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Differential Modulation of Human Fibroblast and Keratinocyte Growth by the Protein Kinase C Inhibitor GF 109203X

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SUMMARY

Protein kinase C (PKC) is known to be involved in cellular proliferation and differentiation. In this work, we have investigated the effects of a novel PKC inhibitor, GF 109203X, on normal human fibroblast and keratinocyte growth. GF 109203X selectively inhibited PKC activity extracted from either fibroblasts (IC_{50} = 0.01 μM) or keratinocytes (IC_{50} = 0.4 μM). The inhibitory effects of GF 109203X on total PKC activity and Ca^{2+}-independent PKC activity were similar. Nevertheless, in keratinocytes Ca^{2+}-independent PKC activity represented 95% of total PKC activity, whereas in fibroblasts it corresponded to only 32% of total PKC activity. GF 109203X also inhibited a cellular function related to PKC activity in living fibroblasts and keratinocytes; it blocked the inhibitory effect of 12-*O*-tetradecanoylphorbol-13-acetate on ^{125}I-epidermal growth factor binding. GF 109203X inhibited fibroblast growth, in terms of tritiated thymidine incorporation and cell counts, in a dose-dependent manner. We also observed that GF 109203X at 1 μM inhibited serum stimulation of expression of mRNA for c-*fos* and c-*jun*, which are usually involved in cellular proliferation. These results suggest that PKC stimulates fibroblast growth. In contrast, GF 109203X stimulated keratinocyte growth. We also observed that GF 109203X inhibited c-*fos* and c-*jun* mRNA expression in these cells. In fact, in keratinocytes these proto-oncogenes would be involved in the cellular differentiation process rather than in cellular proliferation. This suggests that the inhibition of PKC favors keratinocyte proliferation probably by inhibiting their differentiation. Thus, using GF 109203X, we show that PKC is involved differently in human fibroblast and keratinocyte growth.

PKC plays a central role in the transduction of a variety of external signals (growth factors, hormones, etc.) at the intracellular level (1). PKC belongs to a family of phospholipid-dependent protein serine/threonine kinases activated especially by DAG (2). This protein kinase is known to regulate a variety of cell functions, such as proliferation and differentiation (3). The involvement of PKC in skin homeostasis is now well established. Indeed, considering that this kinase is a receptor for phorbol ester tumor promoters (4), it may play an essential role in tumor promotion in human skin. Moreover, a PKC defect seems to be involved in a number of skin diseases, e.g., psoriasis and keloids (5–7).

To evaluate the role of PKC in cellular functions, studies have mainly been carried out using phorbol esters (especially TPA). TPA has a structure very similar to that of DAG; however, it may sometimes be unsuitable for the study of PKC activation, because DAG is present only transiently in membranes, whereas TPA is hardly degraded (1). Another approach to study the role of PKC in cells is to use inhibitors of PKC. Until now, the main inhibitors used have been staurosporine and H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], but they are not very selective and inhibit other protein kinases (8, 9). Recently, a new inhibitor has been synthesized, i.e., GF 109203X, which belongs to the bisindolylmaleimide PKC inhibitor family (10). This inhibitor family has been used to demonstrate the involvement of PKC in different cellular responses (10, 11, 12).

In the present study, we have used this PKC inhibitor (GF 109203X) to analyze the involvement of PKC in human fibroblast and keratinocyte growth. We have evaluated in cell-free assays the selectivity of GF 109203X for total PKC and Ca^{2+}-independent PKC activity. We have also verified its effects, in living cells, on a cellular function modulated by PKC. In a second series of experiments, we have studied the effects of GF 109203X on the growth of these two human skin cell types.

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ABBREVIATIONS: PKC, protein kinase C; DAG, diacylglycerol; EGF, epidermal growth factor; FCS, fetal calf serum; PBS, phosphate-buffered saline; PKA, protein kinase A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EMEM, Earle's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Materials and Methods

Reagents

EMEM and FCS were from GIBCO. GF 109203X was kindly supplied by Glaxo Laboratories. The stock solution was prepared in dimethylsulfoxide at 5 mM, stored at 4°C, and diluted in culture medium just before each use. We verified that the highest final concentration of dimethylsulfoxide in culture medium (0.2%) did not modify cell growth.

The PKC-α isoenzyme from rat cerebellum was kindly provided by R. Clegg (HANNAH Research Institute, Ayr, Scotland, UK). PKC and PKA assay kits were from GIBCO, and TPA and chemicals used for the tyrosine kinase assay were from Sigma Chemical Co. [γ-^{32}P]ATP (3000–5000 cpm/pmol) was obtained from ICN. ^{125}I-EGF (100 μCi/ml) was purchased from Amersham and EGF (from mouse submaxillary glands) was from Boehringer. [^{3}H]Thymidine (27 Ci/mmol) was from CEA and [α-^{32}P]dCTP (10 mCi/ml) was purchased from Amersham.

