyte cultures to investigate *hr* expression. We found that an up-regulation of *hr* mRNA is associated with keratinocyte differentiation, and protein kinase C (PKC) activation is required for this regulation.

MATERIALS AND METHODS

Primary keratinocyte culture. Primary mouse keratinocytes were isolated and cultured according to a method established previously (14). Briefly, skins were harvested from 2- to 3-day-old C57BL6 mice and placed in 0.25% trypsin solution (Life Technologies, Rockville, MD). After digestion overnight at 4°C, the epidermis was mechanically separated from the dermis, and epidermal cells were released by gently grinding on a 50 μm tissue sieve (Bellco Glass, Vineland, NJ). The cells were cultured in 24-well plates, or in 10-cm dishes coated with 1% collagen solution (Cohesion, Palo Alto, CA), and

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grown in a low calcium medium containing 0.045 mM Ca2+ supplemented with 4% Chelex-treated FBS and 7.5 ng/ml epidermal growth factor (EGF) at 34°C and 7% CO_2. The cultured keratinocytes were treated with different agents as detailed in each experiment.

[3H]Thymidine incorporation assay. Primary keratinocytes were cultured in 24-well plates until 90% confluence. The cells were treated with different doses of phorbol-12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO) for 6 h and then pulse-labeled with 1.0 μCi/ml of [*methyl*-3H]thymidine (Amersham Pharmacia, Princeton, NJ) for 2 h. After wash sequentially with 0.5 ml of PBS twice and 0.5 ml of 10% TCA twice, the cells were lysed with 0.2 N NaOH. The lysates were added into 10 vol of ScintSafe 30% scintillation cocktail (Fisher Scientific, Itasca, IL), and counted for radioactivity using a liquid scintillation counter (Packard Instrument, Downers Grove, IL).

RNA isolation and Northern blot. Total RNA was isolated from primary keratinocytes using the Trizol reagent (Life Technologies) according to manufacturer's instructions. For Northern analysis, total RNA (25 μg/lane) was separated on a 1.2% agarose gel containing 0.6 M formaldehyde (15), transferred onto a nylon membrane (MSI, Westborough, MA) and crosslinked by UV light using a UV crosslinker (Bio-Rad, Hercules, CA). Hybridizations were performed according to the method described by Church and Gilbert (16) with cDNA probes labeled with [32P]dATP (New England Nuclear, Boston, MA) using the Prime-a-gene labeling system (Promega, Madison, WI). Murine *hairless* and involucrin cDNA probes were provided by Drs. J. Stoye (National Institute for Medical Research, London, England) and G. P. Dotto (Massachusetts General Hospital, Charlestown, MA), respectively. After hybridization, properly washed membranes were exposed to X-ray films at −80°C for autoradiography. The relative amount of mRNA was quantitated using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Variations in RNA loading were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Statistical analysis. Quantitative data were obtained from at least three independent experiments. Results were analyzed with Student's *t* test to assess significance. *P* values of 0.05 or lower were considered statistically significant.

RESULTS

It is known that PMA, a potent PKC activator (17, 18), is a strong inducer of keratinocyte differentiation (19). Thus, we first examined the effect of PMA on DNA synthesis in primary mouse keratinocytes by 3H-thymidine incorporation assays. As shown in Fig. 1, PMA inhibited DNA synthesis in these cells in a dose-dependent manner, with the maximal inhibition (60%) seen at 60 nM.

We then examined the mRNA levels of the cyclin-dependent kinase inhibitor p21Cip1/WAF1 (p21), *hr*, and the keratinocyte differentiation marker involucrin (inv) genes in mouse primary keratinocytes treated with different doses of PMA. As shown in Fig. 2, PMA up-regulated the expression of p21, *hr* and inv mRNAs in a dose-dependent manner, with the maximal effect seen at 60 nM. At this concentration, the mRNA level of p21, *hr* and inv was increased approximately 10-, 4-, and 15-fold over untreated controls, respectively (Figs. 2B–2D). Thus, 60 nM was used as the optimal concentration in the following experiments.

