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Phorbol ester-stimulated phosphorylation of keratinocyte transglutaminase in the membrane anchorage region

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The membrane-bound transglutaminase of cultured keratinocytes became radioactively labelled upon addition of $[^{32}P]P_i$ to the medium. Transglutaminase phosphorylation was also demonstrable using particulate material isolated from cell homogenates. Compatible with mediation of the labelling by protein kinase C, the degree of phosphorylation in intact cells was stimulated approx. 5-fold in 4 h on treatment with the tumour-promoting phorbol ester phorbol 12-myristate 13-acetate, but not by phorbol. The extent of labelling was virtually unaffected by cycloheximide inhibition of protein synthesis, indicating that it arose primarily through turnover of phosphate in the membrane-bound enzyme. Phosphoamino acid analysis detected labelling only of serine residues. Most of the label was removed by trypsin release of the enzyme from the particulate fraction of cell homogenates, which deletes a membrane anchorage region of $\approx$ 10 kDa. Upon trypsin treatment of the enzyme after immunoprecipitation, the phosphate label was recovered in soluble peptide material with a size of several thousand Da or less. Indicative of fragmentation of the membrane anchorage region, this material was separable by h.p.l.c. into two equally labelled peptides. Moreover, when the enzyme was labelled with $[^{3}H]$palmitate or $[^{3}H]$myristate, the fatty-acid-labelled peptide material required non-ionic detergent for solubilization and was separable from the phosphate-labelled material by gel filtration. Phorbol ester treatment of cultured keratinocytes in high- or low-Ca^{2+}-containing medium was not accompanied by an appreciable protein-synthesis-independent change in transglutaminase activity. Independent of possible alteration of the intrinsic catalytic activity of the enzyme, phosphorylation may well modulate its interaction with substrate proteins, a potential site for physiological regulation.

INTRODUCTION

During the terminal stages of keratinocyte differentiation, in the epidermis and in culture, a protein envelope is formed (Matoltsy & Balsamo, 1955; Sun & Green, 1976). Stabilized by extensive ε-(γ-glutamyl)lysine isopeptide cross-linking involving 15–20 % of constituent lysine residues (Rice & Green, 1977), this structure contributes to the cohesiveness of cornified epithelia. Localization of the envelope at the cell periphery occurs through the action of a membrane-bound transglutaminase (Simon & Green, 1985; Thacher & Rice, 1985). This enzyme is distinct from the well-studied tissue transglutaminase, which can also be expressed by keratinocytes exposed to retinoids (Lichti *et al.*, 1985; Rubin & Rice, 1986), but which is unnecessary for envelope formation (Thacher *et al.*, 1985). While envelope structures can be induced in cultured cells by permeabilization to Ca^{2+} (Rice & Green, 1979), they are not identical with those which form spontaneously in these cells, probably due to the abrupt onset of cross-linking instead of the gradual process which occurs ordinarily (Warhol *et al.*, 1985). The transglutaminase has access to a variety of potential substrate proteins, perhaps varying with the physiological state of the cell, which are capable of being cross-linked upon activation of the enzyme in intact keratinocytes or in extracts (Simon & Green, 1984; Michel *et al.*, 1987; Nagae *et al.*, 1987). Understanding how such interactions are modulated is of importance for elucidating the proper sequence of events resulting in envelope formation in the epidermis.

Phorbol diester tumour promoters considerably alter intracellular processes in epithelial targets such as keratinocytes. In culture, for example, phorbol 12-myristate 13-acetate (PMA; 'TPA') greatly reduces the colony-forming ability of cultured normal human epidermal cells and elicits the formation of cross-linked envelopes (Parkinson *et al.*, 1983; Wille *et al.*, 1985), as originally demonstrated with mouse epidermal cells (Yuspa *et al.*, 1982). This action, which resembles terminal differentiation in this cell type, is highly likely due to the stimulation of protein kinase C by PMA as opposed to the generation of active oxygen species (Gabrielson *et al.*, 1988). Since envelope formation can be stimulated by keratinocyte permeabilization without a need for altered gene expression (Rice & Green, 1979), additional characterization of PMA-stimulated events would help clarify the role of protein kinase C in this process. The activity of keratinocyte transglutaminase is reported to be considerably increased in mouse epidermal cultures treated for 6–12 h with PMA (Jeng *et al.*, 1985). The possibility that this phenomenon might reflect in part post-translational phosphorylation prompted the present study. This transglutaminase is already known to be subject to another post-translational modification, namely anchorage in the membrane as a consequence of fatty acid acylation (Chakravarty & Rice, 1989).