Cell Cultures

Fibroblast and keratinocyte cultures were established from skin explants obtained, during breast plastic surgery, from 18–45-year-old healthy donors. The fibroblasts were grown in EMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml amphotericin B, in 5% CO_2/95% air, at 37°C. Just after confluency, fibroblasts (between passages 4 and 7) were removed by treatment with 0.05% trypsin/0.02% EDTA and were suspended in culture medium at the concentration defined for each type of experiments.

Keratinocyte primary cultures were established according to the usual procedure, with minor modifications (13). Briefly, skin was cleaned of excess deep dermis and subcutaneous fat, cut into thin pieces, rinsed in calcium-free PBS, and incubated with 0.25% trypsin/0.02% EDTA overnight at 4°C. The epidermis was then separated from the dermis with forceps and incubated for 30 min in PBS at 37°C. The undigested pieces of epidermis were discarded and epidermal cells were recovered in the same culture medium as that used for fibroblasts (differentiative high-calcium medium, with a Ca^{2+} concentration of 1.7 mM). Keratinocytes were then seeded at a defined concentration according to experiments.

Enzyme Preparations and Protein Kinase Assays

Confluent cultures of human fibroblasts or keratinocytes, cultivated in the medium describe above, were washed three times with PBS. Cells were scraped, recovered in PBS, and centrifugated for 10 min at 800 × *g*. The pellets were kept at −80°C until protein kinase assays.

PKC, PKA, and casein kinase II assays. Extraction of total PKC and PKA from human fibroblasts and keratinocytes and determination of the kinase activities were performed according to the protocols defined in the GIBCO assay kits. In addition, Ca^{2+}-independent PKC activity was determined in the presence of 5 mM EGTA in three experiments. Casein kinase II was purified from bovine brain grey matter and tested as described (14). All samples were assayed in duplicate.

Preparation of crude membranes and protein tyrosine kinase assay. The frozen cells were transferred to a glass/glass conical homogenizer and homogenized by hand in 0.8 ml of 50 mM HEPES buffer, pH 7.4, containing 10% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml levels each of aprotinin, antipain, leupeptin, pepstatin A, and chymostatin. The homogenate was centrifugated at 105,000 × *g* for 60 min and the supernatant fraction was removed. The pellet was solubilized in tyrosine kinase buffer and was used as a crude membrane preparation.

The tyrosine kinase activity was determined in medium (50-μl final volume) made up of 50 mM HEPES buffer, pH 7.4, containing 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 150 mM NaCl, about 10 μg of enzyme protein, 1 mg/ml tyrosine synthetic substrate poly(Glu,Tyr) (4:1), and 10 μM [γ-^{32}P]ATP. The reaction was started by addition of [γ-^{32}P]ATP, incubated for 5 min at 30°C, and stopped by spotting of 40-μl aliquots on Whatman P-81 paper. The papers were washed with 1% phosphoric acid containing 1 mM sodium pyrophosphate. This wash was repeated five times, and then papers were washed twice more with 95% ethanol. The strips were dried, and $^{32}P_i$ incorporation was determined by liquid scintillation counting. The tyrosine kinase activity was the difference between samples with and without poly(Glu,Tyr). All samples were assayed in duplicate.

Measurement of ^{125}I-EGF Binding

EGF binding assays were performed in 24-well tissue culture dishes seeded with 2 × 10^4 fibroblasts/well or 1 × 10^6 keratinocytes/well, in the medium described above. Confluent cultures were incubated for 20 min at 37°C with different concentrations of GF 109203X. Then TPA (100 ng/ml) was added and cells were further incubated for 1 hr (for fibroblasts) or 2 hr (for keratinocytes).

After the incubation, the EGF binding assays were performed as reported previously (15). In brief, cells were washed twice with ice-cold binding buffer (EMEM containing 0.1% bovine serum albumin and 20 mM HEPES, pH 7.5) and incubated at 0–4°C for 4 hr in 1 ml of binding buffer containing 20 nCi/well ^{125}I-EGF. The binding of ^{125}I-EGF was evaluated at 4°C to avoid receptor internalization. For the estimation of nonspecific binding, 1 μg/ml unlabeled EGF was added to the medium. After the incubation, cultures were washed three times with ice-cold binding buffer and harvested in 1 ml of lysis buffer (0.1% sodium dodecyl sulfate, 1 M NaOH). The radioactivity was determined with a γ counter (CG 4000; Intertechnique).