Figure 3 shows the temporal change of p21, *hr* and inv mRNA levels in keratinocytes treated with 60 nM

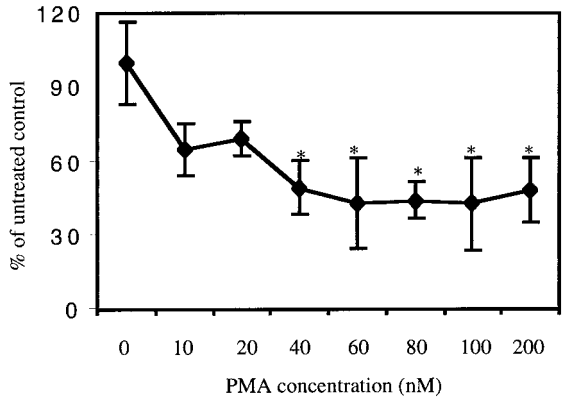

FIG. 1. Effect of PMA on the proliferation of mouse primary keratinocytes. Mouse keratinocytes were cultured in 24-well plates and treated for 6 h with different doses of PMA as indicated. Cells were then labeled with 1.0 μCi/ml of [3H]thymidine for 2 h before lysis. Incorporated radioactivity was counted with a scintillation counter. Each value was determined in triplicates, and results were averaged from three independent experiments. Data were expressed as mean percentage ± SD of untreated control cells. *$P < 0.01$ compared with untreated controls.

of PMA for various times. The up-regulation of p21 reached the maximal level at 2 h after PMA treatment, whereas the induction of *hr* and inv mRNAs were evident at 2 and 4 h, respectively, reaching the maximum at 6 h. After 6 h, the levels of these mRNAs gradually declined to the basal levels (Figs. 3A–3D).

To assess whether RNA synthesis is required for the up-regulation of *hr* and involucrin, the keratinocytes were co-treated with PMA and actinomycin D, a DNA-primed RNA polymerase inhibitor. As shown in Fig. 4, both the *hr* and inv mRNA inductions by PMA were blocked by actinomycin D in a dose-dependent manner, suggesting that a transcriptional mechanism may be involved in these inductions. Interestingly, when the cells were cotreated with PMA and cycloheximide, an inhibitor of protein translation, the induction of inv mRNA was completely blocked (Figs. 5A and 5C), but an increase in the *hr* mRNA level was observed in the cotreatment or in cycloheximide treatment alone, with a magnitude even higher than the PMA treatment (Figs. 5A and 5B). Indeed, cycloheximide alone increased the *hr* mRNA level in a time-dependent manner, which was evident at as early as 30 min after the treatment (Fig. 5D). This concentration of cycloheximide (20 μg/ml) has previously been shown to block protein synthesis by >95% in cultured mouse keratinocytes (20). Thus, the up-regulation of inv mRNA by PMA is dependent on new protein synthesis, whereas protein synthesis appears to reduce the *hr* mRNA level in keratinocytes.

To test whether the induction of *hr* mRNA by PMA involves the activation of the PKC signaling pathway, the expression of *hr* mRNA was examined in cultures co-treated with PMA and the PKC inhibitor