EXPERIMENTAL

Cell culture

Normal human epidermal cells were propagated according to standard methods (Allen-Hoffman & Rheinwald, 1984) by co-cultivation with a feeder layer of lethally irradiated mouse 3T3 cells. The cells were grown in a 3:1 mixture of Dulbecco–Vogt Eagle's and Hams F-12 media supplemented with fetal bovine serum (5 %), cortisol (0.4 μg/ml), epidermal growth factor (10 ng/ml), adenine (0.18 mM), tri-iodothyronine (20 pM), insulin

Abbreviations used: PMA, phorbol 12-myristate 13-acetate ('TPA'); Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK').

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(5 μg/ml), transferrin (5 μg/ml) and antibiotics. Cholera toxin (10 ng/ml) was added upon inoculation of the cultures, but not at subsequent medium changes. Certain experiments employed Dulbecco–Vogt Eagle's medium prepared without added $CaCl_2$, which was approx. 25 μM in Ca^{2+} (Rubin & Rice, 1986).

Phosphate labelling

Each 10 cm culture, confluent for approx. 1 week, was incubated for 4 h with 5 ml of Dulbecco–Vogt Eagle's medium prepared without added phosphate and supplemented with fetal bovine serum (5%), 10 mM-Hepes buffer, pH 7.2, $[^{32}P]P_i$ (1 mCi) and, in some cases, other reagents as indicated. The culture was rinsed twice with iso-osmotic neutral saline, scraped from the dish and stored frozen overnight. Before labelling, samples of newborn-human skin were largely freed of subcutaneous fat and vascular connective tissue by dissection. They were then incubated for 15 min in 4 ml of low phosphate medium with serum (two foreskins per dish), after which 2 mCi of $[^{32}P]P_i$ was then added, and 15 min later PMA was added to 1 μM. After a further 4 h, the samples were rinsed in iso-osmotic neutral saline and frozen overnight.

Cell-free phosphorylation

Particulate material was isolated by homogenization of frozen cultures in 20 mM-Tris/HCl buffer (pH 7.5)/1 mM-EDTA with subsequent high-speed centrifugation (100000 *g*, 1 h) and was then resuspended in 1 ml of Tris/HCl (pH 8.0)/8 μM-$MgCl_2$/5 μM-$CaCl_2$ containing 50 μCi of $[\gamma\text{-}^{32}P]$ATP and 10 μg each of antipain and leupeptin. After incubation at 30 °C for 30 min, EDTA was added to 0.1 M, sodium molybdate to 10 mM, and the transglutaminase was solubilized with Emulgen and immunoprecipitated.

Immunoprecipitation

Each labelled culture or tissue sample was homogenized in 6 ml of 50 mM-Tris/HCl (pH 8.0)/10 mM-sodium molybdate/1 mM-EDTA. The particulate material was then isolated by high-speed centrifugation (100000 *g* for 1 h), resuspended in 6 ml of 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/0.3% Emulgen 911 non-ionic detergent and stirred at 4 °C for 2 h. [Alternatively the transglutaminase was solubilized from the isolated particulate material by mild digestion, i.e. suspension for 5 min in 2 ml of 20 mM-Tris/HCl, pH 8.0, containing 50 μg of tosylphenylalanylchloromethane (Tos-Phe-CH_2Cl; 'TPCK')-treated trypsin (Worthington Biochem Co., Freehold, NJ, U.S.A.), followed by addition of 150 μg of soybean trypsin inhibitor.] The solubilized material was clarified by high-speed centrifugation (as above) and to the supernatant were added first 9 μg of B.Cl monoclonal antibody (Thacher & Rice, 1985) and NaCl to 0.2 M, and then, after 1 h at 4 °C, 5 mg of Protein A–Sepharose. After 45 min at room temperature, the immune complexes were recovered by centrifugation and rinsed three times in immunoprecipitation buffer. In most experiments the transglutaminase was resolubilized in SDS, electrophoresed in 10% (w/v) polyacrylamide gels (Laemmli, 1970), stained with Coomassie Blue, submitted to autoradiography and the autoradiogram scanned by laser densitometry.