Quantification of Cell Growth

Microplates (96 wells) were seeded with 1 × 10^4 fibroblasts/well or 1 × 10^5 keratinocytes/well. Cultures were treated 2 hr after seeding. Dose-response experiments were performed with 0.1–10 μM GF 109203X and were analyzed after 72 hr. Cell growth was evaluated by counting cells and by measuring the rate of [^{3}H]thymidine incorporation. Cells were recovered by trypsin treatment and counted. To measure the rate of [^{3}H]thymidine incorporation, parallel cultures were labeled for the last 4 hr with 4 μCi/ml [^{3}H]thymidine. Then, cells were recovered as described above and were collected on Whatman GF/C filters with a cell harvester (Skatron; Omnium Scientific Industriel). Radioactivity was measured in a liquid scintillation counter (LKB 1212 Rackbeta).

Quantification of c-*fos* and c-*jun* mRNA Expression by Northern Blot Analysis

Plates (150-cm diameter) were seeded with 3 × 10^6 fibroblasts/plate or 4 × 10^6 keratinocytes/plate and, after 3 days, were incubated for 24 hr in EMEM containing 0.1% FCS. The culture medium was removed and cells were treated with EMEM containing 10% FCS, with or without 1 μM GF 109203X, for various times. Total cytoplasmic RNA was isolated by the method of Chomczynski and Sacchi (16). RNA samples were electrophoresed on 1% denaturing agarose gels containing 5.4% formaldehyde. RNA samples were transferred to GeneScreenPlus membranes by capillary transfert. The membranes were air dried and baked under vacuum at 80° for 2 hr. The molecular probes used were a 2.5-kilobase *Nco*I/*Bam*HI fragment of pc-*fos*-human 1, a 1.3-kilobase *Pst*I fragment of human GAPDH (generous gifts from T. Krieg, Lab. Dermatology, Universität Köln, Germany), and a 1.4-kilobase *Hind*III/*Eco*RI fragment of human c-*jun* (a generous gift from N. Basset-Séguin, Laboratoire de dermatologie, Moléculaire CNRS/CRBM, Montpellier, France). After denaturation, the cDNA probes were labeled with [α-^{32}P]dCTP using a random priming kit (United States Biochemical Corp.). Unincorporated label was separated from labeled DNA using a Elutip-d column. Prehybridization, hybridization, and subsequent washes were performed according to the GeneScreenPlus manufacturer's recommendations. Autoradiography (Kodak X-OMAT X-ray film) was then performed with intensifying screens at −80°C for 24–72 hr.

Results

Effects of GF 109203X on human fibroblast and keratinocyte PKC activity in cell-free assays. We tested the effects of GF 109203X on the activity of PKA, PKC, and membrane tyrosine kinases extracted from confluent human fibroblasts and keratinocytes. PKA activity from human fibroblasts and keratinocytes was not affected by GF 109203X at up to 5 μM, and GF 109203X was inactive against membrane tyrosine kinases ($IC_{50} > 50$ μM). In addition, GF 109203X at 5 μM inhibited only 30% of the activity of casein kinase II, another growth-related serine/threonine protein kinase.

The levels of PKC activity showed significant differences between cells of different origins. First, the total PKC activity in fibroblasts was much higher than that in keratinocytes (Table 1). Second, compared with fibroblasts, the PKC activity from keratinocytes was inhibited at much higher GF 109203X concentrations (Table 1). Interestingly, the assay with the most potent but poorly selective PKC inhibitor, staurosporine, showed the same dependence. Staurosporine at 10 nM completely suppressed PKC activity in fibroblasts, whereas in keratinocytes its IC_{50} was 20 nM. The same observation was also made with the PKC pseudosubstrate inhibitor peptide PKC(19–36) (GIBCO assay kit); at a concentration of 20 μM, it completely inhibited PKC activity in fibroblasts, whereas at the same concentration it inhibited only 40% of PKC activity in keratinocytes.

In all experiments, we used purified PKC-α isoenzyme from rat cerebellum as a positive control. GF 109203X was a potent inhibitor of PKC-α ($IC_{50} = 20$ nM). Staurosporine at 10 nM and PKC pseudosubstrate inhibitor peptide at 20 μM completely suppressed PKC-α activity.

