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Regulation of TGF-α Expression in Human Keratinocytes: PKC-Dependent and -Independent Pathways

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Transforming growth factor–α (TGF-α) is an autocrine growth factor for epidermal keratinocytes that can induce its own expression (autoinduction). Because the regulation of this process may be important for the control of epidermal growth, we examined the roles of EGF receptor tyrosine kinase and protein kinase C (PKC) in TGF-α autoinduction in cultured human keratinocytes. Antiphosphotyrosine immunoblot analysis demonstrated that EGF and TGF-α rapidly and markedly stimulated tyrosine phosphorylation of a 170 kDa protein in growth factor–deprived keratinocytes. This protein was identified as the EGF receptor by immunoprecipitation using anti-EGF receptor mAbs. Tyrosine phosphorylation and TGF-α mRNA accumulation in response to EGF and TGF-α were both inhibited by a monoclonal antibody against the EGF receptor and by the EGF receptor tyrosine kinase inhibitor RG50864, demonstrating the involvement of the tyrosine kinase activity of the receptor in TGF-α autoinduction. The monoclonal antibody inhibited keratinocyte growth and TGF-α autoinduction with similar potency (IC_{50} ~ 0.1 μg/ml). TGF-α and the PKC activator tetradecanoyl phorbol 12-myristyl, 13-acetate (TPA) had similar effects on TGF-α steady-state mRNA levels, suggesting that PKC activation might be a downstream mediator of TGF-α autoinduction. However, down-regulation of more than 90% of keratinocyte PKC activity by bryostatin pretreatment abrogated the induction of TGF-α mRNA in response to TPA without affecting the autoinductive response or EGF-stimulated tyrosine phosphorylation. These results indicate that EGF receptor and PKC stimulate TGF-α gene expression by different pathways, and suggest that PKC is not required for TGF-α autoinduction in this system. Moreover, the fact that EGF-stimulated tyrosine phosphorylation and TGF-α autoinduction were not potentiated after PKC down-regulation suggests that PKC does not exert a tonic inhibitory influence on EGF receptor tyrosine kinase activity in normal human keratinocytes.

The growth factor receptor–associated tyrosine kinases mediate the pleiotypic effects of their ligands, including cell proliferation (Hanks et al., 1988). Transfection studies have demonstrated that the tyrosine kinase activity of the epidermal growth factor (EGF) receptor mediates the pleiotypic response to EGF in fibroblasts and hematopoietic cells (Honegger et al., 1987; Chen et al., 1987; Pierce et al., 1988). However, transforming growth factor–α (TGF-α), and not EGF, appears to be the cognate EGF receptor ligand produced in an autocrine fashion in a variety of epithelial tissues (Koyama and Podolsky, 1989; Cartlidge and Elder, 1989; Malden et al., 1989; Mead and Fausto, 1989; Markowitz et al., 1990). TGF-α binds to the EGF receptor with high affinity (Massague, 1983; Derynck et al., 1984), and overexpression of TGF-α in transgenic mice results in epithelial hyperplasia in several epithelial tissues (Matsui et al., 1990; Jhappan et al., 1990; Sandgren et al., 1990). TGF-α is markedly overexpressed in the skin lesions of psoriasis, an inflammatory and hyperplastic skin disease (Gottlieb et al., 1988; Elder et al., 1989a; Higashiyama et al., 1991). Recently, targeted overexpression of TGF-α in murine epidermis has been shown to cause epidermal hyperplasia and scaling similar to that seen in psoriatic lesions (Vassar and Fuchs, 1991). TGF-α and EGF both stimulate TGF-α expression (autoinduction) in murine and human keratinocytes (Coffey et al., 1987, 1988). Since TGF-α is potently mitogenic for keratinocytes (Coffey et al., 1987; Barrandon and Green, 1987; Elder et al., 1989b), increased EGF receptor tyrosine kinase activity could explain both the hyperplasia and the overex-

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pression of TGF-α which occur in psoriasis. In fact, increased EGF-dependent tyrosine kinase activity has been observed in psoriatic lesions (Gentleman et al., 1984).

One candidate for altered regulation of EGF receptor tyrosine kinase activity in psoriasis is protein kinase C (PKC). PKC has been reported to inhibit EGF receptor tyrosine kinase activity via phosphorylation of a threonine residue at position 654 of the receptor (Friedman et al., 1984; Davis, 1988; Livneh et al., 1988; Decker et al., 1990). Thus, PKC may normally act as a negative regulator of EGF receptor activation. PKC activity is significantly reduced in psoriatic epidermis (Horn et al., 1987). By this concept, reduced PKC activity in psoriasis would potentiate keratinocyte responses to EGF receptor activation, including proliferation and expression of the EGF receptor agonist, TGF-α.

Alternatively, it is also possible that activation, rather than down-regulation, of PKC is responsible for increased TGF-α expression in psoriasis. Diacylglycerol (DAG) and tetradecanoyl phorbol acetate (TPA), two agents which activate PKC, stimulate keratinocyte production of TGF-α mRNA and protein (Pittelkow et al., 1989). Moreover, several lines of evidence suggest that chronic PKC activation occurs in psoriasis. Phospholipase C (PLC) activity is markedly increased in psoriatic lesions (Bartel et al., 1987; Bergers et al., 1990; Fisher et al., 1990), as is the content of its product, the PKC activator diacylglycerol (Fisher et al., 1990). It has been suggested that down-regulation of PKC in response to chronic activation could explain the reduced PKC levels characteristic of psoriatic epidermis (Fisher et al., 1990).

One possible stimulus for PKC activation in psoriasis is TGF-α itself. Ligand binding results in tyrosine phosphorylation and activation of phospholipase C-gamma (PLC-gamma) by EGF receptor tyrosine kinase in A431 cells (Wahl et al., 1988; Margolis et al., 1989; Nishibe et al., 1990). Consistent with this model, EGF was found to activate phosphoinositide turnover and stimulate phosphorylation of the PKC-specific substrate p80 in the immortalized murine keratinocyte line, Balb/MK (Moscat et al., 1988). These results, coupled with the observation that direct activators of PKC are known to stimulate expression of TGF-α (Pittelkow et al., 1989), suggest that PKC might be a distal component of the signal transduction pathway mediating TGF-α autoinduction, and other cellular responses to TGF-α as well.

In order to address these interrelated issues, we have determined whether TGF-α autoinduction requires activation of the EGF receptor tyrosine kinase in human keratinocytes, and whether PKC activation is involved in this process as a postreceptor event. In addition, in order to determine whether PKC actually acts as a negative regulator of the EGF receptor tyrosine kinase in intact epidermal cells, we have determined whether experimental reduction in PKC activity can potentiate EGF receptor tyrosine kinase activation and autoinduction of TGF-α in human keratinocytes.