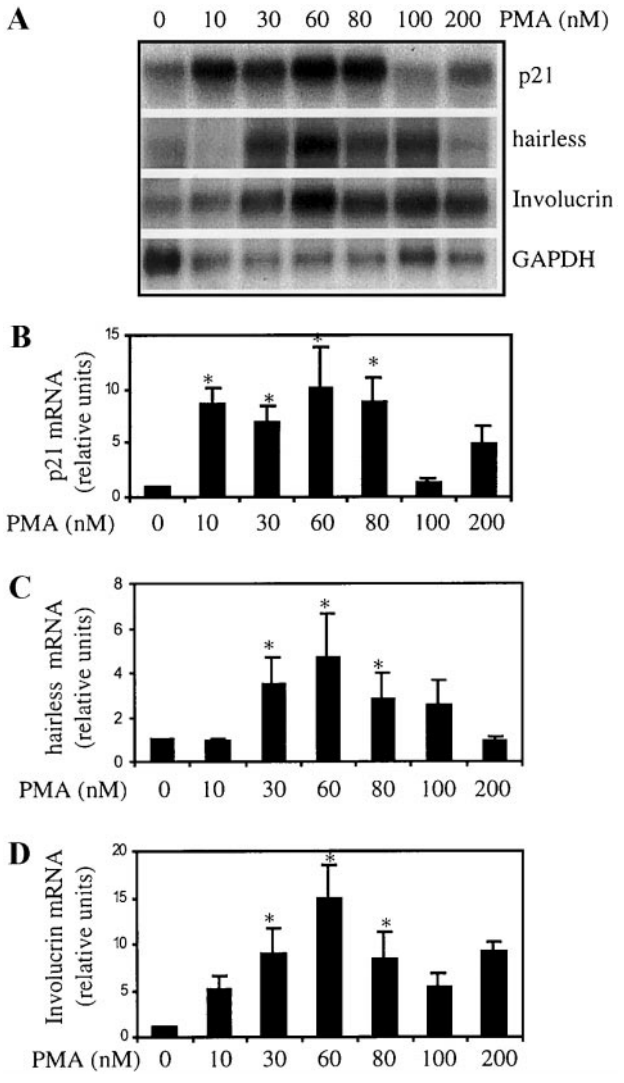

FIG. 2. Dose-dependent induction of p21, involucrin, and *hr* mRNA expression by PMA. (A) Representative Northern blot. Mouse keratinocytes were treated with different doses of PMA as indicated for 6 h. Total RNA was prepared, and Northern blots were hybridized sequentially with *hr*, involucrin, p21, and GAPDH cDNA probes, respectively. (B–D) Quantitation of p21, *hr*, and involucrin mRNA levels as indicated. The data were obtained from three independent experiments. $*P < 0.05$ compared with untreated controls.

GF109203X (21). As shown in Fig. 6, GF109203X blocked PMA-induced *hr* and inv mRNA expressions in a dose-dependent manner, with the maximal effect seen at 5 μM. These results strongly suggest that PKC activation is required for the up-regulation of *hr* and involucrin in mouse keratinocytes.

DISCUSSION

The observation that *hr* gene mutations lead to hair loss in both mice and humans (22) suggests that the *hr* gene plays a crucial role in hair growth. As hair growth involves massive cell proliferation and differentiation (23), and keratinocytes express a high level of *hr* (2), we ask whether *hr* expression is linked to keratinocyte differentiation. To this end, we have examined the expression of *hr* mRNA, in parallel with the expression of p21 and involucrin, in mouse keratinocytes treated with PMA, as PMA is a potent inducer of keratinocyte differentiation (19, 24). p21, the cyclin-dependent kinase inhibitor, has been shown to inhibit cell growth and promote differentiation (25), whereas involucrin is a differentiation marker of keratinocytes, as it is expressed in the granular layer of the epidermis and is cross-linked with loricrin and other proteins into insol-