The effects of GF 109203X on total PKC activity were also compared with its effects on Ca^{2+}-independent PKC activity from both human fibroblasts and keratinocytes; this also allowed us to determine the effects of GF 109203X on Ca^{2+}-dependent PKC activity. First, we observed in fibroblasts that, in the presence of 5 mM EGTA, the Ca^{2+}-independent PKC activity corresponded to about 32% (the difference, 68%, could be considered as the Ca^{2+}-dependent PKC activity). In contrast, in keratinocytes the Ca^{2+}-independent PKC activity represented 95% of the total PKC activity (yielding only 5% Ca^{2+}-dependent PKC activity) (Table 2). Second, we observed that in fibroblasts GF 109203X at 100 nM inhibited 86% of the total PKC activity and 78% of the Ca^{2+}-independent PKC activity, whereas in keratinocytes the inhibition was only about 15% for both activities (Table 2).

These cell-free assay experiments emphasized the selectivity of GF 109203X for human fibroblast and keratinocyte PKC activity. Moreover, we observed that Ca^{2+}-independent and -dependent PKC activity contributed in different proportions to the total PKC activity in fibroblasts and keratinocytes. In addition, according to the cell type, GF 109203X showed similar efficiencies for total PKC and Ca^{2+}-independent PKC activities.

TABLE 1

Inhibition of total PKC activity by GF 109203X

PKC assays were performed as described in Materials and Methods. Values are means ± standard errors from three independent experiments.

	Total PKC Activity	IC_{50}
	pmol/min/10⁶ cells	*μM*
Human fibroblasts	21.94 ± 2.33	0.01 ± 0.004
Human keratinocytes	1.96 ± 0.11	0.40 ± 0.05

TABLE 2

Inhibition of total and Ca^{2+}-dependent PKC activity by GF 109203X

PKC assays were performed as described in Materials and Methods. Values are means ± standard errors from three independent experiments.

	PKC Activity	
	Total	Ca^{2+}-independent
	pmol/min/10⁶ cells	
Human fibroblasts		
Control	23.54 ± 2.13	7.53 ± 0.08
GF 109203X (100 nM)	3.3 ± 0.10	1.66 ± 0.05
Human keratinocytes		
Control	2.08 ± 0.16	1.98 ± 0.04
GF 109203X (100 nM)	1.77 ± 0.11	1.62 ± 0.03

Effects of GF 109203X on a cellular function modulated by PKC, EGF binding. PKC activation by TPA leads to rapid inhibition of EGF binding, which is attributed to PKC-mediated phosphorylation of the EGF receptor (17, 18). In confluent human fibroblast and keratinocyte cultures, TPA (100 ng/ml) reduced the EGF binding capacity at least to 20% of the control level for fibroblasts and at least to 50% for keratinocytes (Fig. 1). GF 109203X alone (0.5–8 μM) had no effect on ^{125}I-EGF binding to fibroblasts and keratinocytes. However, GF 109203X reversed, in a dose-dependent manner, the inhibitory effect of TPA on EGF binding to fibroblasts and keratinocytes (Fig. 1). These experiments show that GF 109203X inhibits a cellular function modulated by PKC in intact living human fibroblasts and keratinocytes.

Effects of GF 109203X on fibroblast and keratinocyte growth. The dose-dependent response for the effects of GF 109203X (0.1–10 μM) on fibroblast growth was analyzed 72 hr after treatment. The number of fibroblasts decreased slightly as the concentration of GF 109203X increased (Fig. 2A), and [^{3}H]thymidine incorporation decreased in a dose-dependent manner from 1 μM (Fig. 2B).

The dose-dependent response for the effects of GF 109203X on keratinocyte growth, 72 hr after treatment, showed a concentration-dependent increase in the number of keratinocytes (Fig. 3A). [^{3}H]Thymidine incorporation was also increased in a concentration-dependent manner up to 5 μM. A sharp drop in [^{3}H]thymidine incorporation was observed, despite the increase in the number of keratinocytes, at 10 μM GF 109203X (Fig. 3B). The high cell density and low [^{3}H]thymidine incorporation at this concentration probably reflected the confluency of the cultures at this time.

These experiments showed that GF 109203X had opposite effects on fibroblast and keratinocyte growth. GF 109203X inhibited fibroblast growth and stimulateed keratinocyte growth. These effects on fibroblast and keratinocyte growth have also been observed in kinetic experiments over 3 days using GF 109203X at 1 μM. This inhibitor concentration was then used in the following experiments.