MATERIALS AND METHODS

Reagents

Bryostatin 1 was generously provided by Dr. G. Petit, University of Arizona, and the tyrphostins RG 50864 and 50862 were kindly supplied by Rorer Biotechnology, King of Prussia, PA. Recombinant human TGF-α was provided by Dr. R. Derynck, Genentech, Inc., South San Francisco, CA, or purchased from Collaborative Research. TGF-β1 from bovine bone was the kind gift of Dr. L. Ellingsworth, Collagen Corp., Palo Alto, CA. Preparation and characterization of the anti-EGF receptor monoclonal antibody 225 IgG has been described (Kawamoto et al., 1983; Gill et al., 1984; Sunada et al., 1986).

TPA and the monoclonal antibody MOPC 21 were from Sigma, and bovine insulin bovine pituitary extract and murine submaxillary gland epidermal growth factor were from Clonetics. Recombinant human cytokines IL-6 and GM-CSF were purchased from Amgen, IL-2, PDGF, and IFN-gamma from Collaborative Research, and IL-1α from Dainippon. All other biochemicals were of reagent grade.

Keratinocyte culture

Cultured adult human keratinocytes were propagated from keratome biopsies of human skin as described (Tsao et al., 1982; Wille et al., 1984), using a commercially available modification of MCDB 153 medium optimized for high density keratinocyte growth (Keratinocyte Growth Medium, Clonetics). Secondary cultures in the second to fourth passage were deprived of polypeptide growth factors at 40 to 50% confluence by incubation in MCDB 153 or Keratinocyte Basal Medium (Clonetics) for 48 hours prior to agonist treatment as described (Coffey et al., 1987). During this period, cell confluence increased to 80 to 90%. For analysis of TGF-α mRNA levels, cells were treated with natural murine EGF (Clonetics) or human recombinant TGF-α (Collaborative Research) at various doses for various times as described in the figure legends. Unless otherwise specified, the standard stimulation with either agent was 4 hours of treatment with 20 ng/ml EGF or TGF-α.

Clonal growth assays were performed as described (Tsao et al., 1982; Wille et al., 1984) except that Keratinocyte Basal Medium (Clonetics) was used instead of MCDB 153 and cells were plated at 2,000 rather than 400 cells per 60 mm dish.

RNA extraction and analysis

RNA was isolated by guanidinium isothionate with (Chomczynski and Sacchi, 1987) or without acid phenol extraction as described (Nickoloff et al., 1989). Methods for blot hybridization and washing, probe preparation and labelling, and laser densitometry methods have been described (Elder et al., 1990). Briefly, 20 μg total RNA was separated by electrophoresis in 1% agarose gels, transferred to derivatized nylon membranes (Zeta-Probe, Bio-Rad, Richmond, CA), and hybridized for 18–24 hours against 1×10^6 cpm/ml random-primed probe in 50% formamide, 5X SSC, 50 mM sodium phosphate (pH 7.0), 1X Denhardt's solution, 250 μg/ml yeast tRNA, 100 μg/ml denatured sonicated salmon sperm DNA, and 1% SDS. After autoradiography at −70°C using intensifying screens, band intensities were quantitated by laser densitometry using an LKB model 2202 densitometer coupled to a Hewlet-Packard 3390A integrator. Only bands which fell in the

linear range of densitometric responsiveness, as determined by loading different amounts of input RNA, were used. Unless otherwise indicated, data which have been quantitated by densitometry are normalized to the reference gene, cyclophilin, and expressed as fold-change relative to control untreated samples as described (Elder et al., 1990).

Protein kinase C assay

Total cellular PKC activity was measured in cultured keratinocytes deprived of growth factors for 30 hours, and bryostatin or DMSO vehicle was added for an additional 18 hours. Cells were harvested by scraping on ice in 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol, and 0.01% leupeptin, and disrupted by sonication. Total cellular PKC was extracted by addition of 1% Triton X-100 and partially purified by ion exchange chromatography (Kikkawa et al., 1982). Total PKC activity was determined by subtracting the histone phosphorylation obtained in the absence of calcium, phosphatidylserine, and TPA from that obtained in their presence (Fisher et al., 1987).

Western blot assay for tyrosine phosphorylation

Keratinocytes grown in 100 mm dishes were depleted of growth factors for 48 hours and treated with various doses of EGF or TGF-α as described above. After various periods of time (usually 5 minutes), cells were lysed for determination of tyrosine phosphorylation essentially as described (Honegger et al., 1990). Cells were rapidly placed on ice and washed once with ice-cold Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Gibco) containing 10 μM sodium orthovanadate, then scraped into 500 μl lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 200 μM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 4 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were passed three times through a sterile 21G needle and spun in a microfuge at 4°C for 5 minutes. Supernatants were either stored at −20°C for up to one week or analyzed directly. Forty to 100 μg protein (as determined by the Bradford dye-binding assay, Bio-Rad, Richmond, CA) were subjected to SDS-PAGE using precast gels (Novex) according to the manufacturer's instructions. Samples were transferred to 0.22 μ nitrocellulose sheets using a semi-dry blotting apparatus (Bio-Rad) according to manufacturer's instructions. Filters were blocked in 5% nonfat dry milk in PBS containing 0.1% NaN_3 (blotto) for 1 to 4 hours at 20°C or overnight at 4°C, then incubated in the antiphosphotyrosine mAb PY20 (Oncogene Sciences) at 2 μg/ml in blotto overnight at 4°C or for 4 hours at 20°C. Filters were then washed 3 × 10 minutes at 20°C in TBS/Tween (10 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% Tween-20), then incubated for 1 hour at 20°C in 4 μg/ml rabbit anti-mouse IgG (Cappell Research Products, Durham, NC) in blotto. After 3 × 10 minute washes in TBS/Tween, filters were incubated in ^{125}I staph A protein (10 μCi/ml, >30 μCi/μg, ICN, Costa Mesa, CA) in blotto, washed 3 × 10 minutes in blotto, dried briefly, and autoradiographed using intensifying screens.

TABLE 1. Ligand specificity of TGF-α induction[1]

Agonist[2]	n	Concentration	Induction[3]	95% C.I.[4]
TGF-α	39	20 ng/ml	5.0	3.7-6.3
EGF	19	20 ng/ml	3.7	2.4-5.1
Insulin	4	5 μg/ml	1.5	0.6-2.4
IGF-1	4	50 ng/ml	1.5	0.3-2.7
BPE	4	0.4%	1.3	0.4-2.3
TGF-β	4	2.5 ng/ml	1.1	0.4-1.8
IL-6	4	20 ng/ml	1.4	0.6-2.2
GM-CSF	4	20 ng/ml	1.3	0.6-2.0
PDGF	4	5 U/ml	0.7	0.2-1.2
bFGF	3	2 ng/ml	1.1	0.4-1.8
IL-1α	3	100 U/ml	0.7	0.4-1.1
IL-2	2	100 U/ml	0.6	<0-1.6
IFN-gamma	2	100 U/ml	0.5	<0-1.3