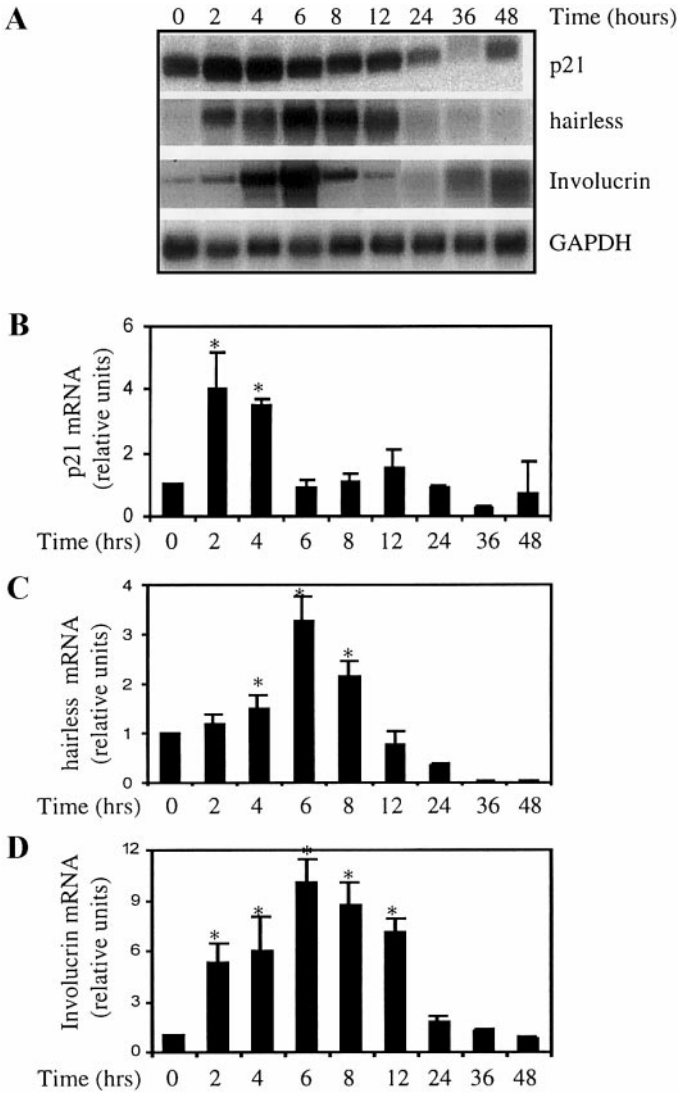

FIG. 3. Time course of p21, *hr*, and involucrin mRNA induction by PMA. (A) Representative Northern blot. Primary keratinocyte cultures were treated with 60 nM of PMA for different time as indicated. Total RNA was isolated and subject to Northern blot analyses with cDNA probes as indicated. (B–D) Quantitation of p21, *hr*, and involucrin mRNA levels, respectively. Quantitative results were obtained from three independent experiments. $*P < 0.05$ compared with untreated controls.

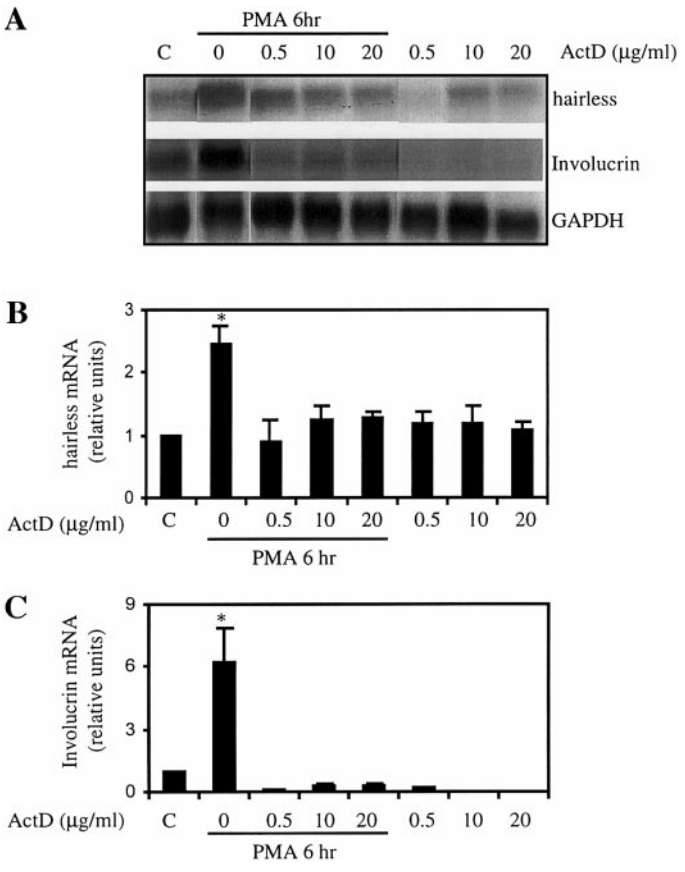

FIG. 4. Effect of actinomycin D on the up-regulation of *hr* and involucrin mRNA expression. (A) Primary keratinocyte cultures were pretreated with the indicated doses of actinomycin D for 30 min and then 60 nM of PMA was added into the media. The treatment was continued for 6 h. Total RNA was isolated and subject to Northern analyses with *hr* and involucrin cDNA probes. (B and C) Quantitative data of *hr* and involucrin mRNA were obtained from three independent experiments. ActD, actinomycin D. $^*P < 0.01$ compared with untreated controls.

uble cornified envelope by transglutaminase during keratinocyte differentiation (26). Since the *hr* mRNA expression was highly induced by PMA, in parallel with the induction of p21 and involucrin, we conclude that an up-regulation of the *hr* gene expression is closely associated with the differentiation of mouse keratinocytes. Whether the *hr* induction is a prerequisite for or a consequence of keratinocyte differentiation still needs further investigations. It is conceivable that, as a putative transcriptional factor (2), *hr* may be important for the regulation of other genes involved in keratinocyte differentiation.