Effects of GF 109203X on c-*fos* and c-*jun* mRNA expression. The proto-oncogenes c-*fos* and c-*jun* are immediate early genes involved in cellular proliferation and differentiation (19). Moreover, the expression of these proto-oncogenes is controlled by PKC (19, 20). Cells were growth stimulated with culture medium containing 10% FCS, in the absence or presence of GF 109203X at 1 μM. The levels of c-*fos* and c-*jun* mRNA were analyzed at different times after

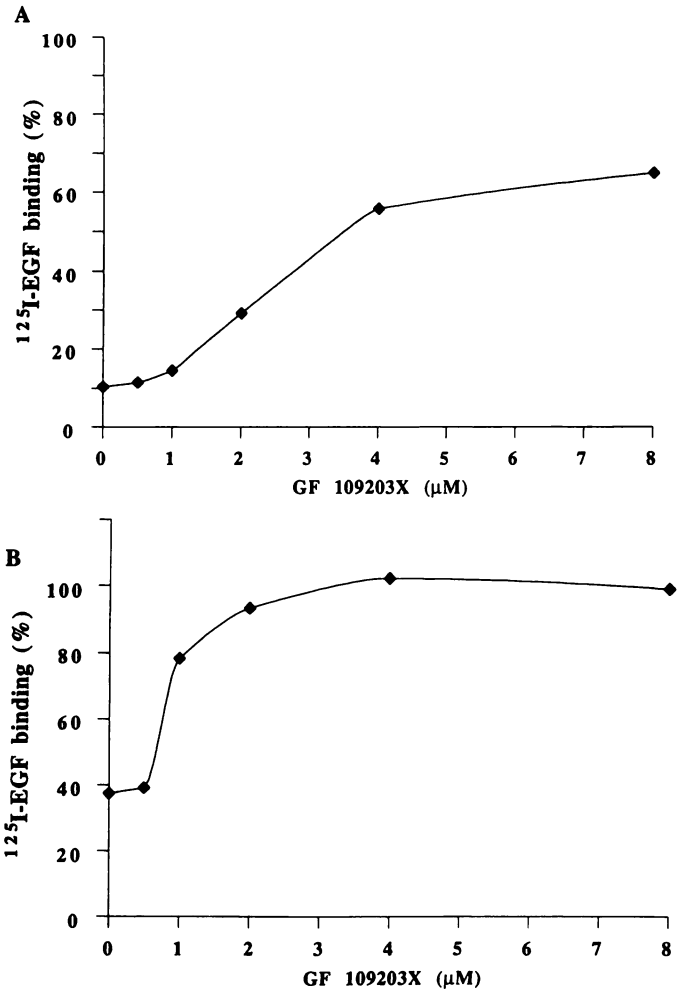

Fig. 1. Effects of GF 109203X on the inhibition of ^{125}I-EGF binding to human fibroblasts (A) and keratinocytes (B). Confluent cultures were treated with increasing concentrations of GF 109203X. After 20 min, cells were further incubated with TPA (100 ng/ml) for 1 hr (for fibroblasts) or 2 hr (for keratinocytes). The results are expressed as percentage of control values for specific binding (fibroblasts, 529 ± 63 cpm/well; keratinocytes, 192 ± 21 cpm/well). Nonspecific binding was about 8% for fibroblasts and 40% for keratinocytes. *Points*, means of triplicate wells. Similar experiments were repeated three times with different fibroblast and keratinocyte strains, and the results obtained were reproducible.