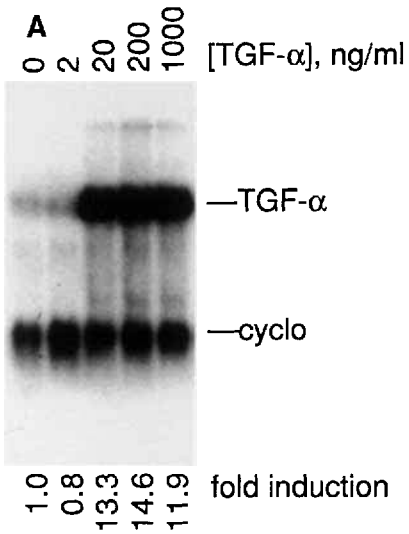
[1]Quiescent keratinocytes were treated for 4 hours with the indicated concentrations of the agonists shown.
[2]Abbreviations: bFGF, basic fibroblast growth factor; BPE, bovine pituitary extract; GM-CSF, colony stimulating factor (granulocyte-macrophage); IFN-gamma, interferon-gamma; IGF-1, insulinlike growth factor-1; IL-1α, interleukin 1-alpha; IL-2, interleukin-2; IL-6, interleukin 6; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.
[3]Mean fold-induction relative to untreated quiescent keratinocytes, after normalization to cyclophilin.
[4]95% confidence interval. 95% confidence intervals were calculated from the standard errors of the induction values.

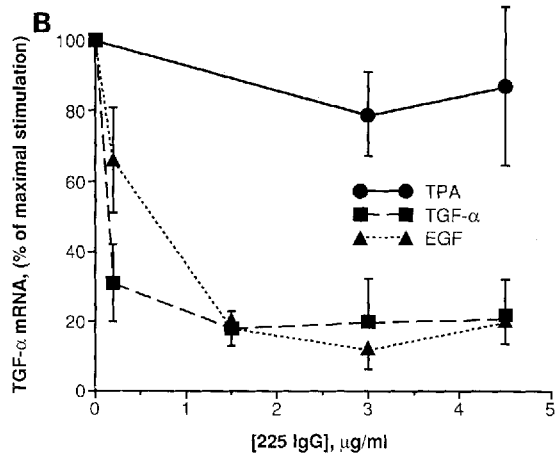
Immunoprecipitation

Keratinocytes in 100 mm dishes were treated with EGF (200 ng/ml) for 5 minutes, washed with ice-cold Ca^{++}, Mg^{++}-free PBS containing 10 μM $NaVO_4$, and lysed in 500 μl ice-cold RIPA buffer as described (Sunada et al., 1990). Immunoprecipitation was carried out by adding 2 μg of the anti-EGF receptor mAbs 528, 225 (Sunada et al., 1986) or 05-101 (UBI) to 250 μl of RIPA lysate for 30 minutes at room temperature followed by 5 μg of rabbit anti-mouse (RAM) IgG (Cappel) for 15 minutes. 50 μl of 10% Pansorbin was then added, and incubation continued an additional 30 minutes. After pelleting in a microfuge, the pellet was washed twice in 10 mM Hepes (pH 7.4), 0.5% Triton X-100, 5% glycerol as described (Sunada et al., 1990). Electrophoresis, blotting, and decoration with PY20 were as described above, except that the incubation with RAM IgG secondary antibody was omitted.

RESULTS

We assessed the specificity of TGF-α mRNA induction in response to various polypeptides to investigate the involvement of the EGF receptor in the process of TGF-α autoinduction. Growth factor–deprived keratinocytes were stimulated for 4 hours with a variety of growth factors and cytokines and induction of TGF-α transcripts was assessed by RNA blot hybridization. Of the 13 factors tested, only TGF-α and EGF significantly induced TGF-α mRNA (Table 1). This ligand specificity strongly suggests that TGF-α autoinduction is mediated by the EGF receptor. In order to further substantiate this conclusion, we measured the effects of 225 IgG, a well-characterized anti-EGF receptor monoclonal antibody (Kawamoto et al., 1983; Gill et al., 1984; Sunada et al., 1986), on TGF-α autoinduction and EGF receptor tyrosine phosphorylation in keratinocytes. At 4°C, 225 IgG competitively inhibits EGF and TGF-α binding,





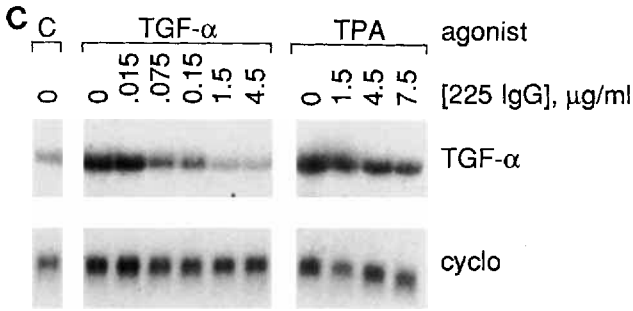


Fig. 1. Inhibition of TGF-α autoinduction by mAb 225 IgG. **A:** Dose response of TGF-α autoinduction. Growth factor–depleted keratinocytes were incubated with the indicated concentrations of TGF-α for 4 hours, followed by lysis and RNA blot analysis against TGF-α and cyclophilin probes. Fold-induction of TGF-α mRNA after normalization to cyclophilin is shown below the autoradiogram. **B:** Autoinduction of TGF-α mRNA is inhibited by mAb 225 IgG. Growth factor–depleted keratinocytes were incubated with the indicated concentrations of mAb 225 IgG for 30 minutes, followed by addition of TPA (20 nM), TGF-α (20 ng/ml) or EGF (20 ng/ml). RNA was harvested after 4 hours and analyzed by blot hybridization. Data are expressed as a percentage of the stimulation over baseline obtained for each agonist in the absence of 225 IgG. Data shown represent mean ± SEM of three or more determinations. **C:** RNA blot hybridization of a representative experiment. *C* indicates no addition of agonist.

while at 37°C, it causes EGFR internalization in intact cells but does not stimulate EGFR autophosphorylation (Sunada et al., 1986).

Figure 1A demonstrates the dose response of TGF-α mRNA accumulation in response to exogenous TGF-α in cultured keratinocytes (autoinduction). This response has previously been shown to correlate with the accumulation of TGF-α protein in the culture supernatant under these conditions (Coffey et al., 1987). Maximal induction was obtained at a dose of 20 ng/ml TGF-α, and this dose was used for subsequent experiments. Pretreatment with 225 IgG for 30 minutes potently and markedly inhibited TGF-α mRNA accumulation in response to both TGF-α and EGF. In contrast, the antibody did not inhibit accumulation of TGF-α mRNA accumulation in response to TPA (Fig. 1B,C).