A large body of evidence has demonstrated that PKC plays an important role in keratinocyte differentiation (27–29). For instance, PKC activation is required for the PMA-induced expression of the granular markers loricrin, filaggrin and transglutaminase, for the formation of cornified envelopes (19, 24, 30), as well as for the down-regulation of spinous markers keratin 1 and 10 (31). Here we demonstrate that the PKC inhibitor GF109203X abolishes the stimulatory effect of PMA on both *hr* and involucrin mRNA expression, indicating the requirement of PKC activation for the up-regulation of these genes. PKC is a family of serine/threonine kinases composed of multiple isoforms of distinct

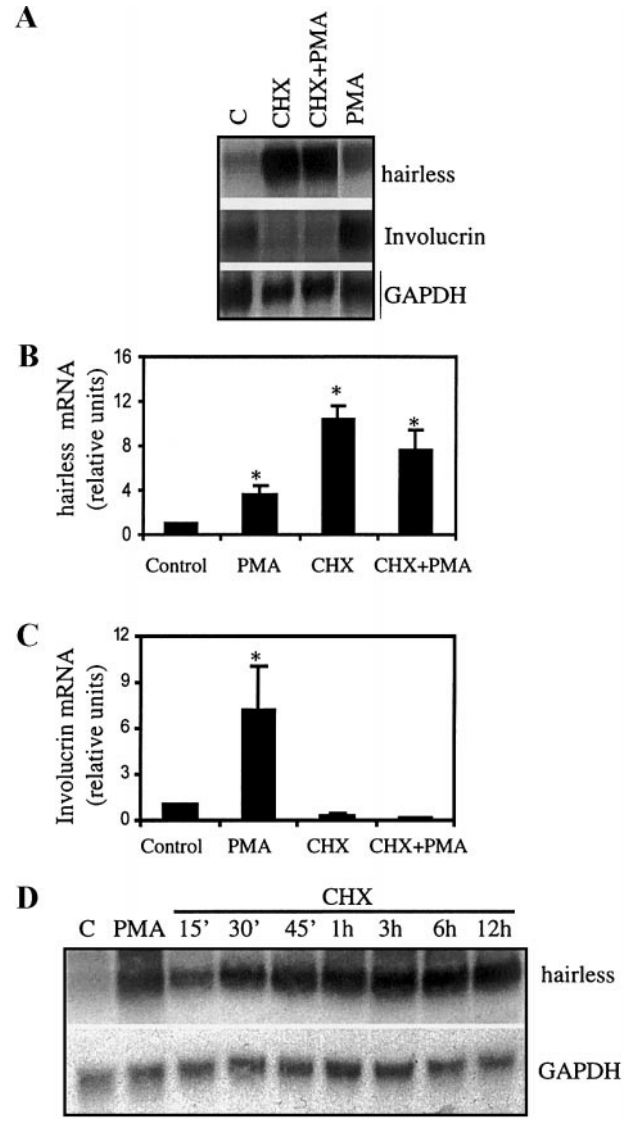

FIG. 5. Increase of *hr* mRNA by cycloheximide treatment. (A) Primary keratinocytes were treated for 6 h with 20 μg/ml of cycloheximide alone, with 60 nM of PMA alone, or cotreated with 20 μg/ml of cycloheximide and 60 nM of PMA for 6 h after a 30-min pretreatment with cycloheximide. Total RNA was extracted, and Northern blots were performed using *hr* and inv cDNA probes. (B and C) Quantitative results of *hr* and inv mRNA levels were obtained from three independent experiments. $^*P < 0.05$ compared with untreated controls. (D) Time course of *hr* mRNA induction by cycloheximide treatment. Primary keratinocytes were treated with 60 nM of cycloheximide alone for the indicated times. Total RNA (25 μg/lane) was analyzed by Northern blot. CHX, cycloheximide treatment; C, untreated control; PMA, 6 h of PMA treatment.