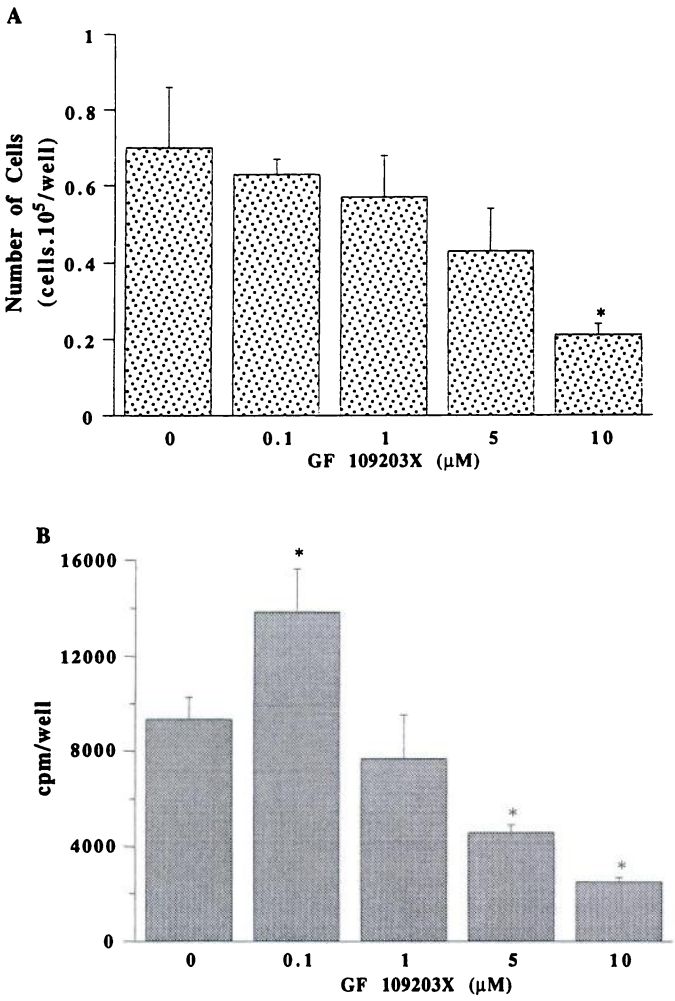

Fig. 2. GF 109203X concentration-response for fibroblast growth. A, The number of fibroblasts was determined 72 hr after treatment. B, Tritiated thymidine was added 4 hr before measurement of its incorporation into DNA, 72 hr after treatment. Similar experiments were repeated with three different strains of fibroblasts and the results obtained were reproducible. *Brackets*, standard error for three samples. *, $p < 0.05$, treated versus control, by Student's *t* test for unpaired values.

the stimulation (30 min or 1, 2, or 4 hr). The kinetics of the expression of c-*fos* and c-*jun* mRNA were different. Whereas the expression of c-*fos* was detected only during the first 2 hr, that of c-*jun* was still observed 4 hr after FCS stimulation. Moreover, the expression of c-*fos* mRNA in keratinocytes was very low, relative to that in fibroblasts. In fibroblasts the stimulation by FCS clearly increased c-*fos* and c-*jun* mRNA expression 1 hr after treatment, whereas GF 109203X decreased the levels of c-*fos* and c-*jun* (Fig. 4A). In keratinocytes the same observations were made, despite the very low levels of c-*fos* expression (Fig. 4B). GAPDH mRNA expression was used as a reference for the mRNA concentration loaded.

Discussion

The involvement of PKC in cell growth and differentiation is now well established (3). However, it seems to have opposite effects; PKC is involved in the mitogenic effects of some agents (21) and the growth-inhibitory and differentiation-inducing effects of others (22). To evaluate the role of PKC in cell growth, pharmacological inhibitors of this kinase can be used. The most commonly used inhibitors have been staurosporine and H7, but they are not very selective and even seem to have some agonist effects towards PKC (23, 24).

In this work we used a new PKC inhibitor, GF 109203X, to study the involvement of PKC in human fibroblast and keratinocyte growth. This inhibitor belongs to the bisindolylmaleimide PKC inhibitor family (10–12). The selectivity of the inhibitor, GF 109203X, was first observed with PKC purified from bovine brain (10). We demonstrate here that GF 109203X also selectively inhibits total PKC activity extracted from normal human fibroblasts (IC_{50} = 0.01 μM) or keratinocytes (IC_{50} = 0.4 μM).

The different amounts and the different inhibitor sensitivities of the total PKC activities in fibroblasts and keratinocytes could be due to differences in the PKC isoenzyme contents of these cell types. Indeed, PKC isoenzymes are distributed differently in different cell types (25) and possess distinct prop-