As shown in Figure 2A, EGF and TGF-α rapidly stimulated tyrosine phosphorylation of a band of Mr ~ 170 kDa in growth factor–depleted human keratinocytes. Persistent phosphorylation of this band was noted in the continued presence of TGF-α agonist for up to 4 hours. The 170 kDa band was demonstrated to be the EGF receptor by co-migration with a band present in EGF-stimulated A431 control membranes (Fig. 2A–C), and by immunoprecipitation of a band of 170 kDa with three different anti-EGF receptor mAbs (Fig. 2B). As shown in Figure 2C, EGF-stimulated tyrosine phosphorylation of the 170 kDa band was completely inhibited by 30 minutes preincubation with 5 μg/ml of the anti-EGF receptor mAb 225 IgG, while the presence of the same concentration of the isotype control mAb MOPC 21 had no effect. As previously reported in A431 cells (Sunada et al., 1986), treatment of keratinocytes with 225 IgG alone did not result in tyrosine phosphorylation of the 170 kDa band (Fig. 2B).

As shown in Figure 3, mAb 225 IgG also inhibited clonal keratinocyte growth with a potency similar to its effects on autoinduction (IC_{50} approximately 0.1 μg/ml). The isotype control antibody, MOPC 21, was inactive in inhibiting clonal keratinocyte growth (data not shown).

To directly demonstrate a requirement for the tyrosine kinase activity of the EGF receptor in keratinocyte TGF-α autoinduction, we treated growth factor–depleted keratinocytes with the tyrphostin RG50864, a specific inhibitor of EGF receptor tyrosine kinase activity (Lyall et al., 1989; Posner et al., 1989). As shown in Figure 4A, RG50864 inhibited EGF- and TGF-α–stimulated tyrosine phosphorylation in growth factor–depleted keratinocytes at 40 and 120 μM. The related tyrphostin, RG50862 (Lyall et al., 1989) was less active, inhibiting tyrosine phosphorylation at 120 μM but not at 40 μM. In some experiments, RG50864 alone demonstrated a partial agonist effect (Fig. 4A, left panel). As shown in Figure 4B and C, RG50864 displayed a dose-dependent inhibition of TGF-α mRNA accumulation in response to increasing amounts of exogenous TGF-α. Relatively high concentrations of RG50864 (>40 μM)

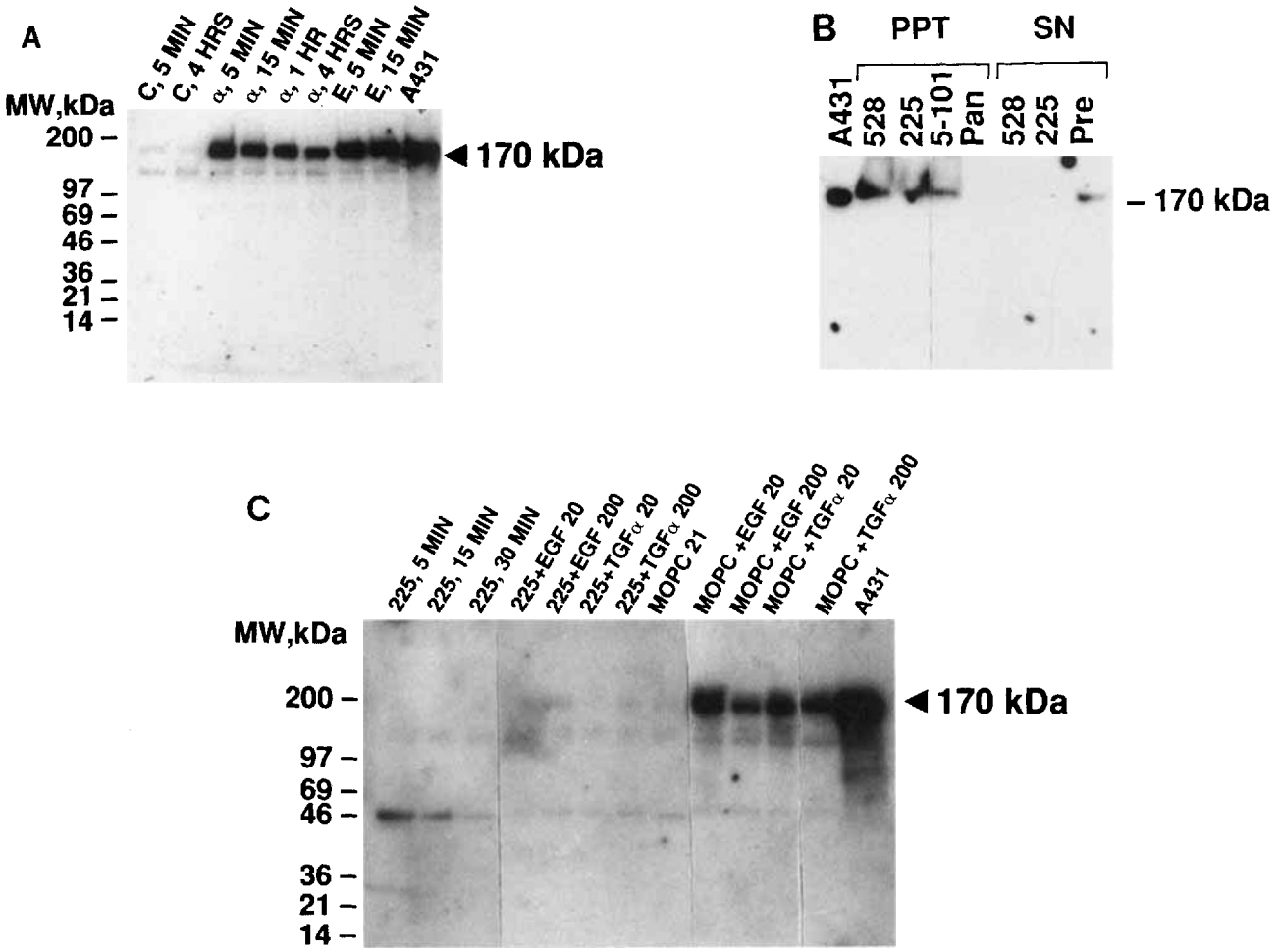


Fig. 2. Immunoblot analysis of tyrosine phosphorylation in intact human keratinocytes. **A:** Time course of EGF- and TGF-α–stimulated tyrosine phosphorylation. Growth factor–depleted keratinocytes were treated with EGF or TGF-α for the times indicated, then lysed as described in Materials and Methods. Gradient gels of 8–16% SDS-PAGE were loaded with 40 μg keratinocyte lysate protein per lane (approximately 2×10^5 cell equivalents), immunoblotted, and decorated with PY20 antiphosphotyrosine mAb as described in Materials and Methods. Mobilities of molecular weight standards are indicated to the left. α, TGF-α, 20 ng/ml; E, EGF, 20 ng/ml; A431, 5 μl solubilized membrane preparation from 5×10^4 EGF-treated A431 cells (UBI). **B:** Immunoprecipitation of EGF receptors. EGF-treated keratinocytes were lysed in RIPA buffer and equal aliquots were immunoprecipitated (PPT) using the anti-EGFR mAbs 528, 225, or 5-101 as described in Materials and Methods. Primary antibody was omitted in the pansorbin control (Pan). Equal aliquots (10 μl/100 μl) of the solubilized immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting against PY20 as described above. Equal aliquots (20 μl/250 μl) of the 528 and 225 post-immunoprecipitation supernatants (SN) and pre-immunoprecipitation lysate (Pre) were run in parallel. A431, EGF-stimulated A431 membranes. **C:** Inhibition of EGF- and TGF-α–stimulated tyrosine phosphorylation by mAb 225 IgG. Growth factor–depleted keratinocytes were treated with 5 μg/ml 225 IgG for 30 minutes, then treated for 5 minutes with the indicated concentrations of EGF or TGF-α. 225, anti-EGF receptor mAb 225 IgG: MOPC, isotype control mAb MOPC 21. Concentrations of EGF and TGF-α are given in ng/ml. A431, EGF-stimulated A431 membranes.

were required to inhibit both tyrosine phosphorylation and TGF-α autoinduction in keratinocytes (see Discussion).