Phosphoamino acid analysis

Transglutaminase was solubilized from immunoprecipitates with 1 ml of 10% (v/v) formic acid and the Sepharose beads were removed by centrifugation. The freeze-dried supernatant was redissolved in 0.2 ml of 6 M-HCl, hydrolysed for 2 h at 110 °C, dried under vacuum in the presence of NaOH pellets, redissolved in 5 μl of pH 1.9 or 3.5 buffer and submitted to cellulose thin-layer electrophoresis in parallel with phosphoserine, -threonine and -tyrosine standards (Cooper *et al.*, 1983).

Gel filtration of tryptic peptides

In initial experiments, immunoprecipitates of ^{32}P-labelled transglutaminase were dissolved in 0.8 ml of 6 M-guanidinium chloride/0.12 M-Tris/HCl (pH 8.3)/0.05 M-dithioerythritol, incubated for 30 min at 37 °C, alkylated at room temperature for 1 h after addition of iodoacetamide to 0.1 M and dialysed extensively against water. To the dialysis residue was added *N*-ethylmorpholine acetate, pH 8.5, to 0.2 M and 25 μg of Tos-Phe-CH_2Cl-treated trypsin. The sample was incubated at 37 °C for 1 h, and a second addition of trypsin was made. After a total of 6 h, the digest was freeze-dried, clarified by centrifugation and applied to a 1.1 cm × 27 cm column of Bio-Gel P-6 maintained in 0.1 M-ammonium formate/0.01 M redistilled γ-collidine, pH 7. Fractions were counted by Čerenkov radiation (5 min) and freeze-dried for phosphoamino acid analysis. In some experiments the cultures were labelled with 1 mCi of $[^3H]$palmitic acid or $[^3H]$myristic acid, in which case the column buffer contained 0.1% Emulgen. In later experiments, immunoprecipitates of transglutaminase from single cultures labelled as described above with $[^{32}P]P_i$ were resuspended in 0.2 ml of buffer and treated for 15 min with 0.1 μg of Tos-Phe-CH_2Cl-treated trypsin. Before gel filtration, soluble and particulate fractions were separated by centrifugation at 8000 *g* for 3 min. The supernatant and solubilized particulate fractions [extracted with 20 mM-Tris buffer (pH 7.5)/0.3% Emulgen] were analysed separately by gel filtration or h.p.l.c.

H.p.l.c. of tryptic peptides

Typically, the soluble fraction of a tryptic digest of an immunoprecipitate was diluted to 1 ml, passed through a 45 μm-pore-size filter and applied to a Waters DEAE 5PW ion-exchange column. The column was washed with 5 ml of buffer [0.01 M-Tris/HCl (pH 7.5)/1 mM-EDTA] and the peptides were eluted with a 35 ml linear gradient of 0–1 M-NaCl in this buffer at a flow rate of 1 ml/min. The column fractions were counted by Čerenkov radiation (5 min). Recovery of the applied radioactivity was approx. 50%. Salt concentrations in eluted fractions were determined by conductivity.