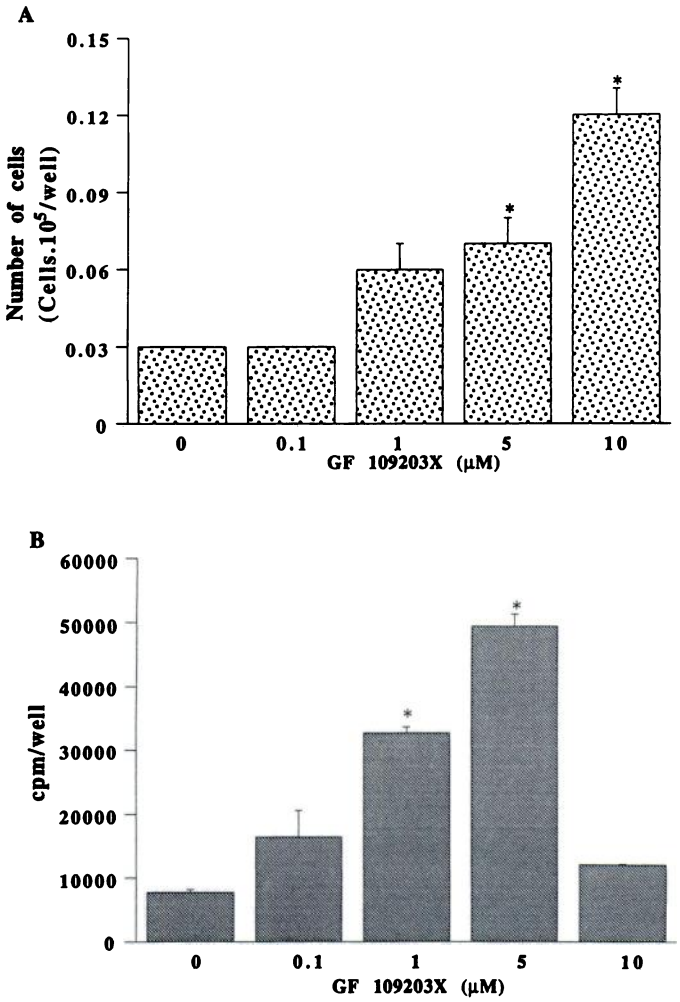

Fig. 3. GF 109203X concentration-response for keratinocyte growth. A, The number of keratinocytes was determined 72 hr after treatment. B, Tritiated thymidine was added 4 hr before measurement of its incorporation into DNA, 72 hr after treatment. Similar experiments were repeated with three different strains of keratinocytes and the results obtained were reproducible. *Brackets*, standard error for three samples. *, $p < 0.05$, treated versus control, by Student's *t* test for unpaired values.

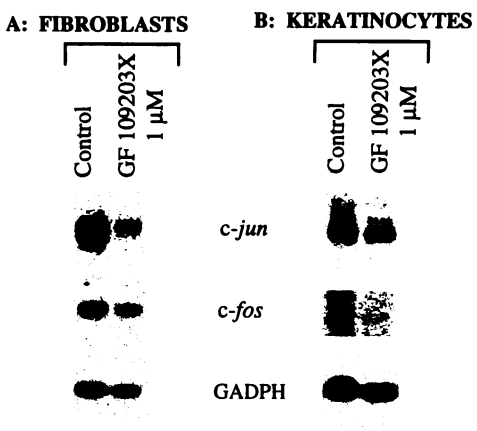

Fig. 4. Effects of GF 109203X (1 μM) on c-*fos* and c-*jun* mRNA expression in quiescent cultures 1 hr after stimulation with FCS. The c-*fos*, c-*jun*, and GAPDH mRNA were identified using ^{32}P-labeled DNA probes. A, Human fibroblasts (each *lane* contains 8 μg of total RNA); B, human keratinocytes (each *lane* contains 5 μg of total RNA).

erties concerning their regulation (2), their substrates (25–27), and even their cellular location (28). Up to now, it has been shown that human keratinocytes express five of the known PKC isoenzymes, i.e., PKC-α, -ϵ, -ζ, -δ, and -η, and human fibroblasts express four PKC isoenzymes, i.e., PKC-α, -ϵ, -ζ, and -δ (29). Thus, from the classical group of Ca^{2+}-dependent PKC forms (α, β_1, β_2, and γ), only PKC-α seems to be present in these two cell types and especially in human fibroblasts; the abundant expression of the Ca^{2+}-sensitive PKC isoenzyme PKC-α has also been observed in 3T3 fibroblasts (30). Concerning the Ca^{2+}-independent PKC activity, PKC-η is a PKC isoenzyme specifically expressed in epithelial cells (31).

In our experiments, we observed that Ca^{2+}-independent and -dependent PKC activities contributed differently to the total PKC activity in fibroblasts and keratinocytes. In fibroblasts Ca^{2+}-independent PKC corresponded to about 32% of total PKC activity. In contrast, in keratinocytes we found that total PKC activity was almost completely due to Ca^{2+}-independent PKC (95%). Nevertheless, we also observed that GF 109203X inhibited total PKC and Ca^{2+}-independent PKC activities with similar efficiencies in each cell type. In another study using recombinant PKC isoenzymes (α, β_1, ϵ, ζ, and δ), GF 109203X has been shown to be less effective in inhibiting Ca^{2+}-independent PKC isoenzymes (32). Nevertheless, these results and the high proportion of Ca^{2+}-independent PKC activity in keratinocytes (95%) would explain the weaker sensitivity of total PKC activity to GF 109203X. In contrast, in human fibroblasts we observed a high basic level (32%) and a high sensitivity of Ca^{2+}-independent PKC activity to GF 109203X; a possible explanation could be the existence in human fibroblasts of a specific Ca^{2+}-independent PKC isoenzyme that is very sensitive to GF 109203X.