Because agents which activate PKC can stimulate TGF-α expression (Pittelkow et al., 1989), and because of numerous studies suggesting a link between EGF receptor activation, phospholipase C activation, and increased levels of the potential PKC activators calcium and diacylglycerol (see introduction and Discussion), we wished to determine whether PKC activation was a downstream signal transduction event required for TGF-α autoinduction. If PKC is not a downstream mediator of this process, then it process should not be affected by experimental reduction of PKC activity prior to TGF-α/EGF treatment. Because of the lack of specificity of most protein kinase inhibitors, we chose to assess the PKC independence of TGF-α autoinduction by down-regulation of PKC activity (Tapley and Murray, 1984). TPA is commonly used for this purpose, but is unsuitable for use in keratinocytes because it causes loss of EGF receptors in these cells (Jeng et al., 1985; Jetten et al., 1989) and causes a sustained induction of TGF-α mRNA (Pittelkow et al., 1989). Bryostatin is a macrocyclic lactone which causes membrane translocation of PKC followed by down-regulation of PKC activity (Kraft et al., 1987), with only a transient loss of EGF binding in keratinocytes (Sako et al., 1987; Jetten et al., 1989). To verify that PKC activity was reduced by

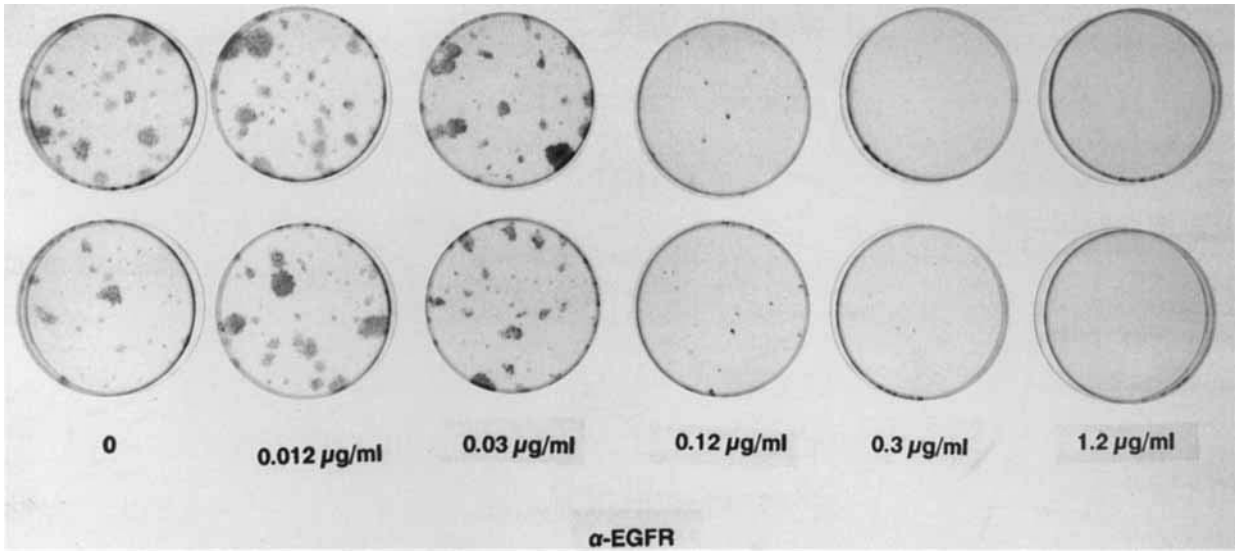


Fig. 3. Inhibition of keratinocyte growth by anti-EGFR IgG. Keratinocytes were plated in Keratinocyte Basal Medium (Clonetics) containing EGF (1 ng/ml), insulin (5 μg/ml), and bovine pituitary extract (0.4%). Twenty-four hours after plating, azide-free 225 IgG in phosphate buffered saline was added to each dish at the indicated concentrations. Duplicate dishes were fixed and stained with crystal violet two weeks later.

bryostatin, growth factor–depleted keratinocytes were treated with various concentrations of bryostatin for 18 hours and PKC activity was determined. After 20 nM bryostatin treatment, PKC activity was reduced by an average of 91% (Fig. 5). Consistent with its inhibitory effect on PKC activity, at this dose bryostatin totally inhibited TPA-stimulated accumulation of TGF-α mRNA (Fig. 6A). Furthermore, bryostatin, unlike TPA, did not itself induce TGF-α mRNA (Fig. 6A). Consistent with the hypothesis that PKC activation is not a downstream mediator of TGF-α autoinduction, accumulation of TGF-α mRNA in response to exogenous TGF-α was not inhibited by bryostatin treatment (Fig. 6B,C).

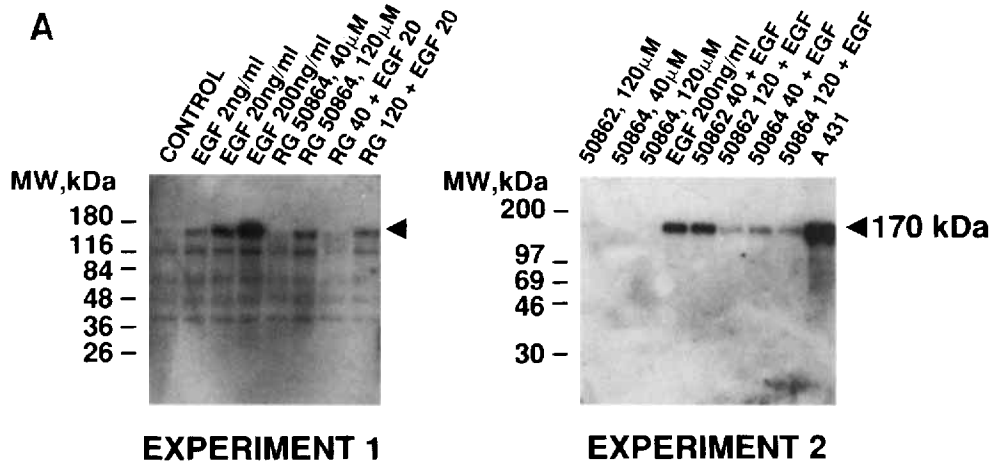
Because PKC has been reported to inhibit EGF receptor tyrosine kinase activity (see introduction), we hypothesized that reduction of PKC activity by bryostatin would potentiate EGF receptor activation in response to ligand as well as TGF-α autoinduction. However, as shown in Figure 6B and C, pretreatment with 2 or 20 nM bryostatin for 18 hours did not alter the dose dependence or increase the magnitude of the induction of TGF-α mRNA in response to exogenous TGF-α. In addition, pretreatment with 20 nM bryostatin failed to potentiate tyrosine phosphorylation of the 170 kDa band on Western blots in response to 20 or 200 ng/ml EGF (Fig. 7).