RESULTS

Phosphate labelling of transglutaminase

When human epidermal cultures were incubated for several hours in the presence of $[^{32}P]P_i$ and the keratinocyte transglutaminase isolated by immunoprecipitation, autoradiograms of the polyacrylamide gels showed a clear band of radioactivity coincident with the Coomassie Blue-stained band of transglutaminase. When the labelling was carried out in the presence in the culture medium of cycloheximide (10 μg/ml), which inhibits protein synthesis in these keratinocytes by 96% (Rice & Green, 1978), the degree of labelling was not reduced (Fig. 1*a*). This finding indicates that the labelling reflected phosphate turnover, a dynamic process, and suggested that it was occurring on the membrane-bound form of the enzyme. To test the latter possibility, the particulate fraction of unlabelled cultures was incubated with $[\gamma\text{-}^{32}P]$ATP and then immunoprecipitated, electrophoresed and autoradiographed. Under the conditions employed, as shown in Fig. 1(*a*), the enzyme was labelled. Initial trials included Ca^{2+} and Mg^{2+} during the incubation with radioactive ATP, but these additions were subsequently found to be unnecessary.

To investigate whether protein kinase C was involved in the observed labelling of transglutaminase, cultures were treated

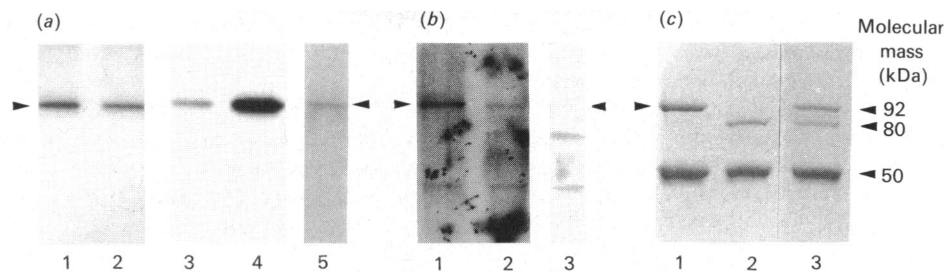

Fig. 1. Gel electrophoresis of immunoprecipitated particulate transglutaminase

For reference, arrowheads near the top of each panel indicate the position of transglutaminase (92 kDa). To the right in (*c*) arrowheads also give the positions of trypsin-released transglutaminase (80 kDa) and the B.C1 monoclonal antibody IgG heavy chain (50 kDa) visible by Coomassie Blue staining. (*a*) Autoradiography of phosphate-labelled transglutaminase from single cultures. The cells were labelled in the presence (1) or absence (2) of cycloheximide or in the absence (3) or presence (4) of PMA, whereas the particulate fraction was labelled by incubation with [γ-^{32}P]ATP (5). (*b*) Autoradiography of transglutaminase from skin organ cultures labelled in the presence (1) or absence (2) of PMA or without PMA or B.C1 monoclonal antibody (3). (*c*) Coomassie Blue staining of transglutaminase from single cultures, where the immunoprecipitates were treated for 15 min with 0 (1), 0.1 (2) or 0.01 (3) μg of Tos-Phe-CH_2Cl-treated trypsin (24 kDa, not visible) before electrophoresis.

with the tumour promoter PMA, which is known to stimulate this kinase. At a PMA concentration of 1 μM, the observed incorporation of ^{32}P after 1 h was double (result not shown) and after 4 h was 5-fold the level observed in the absence of PMA (Fig. 1*a*). The inactive agent phorbol had no stimulatory action (result not shown). The increase in labelling induced by PMA could not be attributed to new protein synthesis, since in the absence of PMA (Fig. 1*a*) the extent of labelling was not reduced by inclusion of cycloheximide (10 μg/ml) in the medium. Moreover, the increased labelling was not due to the increased flux of [^{32}P]P_i into the cells. This was demonstrated by labelling parallel cultures for 2 h with [^{32}P]P_i, after which time the low phosphate medium was changed, PMA was added to one culture, and the incubation continued for another 2 h. The extent and degree of stimulation of the labelling were the same as shown in Fig. 1(*a*), where the [^{32}P]P_i was not removed from the medium before treatment with PMA.

In short-term skin organ culture, keratinocyte transglutaminase was labelled by addition of [^{32}P]P_i to the medium. The autoradiograms exhibited faint radioactive bands corresponding to non-specifically precipitating material. However, the immunoprecipitates clearly showed not only the labelling of the particulate enzyme but also a stimulation of incorporation by PMA similar to that observed in the above serial cultures with feeder-layer support (Fig. 1*b*).