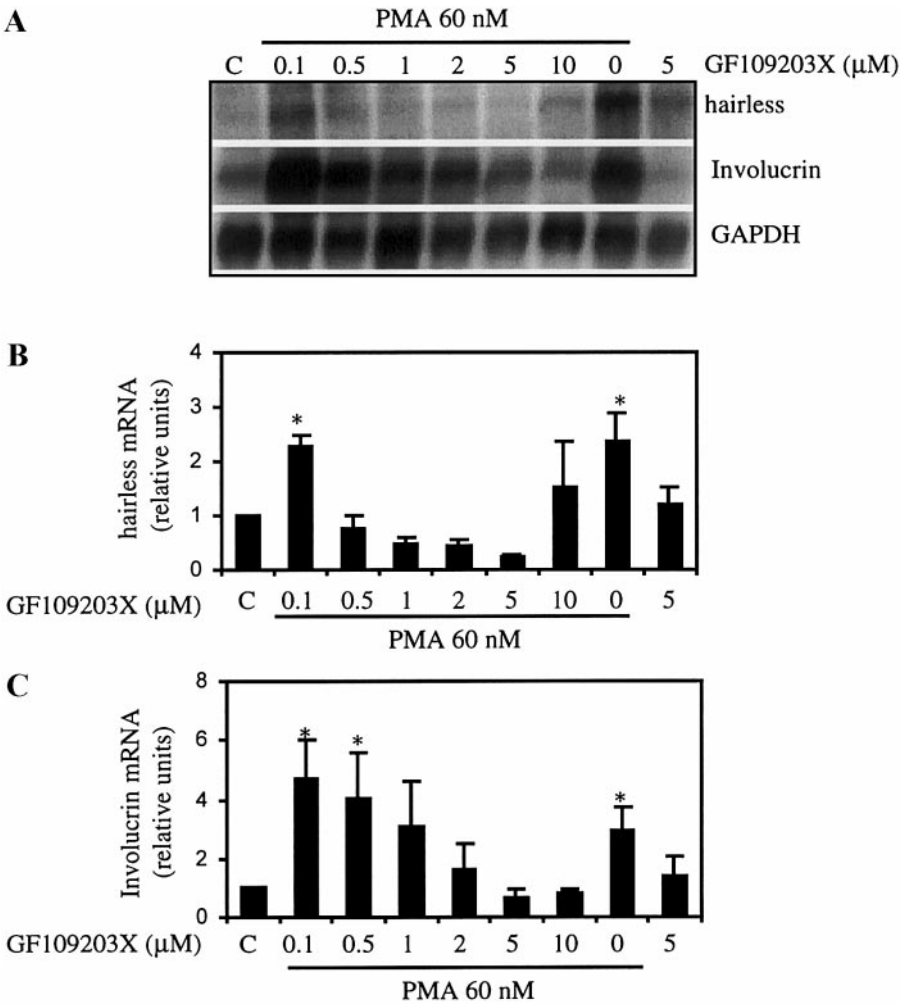

FIG. 6. Inhibition of *hr* and involucrin mRNA induction by the PKC inhibitor GF109203X. (A) Primary keratinocyte cultures were pretreated with the indicated concentrations of GF109203X for 1 h, and 60 nM of PMA was added into the culture. The treatment was continued for 6 h. Northern blot analysis was performed using *hr* and involucrin cDNA probes as indicated. (B and C) Quantitative results for *hr* and involucrin mRNA levels obtained from three independent experiments. $*P < 0.05$ compared with untreated controls.

functions, and five PKC isoforms (α, δ, ε, ζ, and η) have been identified in cultured mouse keratinocytes (32). Specific PKC isoforms have been implicated in the regulation of keratinocyte differentiation markers (29, 33–35). For instance, α and η isoforms are involved in the stimulation of involucrin gene transcription (33), and η isoform is also important for transglutaminase gene transcription (34), whereas the activation of PKC δ may be a signal to turn off the differentiation program (36). Since GF109203X is a non-selective PKC inhibitor, more specific PKC inhibitors are needed in future studies to distinguish the Ca2+-dependent and Ca2+-independent PKC signaling pathways, or to identify the specific PKC isoforms that are involved in the regulation of *hr* gene expression.

The observation that the induction of the *hr* gene in keratinocyte differentiation is dependent on the activation of PKC is reminiscent of the expression pattern of other keratinocyte markers such as involucrin, loricrin, filaggrin and transglutaminase (30, 31, 33). However, the mode of PKC action in the regulation of *hr* and the other markers may be different. For instance, we showed that, in contrast to involucrin mRNA, *hr* mRNA is increased by the protein translation inhibitor cycloheximide, suggesting that new protein synthesis is required for suppressing the *hr* mRNA synthesis or destabilizing the *hr* mRNA transcript in keratinocytes in the steady state. Thus, as in the regulation of involucrin gene (33), PKC may be directly involved in the stimulation of the *hr* gene promoter; alternatively, PKC may directly inhibit the suppressors of *hr* gene expression or mRNA stability. To dissect the regulatory mechanism, an analysis of the *hr* gene promoter as well as an identification of the suppressors are needed in future investigations.

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