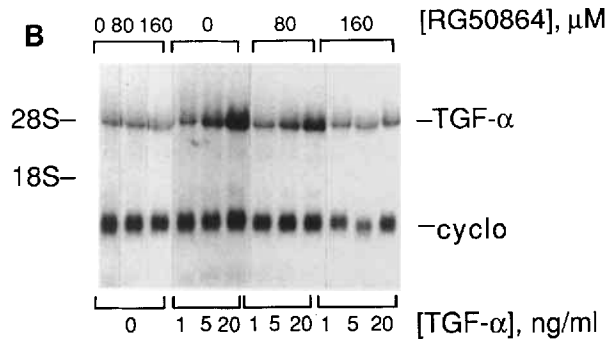
DISCUSSION

Several studies involving transfection of normal and mutant receptors have demonstrated an important role for the tyrosine kinase activity of the EGF receptor in mediating the pleiotypic response to EGF (Honegger et al., 1987; Chen et al., 1987; Pierce et al., 1988). We have hypothesized that one component of this response, the autoinduction of TGF-α mRNA and protein, could be relevant to the pathogenesis of psoriasis (Elder et al., 1989a, and vide supra). However, transfection of EGF receptors cannot be used to assess the role of EGF receptor tyrosine kinase activation in this process in keratinocytes due to endogenous EGF receptor expression in these cells. Therefore, we have tested the specificity of this response for various polypeptide ligands, and made use of specific antibodies and inhibitors to demonstrate that accumulation of TGF-α mRNA in response to exogenous TGF-α or EGF occurs via the EGF receptor and requires its tyrosine kinase activity. In addition, we demonstrate for the first time the pattern of EGF- and TGF-α–stimulated tyrosine phosphorylation in intact human keratinocytes, and compare this immediate (proximal) response to agonist to the delayed (distal) response of TGF-α autoinduction under various conditions.

EGF and TGF-α stimulate tyrosine phosphorylation of a 170 kDa substrate in intact keratinocytes

Figure 2 demonstrates that both EGF and TGF-α rapidly and markedly stimulate tyrosine phosphorylation of a 170 kDa band in detergent lysates of quiescent cultured keratinocytes, consistent with phosphorylation of the EGF receptor. Three different anti-EGFR mAbs immunoprecipitated a band of the same Mr, identifying this band as the EGF receptor (Fig. 2B). Background bands of 40, 47, 84, and about 110 kDa have been variably noted in a number of experiments (Figs. 1, 4, 7); however, the molecular identity of these bands is presently unclear. Utilizing metabolic labelling coupled with immunoprecipitation, Dvir and colleagues (1991) have reported EGF-dependent tyrosine phosphorylation of the EGF receptor as well as additional bands in permeabilized keratinocytes grown on fibroblast feeder layers. However, this is the first description of the pattern of EGF- and TGF-α–stimulated tyrosine phosphorylation in intact normal keratinocytes.





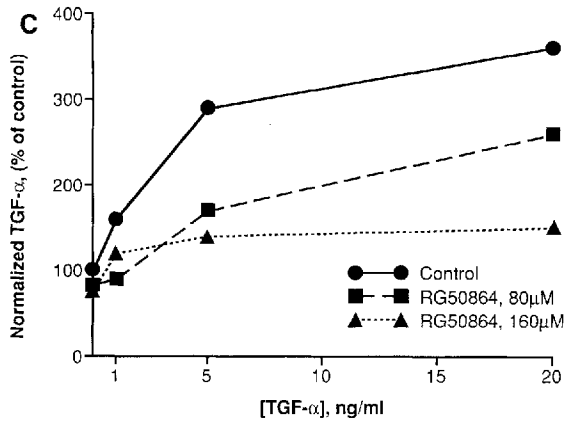


Fig. 4. Inhibition of EGF-stimulated tyrosine phosphorylation and TGF-α autoinduction by tyrphostin. **A:** Immunoblot analysis of tyrosine phosphorylation. Growth factor–depleted keratinocytes were treated with the indicated concentrations of RG50864 or RG50862 for 18 hours. EGF was then added for 5 minutes at 37°C, and cells were lysed for immunoblot analysis as described in Materials and Methods. Identical results have been obtained using TGF-α rather than EGF as agonist (not shown). Results shown are representative of 4 independent experiments. **B:** RNA blot analysis. Quiescent keratinocytes were pretreated for 18 hours with 80 or 160 μM RG50864 prior to addition of the indicated amounts of TGF-α for 4 hours. RNA isolation and blot hybridization analysis were as in Fig. 1A. This autoradiograph is representative of 6 experiments with pretreatment times varying between 2 and 18 hours. **C:** Quantitation of data shown in Fig. 4B (see Materials and Methods). Data are expressed as a percentage of the TGF-α hybridization obtained without TGF-α or tyrphostin treatment. Values shown are normalized to cyclophilin.

In most of our experiments, only a single 170 kDa band demonstrated agonist-dependent tyrosine phosphorylation (Figs. 2, 4). However, we have occasionally observed agonist-dependent phosphorylation of a band of about 110 kDa, the identity of which is presently unknown (Fig. 7). It is possible that differences in culture conditions and/or the use of permeabilized rather than intact living cells may account for the differences between these our studies and those of Dvir et al. However, in neither study was tyrosine phosphorylation of a Mr 145 kDa band observed, as would be expected if PLC-gamma1 were a predominant substrate for the EGF receptor. Tyrosine phosphorylation of a variety of proteins, including but not limited to growth factor receptors, has been shown to stimulate binding to other proteins bearing Src homology (SH) domains (Koch et al., 1991). Our observation that the 170 kDa band is the predominant protein phosphorylated on tyrosine in response to EGF or TGF-α in intact cells suggests that tyrosine phosphorylation of substrates other than the EGF receptor itself may not be central to the mode of EGF/TGF-α action in keratinocytes.