Localization of labelling to the membrane anchorage region

Previous experiments have shown that the 92 kDa transglutaminase can be released from the particulate fraction of cell homogenates by mild trypsin treatment (Thacher & Rice, 1985), which results in a reduction in size by $\approx$ 10 kDa (Chakravarty & Rice, 1989). When the enzyme, labelled with or without PMA treatment, was solubilized in this fashion, the autoradiograms indicated a loss of 60–80% of the radioactivity upon normalization to the amount of Coomassie Blue-stained 80 kDa transglutaminase in the gel. This phenomenon was readily demonstrable by gel filtration of tryptic digests of the enzyme (Fig. 2*a*). First, the labelled transglutaminase was extracted with Emulgen non-ionic detergent from cell particulates, immunoprecipitated, reduced and alkylated under denaturing conditions and extensively digested. Most of the radioactivity was eluted as a broad peak in the middle of the fractionation range (nominally 1–6 kDa) of Bio-Gel P-6. However, when a parallel sample of the labelled enzyme was solubilized instead by mild trypsin treatment and processed as above, little radioactivity was detected in the digest or seen in that peak.

The elution position of the labelled peak in the first instance above corresponds to a size of only several thousand Da at most, considerably smaller than expected for the estimated $\approx$ 10 kDa loss suffered by the intact enzyme upon mild tryptic release. To investigate whether the proteolysis resulted in separation of the

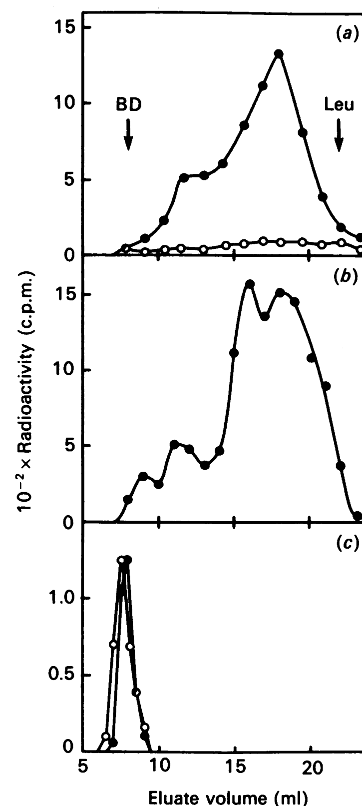

Fig. 2. Bio-Gel P-6 gel filtration of trypsin digests of labelled transglutaminase

(*a*) After Emulgen extraction (●) or mild trypsin release (○), the phosphate-labelled enzyme was immunoprecipitated, denatured, alkylated and extensively digested. 'BD' and 'Leu' represent the elution positions of Blue Dextran and [^{3}H]leucine respectively. (*b*) After Emulgen extraction, the immunoprecipitated enzyme was subjected to brief digestion with 0.1 μg of trypsin. (*c*) After Emulgen extraction, immunoprecipitated [^{3}H]palmitate (○)- and [^{3}H]myristate (●)-labelled transglutaminase were denatured, alkylated and extensively digested. The Emulgen-soluble material, containing the radioactivity, was then chromatographed in parallel runs in Emulgen-containing buffer. In each panel, the radioactivity refers to that in the labelled peptides.

phosphate radioactivity from the acylated fatty acid serving as membrane anchor, samples were labelled with [^{3}H]palmitate or [^{3}H]myristate and treated in the same fashion. Unlike the phosphate-labelled digest, the ^{3}H-labelled material was mostly insoluble after the digestion and required non-ionic-detergent extraction before gel filtration. Consistent with this finding, the ^{3}H label in each case was eluted in the void volume of the column, presumably as a consequence of the peptide being either $\geqslant$ 6 kDa or incorporated into detergent micelles (Fig. 2*c*). Cleavage within the putative $\approx$ 10 kDa anchorage region at least at a residue of arginine likely accounts for this behaviour, since the elution position of the phosphate label was not altered by acylation of the immunoprecipitated transglutaminase amino groups by citraconylation (Dixon & Perham, 1968) before trypsin treatment.