PKC activation in intact living cells was determined by the inhibition of ^{125}I-EGF binding, which occurs in cell cultures exposed to TPA (17, 18). The ability of GF 109203X to prevent TPA inhibition of EGF binding has been previously demonstrated in 3T3 fibroblast cultures (10). In this study, we verified that GF 109203X is able to reverse the effects of TPA on EGF binding to both human fibroblast and keratinocyte cultures. Consequently, GF 109203X is an efficient tool for PKC studies in intact living human fibroblasts and keratinocytes, and we have used it to evaluate the involvement of PKC in the growth of these skin cells.

In our experiments, we observed that GF 109203X reduced the growth of fibroblasts in monolayers, as shown by a decrease in both cell number and [^{3}H]thymidine incorporation, suggesting that PKC could stimulate fibroblast growth. This hypothesis is supported by the increase of Swiss 3T3 fibroblast growth when PKC is activated by phorbol esters (33) or synthetic DAG, i.e., oleoyl-2-acetyl-*sn*-glycerol (21). It is tempting to suggest that PKC-α could play a crucial role in cell growth, because it is expressed universally (2). In addition, overexpression of the PKC-α isoform in fibroblasts results in altered growth regulation and enhanced tumorogenicity (34). Thus, the large proportion of Ca^{2+}-dependent PKC (PKC-α) in fibroblasts, its sensitivity to GF 109203X (10, 32), and the inhibition of human fibroblast growth by GF 109203X are in good agreement with the aforementioned suggestion.

In contrast, GF 109203X led to a significant increase in both keratinocyte number and [^{3}H]thymidine incorporation, suggesting that PKC could inhibit keratinocyte proliferation. The

involvement of PKC in keratinocyte proliferation has already been suggested by experiments using TPA. However, in keratinocyte cultures this compound generates many divergent biochemical changes, which can be divided into two broad categories, i.e., the stimulation of keratinocyte proliferation in cultures (35) and the inhibition of keratinocyte proliferation related to an induction of terminal differentiation (22, 36). In fact, PKC activation may be involved in the initial signaling events for keratinocyte differentiation (37, 38), and GF 109203X, by inhibiting PKC, may have indirectly favored keratinocyte proliferation. This possibility has also been suggested with mouse keratinocytes in experiments using another PKC inhibitor of the same family (12). In addition, in our experiments we observed a predominance of Ca^{2+}-independent PKC activity in keratinocytes, and it is known that the Ca^{2+}-independent PKC-η isoform, present in epithelial tissue, is predominantly expressed in mouse differentiated epithelial cells (31). The differential modulation of fibroblast and keratinocyte growth in the presence of GF 109203X could be due to differences in the PKC isoenzyme contents of the two cell types.

Considering the opposite effects of GF 109203X on fibroblast and keratinocyte growth, we have also analyzed the effects of GF 109203X on the expression of c-*fos* and c-*jun* mRNA in these skin cells. These two proto-oncogenes code for a transcription factor (AP-1) and are among the so-called "immediate early response" genes, which are rapidly and transiently induced by numerous stimuli. Both c-*fos* and c-*jun* are well known to be involved in cellular proliferation. Moreover, they can also be involved in the differentiation process of some cell types, particularly keratinocytes (19, 39, 40).

In our experiments, we observed that GF 109203X inhibited c-*fos* and c-*jun* mRNA expression in fibroblasts. Previous experiments also suggested a role for these two proto-oncogenes in proliferation of 3T3 fibroblasts (41–43). These results and those concerning the effects of GF 109203X on fibroblast growth confirm the positive role of PKC in fibroblast proliferation. However, we also observed that GF 109203X inhibited c-*fos* and c-*jun* mRNA expression in keratinocyte cultures. In fact, these two proto-oncogenes have been shown to be involved in the differentiation process of keratinocytes, rather than their proliferation (19, 39, 40). Thus, we can envision that GF 109203X, by inhibiting the expression of c-*fos* and c-*jun* mRNA in keratinocytes, inhibited their differentiation and, as suggested previously, in turn favored their proliferation.

Interestingly, it is important to note that opposite PKC activities have also been observed in fibroblasts and keratinocytes of patients with psoriasis, which is a disease characterized by hyperproliferation of the epidermis. Indeed, an increase of PKC activity is observed in psoriatic fibroblasts (5), whereas psoriatic epidermis shows abnormally low PKC activity (6). Moreover, in the epidermis of these patients the decrease in PKC activity may be involved in the reduced expression of c-*fos* and c-*jun* proto-oncogenes (44). Thus, alterations of the PKC activity and therefore alterations of c-*fos* and c-*jun* expression would lead to a loss of control of epidermal cell proliferation.

These results show that PKC activity plays an important role in fibroblast and keratinocyte behavior. PKC is involved differently in fibroblast and keratinocyte growth, and these differences seem to be due to variations in PKC isoenzyme composition in each cell type.

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