TGF-α autoinduction requires EGF receptor activation

Of 13 polypeptide ligands tested, only EGF and TGF-α stimulated TGF-α mRNA levels in quiescent keratinocytes (Table 1), which strongly suggests that TGF-α autoinduction is a specific receptor-mediated event. Consistent with this interpretation, autoinduction of TGF-α mRNA (Fig. 1B,C), EGF-dependent tyrosine phosphorylation (Fig. 2C), and keratinocyte growth (Fig. 3) were all markedly inhibited by the anti-EGF receptor monoclonal antibody, 225 IgG. The tyrphostin RG50864 markedly inhibited EGF- and TGF-α–dependent tyrosine phosphorylation (Fig. 4A) and autoinduction of TGF-α mRNA (Fig. 4B,C), strongly suggesting that the tyrosine kinase activity of the EGF

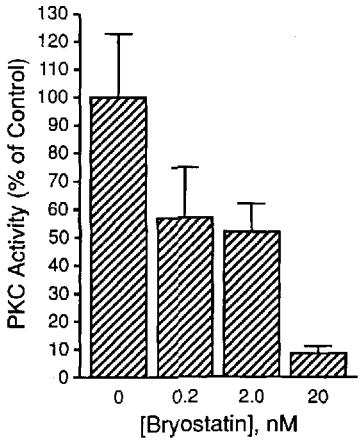


Fig. 5. Down regulation of PKC by bryostatin. Growth factor–depleted keratinocytes were treated with bryostatin and PKC activity was determined as described in Materials and Methods. Bars indicate mean ± SEM of 5 experiments (except 0.2 nM bryostatin, 4 experiments). Data are expressed as a percentage of untreated controls.

receptor is required for this process. The dose response of TGF-α mRNA accumulation (Fig. 1A) was maximal at 20 ng/ml TGF-α (4 nM) and ranged from 1–20 ng/ml (0.2–4 nM), in general agreement with the saturation binding curve for EGF in human KC (K_d = 0.5 nM, saturation at 2 nM) (Nickoloff and Mitra, 1989). These results suggest that high receptor occupancy is required for autoinduction under these conditions. The relatively high concentrations of RG50864 required to inhibit autoinduction and tyrosine phosphorylation in these experiments (Fig. 4) could reflect this requirement for high receptor occupancy, and/or the observation that tyrphostin potency is reduced in late log phase/early stationary phase human keratinocytes (Ferriola et al., 1991).

Two pathways for induction of TGF-α mRNA

Although direct activators of PKC such as TPA and diacylglycerols can clearly cause accumulation of TGF-α mRNA (Pittelkow et al., 1989), several lines of evidence indicate that EGF receptor tyrosine kinase and PKC activation stimulate TGF-α expression by distinct mechanisms. Thus, TGF-α autoinduction was inhibited by the tyrosine kinase inhibitor RG50864 (Fig. 4) but not after down-regulation of over 90% of keratinocyte PKC activity in response to bryostatin (Figs. 5, 6). In contrast, induction of TGF-α in response to TPA treatment was markedly inhibited by bryostatin (Fig. 6B). It is noteworthy that unlike TPA, bryostatin did not stimulate TGF-α mRNA levels (Fig. 6B). This observation is consistent with several reports demonstrating that bryostatins mimic only some but antagonize many of the actions of TPA (Smith et al., 1985; Sako et al., 1987; Kraft et al., 1987; Jetten et al., 1989; Gschwendt et al., 1988).

TGF-α autoinduction may not require PKC activation

Based on the insensitivity of TGF-α autoinduction to bryostatin treatment (Fig. 6), we conclude that activation of PKC is not necessary for TGF-α autoinduction in keratinocytes. The use of other protein kinase inhibitors, such as the isoquinolinesulfonamide H-7, is inappropriate for this purpose, since these inhibitors are not specific for inhibition of protein kinase C (Hidaka et al., 1984). Our results are consistent with several reports demonstrating that EGF does not functionally activate PKC in epithelial cells (Vaartjes et al., 1986; Baliga and Morowitz, 1988; Thompson et al., 1988). While modest elevations of phosphoinositide turnover and/or DAG mass have been observed in response to EGF in murine keratinocytes, hamster fibroblasts, A431 cells, and rat hepatocytes (Moscat et al., 1988; Johnson and Garrison, 1987; Kato et al., 1988; Pessin et al., 1990), activation of PKC in response to EGF has not been detected by direct measurement. In one study (Moscat et al., 1988), a modest (<twofold) increase in phosphorylation of an acidic 80 kDa protein (MARCKS protein), thought to be a specific substrate for PKC phosphorylation (Blackshear et al., 1985), was observed in response to EGF in BALB/MK immortalized murine keratinocytes. However, in many cell lines, serum and/or direct PKC activators such as TPA lead to much larger increases in MARCKS phosphorylation (Blackshear et al., 1991). Moreover, we have been unable to demonstrate significant EGF- or TGF-α–stimulated phosphorylation of MARCKS protein in normal human keratinocytes under conditions in which large increases in tyrosine phosphorylation of the 170 kDa protein have been consistently detected (N. Reynolds, T. Jensen, J. Elder, and G. Fisher, manuscript in preparation). Similarly, exhaustive studies of insulin responses in cells massively overexpressing insulin receptors have failed to convincingly demonstrate PKC activation as a downstream event in the cellular response to insulin (Blackshear et al., 1991). Nevertheless, it remains possible that PKC isoforms whose activity might not be detectable under our assay conditions and which may not be down-regulated by bryostatin could be involved in the transduction of the autoinductive response to TGF-α.

Bryostatin down-regulates PKC but does not potentiate EGF responses

Phosphorylation of ^{654}Thr of the EGF receptor by PKC results in inhibition of EGFR-TK activity, and mutation of this residue leads to loss of phorbol ester inhibition of EGF-stimulated growth (Friedman et al., 1984; Livneh et al., 1988; Decker et al., 1990). Loss of PKC-mediated inhibition of EGFR-TK could explain the increased TGF-α gene expression and proliferation which occur in the context of reduced PKC activity in psoriatic epidermis. However, we were unable to demonstrate potentiation of TGF-α autoinduction (Fig. 6B,C) or of EGF-stimulated tyrosine phosphorylation (Fig. 7) after down-regulation of PKC activity with bryostatin. These results, as well as the inability of bryostatin to stimulate TGF-α mRNA (Fig. 6A) raise the possibility that PKC isoforms not detectable by our assay conditions and/or unresponsive to bryostatin are involved in down-regulation of EGF receptor tyrosine kinase activity in keratinocytes.

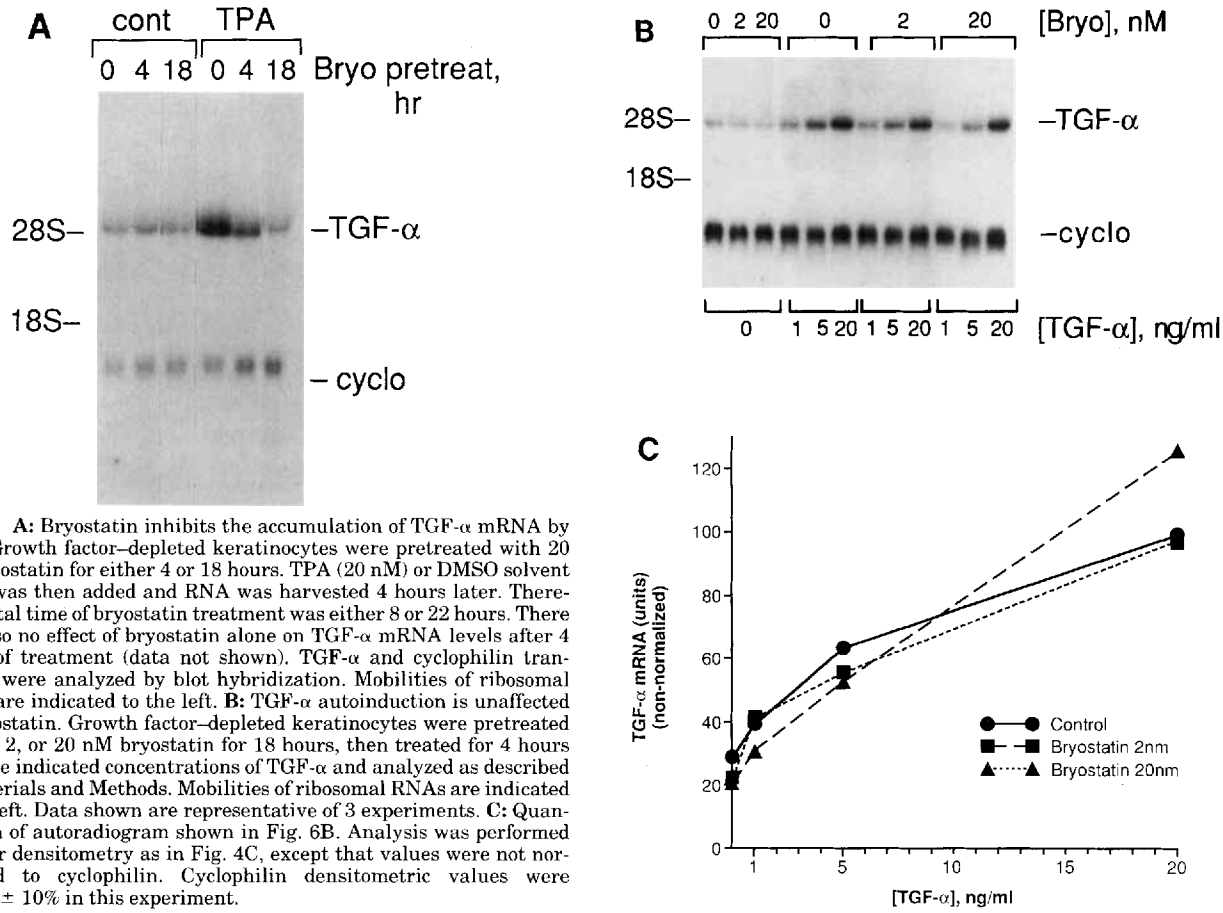


Fig. 6. **A:** Bryostatin inhibits the accumulation of TGF-α mRNA by TPA. Growth factor–depleted keratinocytes were pretreated with 20 nM bryostatin for either 4 or 18 hours. TPA (20 nM) or DMSO solvent (cont) was then added and RNA was harvested 4 hours later. Therefore, total time of bryostatin treatment was either 8 or 22 hours. There was also no effect of bryostatin alone on TGF-α mRNA levels after 4 hours of treatment (data not shown). TGF-α and cyclophilin transcripts were analyzed by blot hybridization. Mobilities of ribosomal RNAs are indicated to the left. **B:** TGF-α autoinduction is unaffected by bryostatin. Growth factor–depleted keratinocytes were pretreated with 0, 2, or 20 nM bryostatin for 18 hours, then treated for 4 hours with the indicated concentrations of TGF-α and analyzed as described in Materials and Methods. Mobilities of ribosomal RNAs are indicated to the left. Data shown are representative of 3 experiments. **C:** Quantitation of autoradiogram shown in Fig. 6B. Analysis was performed by laser densitometry as in Fig. 4C, except that values were not normalized to cyclophilin. Cyclophilin densitometric values were within ± 10% in this experiment.

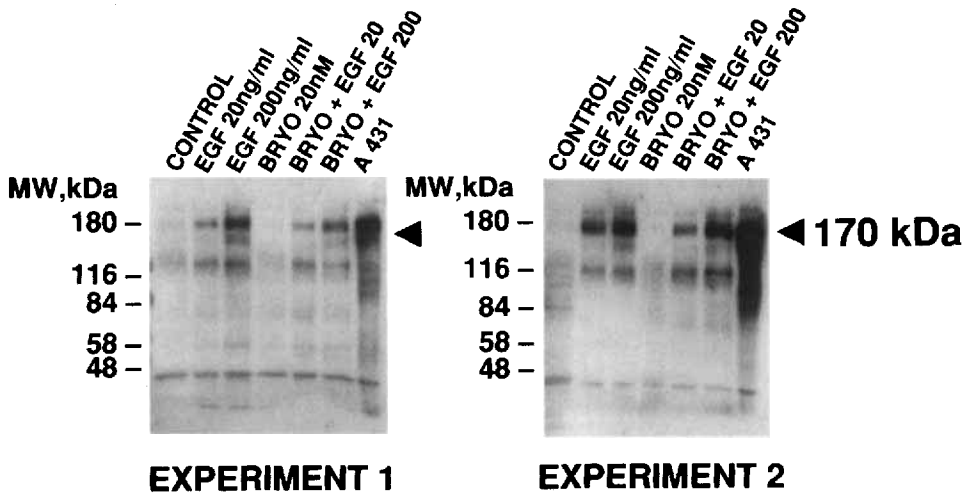


Fig. 7. Immunoblot analysis of bryostatin effects on EGF-stimulated tyrosine phosphorylation in growth factor–depeleted keratinocytes. Cells were pretreated with 20 nM bryostatin for 18 hours, then treated for 5 minutes with the indicated concentrations of EGF. The results of 2 independent experiments are shown.

Summary and perspectives

The results presented here argue for the existence of at least two pathways by which TGF-α can be stimulated in human keratinocytes. TGF-α autoinduction occurs via a pathway which is triggered by activation of the EGF receptor tyrosine kinase in response to EGF or TGF-α, and is not inhibited or potentiated by bryostatin. In contrast, induction of TGF-α by TPA occurs inde-

pendently of EGF receptor activation, and is inhibited by PKC down-regulation in response to bryostatin. It is possible that TGF-α plays important roles in both proliferation and differentiation of the epidermis, as suggested by Finzi et al. (1991). If this is the case, the TGF-α autoinductive pathway may be important in the regulation of keratinocyte growth, while the PKC-dependent pathway of TGF-α activation may be involved in the regulation of keratinocyte differentiation.

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