Further experiments indicated that the digestion could be carried out more simply by treating the immunoprecipitates with a low concentration of trypsin for a short time. It was evident by gel electrophoresis that this procedure yielded the 80 kDa form of transglutaminase without visible degradation of the antibodies (Fig. 1*c*) with or without added Emulgen (0.1 %). Most of the radioactivity was lost from the catalytically active 80 kDa fragment and was not detected above the dye front in autoradiograms of ^{32}P-labelled enzyme, even when the gel was fixed in 10 % (w/v) trichloroacetic acid. Upon gel filtration (Fig. 2*b*), the phosphate label in these trypsin digests was eluted essentially in the same location as after the more extensive digestion. However, the brief trypsin treatment offered considerable initial purification and yielded labelled peptide material suitable for further fractionation.

Several proteolysis experiments suggested the importance of cleavage at arginine for generating the soluble 80 kDa form of transglutaminase. Thus digestion of immunoprecipitates with human plasmin or the bacterial endopeptidase Arg-C gave complete or partial conversion respectively to the 80 kDa form, and also gave partial degradation of the B.C1 monoclonal antibody. Human kallikrein and the bacterial endopeptidase Lys-C also partially degraded the antibody, but did not produce the 80 kDa transglutaminase.

Phosphoamino acid analysis

After immunoprecipitation, phosphate-labelled transglutaminase was subjected to partial acid hydrolysis and thin-layer electrophoresis at pH 1.9 or 3.5 in parallel with phosphoamino acid standards. Whether or not the cells were treated with PMA, only phosphoserine was detected (Fig. 3). In addition, the phosphate-labelled peak material isolated by gel filtration from PMA-treated samples yielded only phosphoserine (results not shown).

H.p.l.c. separation of tryptic peptides

The soluble material generated by trypsin digestion of ^{32}P-labelled immunoprecipitates was separated by anion-exchange column chromatography. Two major peptides containing essentially equal amounts of the label were consistently detected. The possibility that the 15 min digestion period, sufficient for release of the membrane anchorage region, resulted in overlapping peptides from a single labelled residue was tested. As Figs 4(*a*) and 4(*b*) show, increasing the digestion time to 5 h and the temperature from ambient to 37 °C still resulted in two equally labelled peptides, although one peptide shifted in elution position from an estimated 0.1 M- to 0.2 M-NaCl. Two peaks were also observed when the digest was reduced and alkylated before chromatography. This result constitutes presumptive evidence for two phosphorylation sites, but conclusive evidence will require further characterization of the peptides.

Effect of phosphorylation on enzymic activity

Initial experiments showed that the specific activity of the particulate transglutaminase in cultures treated with PMA for 4 h was 40–50 % higher than in untreated controls (Table 1). Little increase in soluble transglutaminase activity (< 5 % of the total in the epidermal-cell strain employed) was observable. Human keratinocyte cultures ordinarily contain a high proportion of differentiating cells, which could have a high background of transglutaminase phosphorylation, even without exposure to PMA. Thus analysis of the treatment was pursued with cells grown in low-Ca^{2+} medium to find out whether the degree of stimulation was more impressive. Under the latter condition,

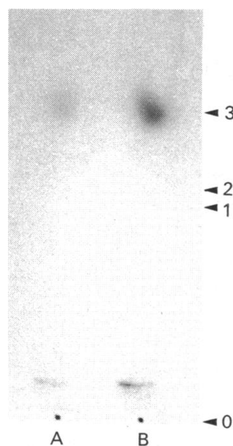

Fig. 3. Phosphoamino acid analysis of phosphate-labelled transglutaminase

Immunoprecipitates from single cultures were labelled in the absence (A) or presence (B) of PMA, acid-hydrolysed, submitted to cellulose thin-layer electrophoresis at pH 1.9 and autoradiographed. The standards, phosphoserine (3), phosphotyrosine (2) and phosphothreonine (1), were electrophoresed in parallel and detected with ninhydrin. The electrophoretic origin (O) is indicated by the black dot at the bottom.

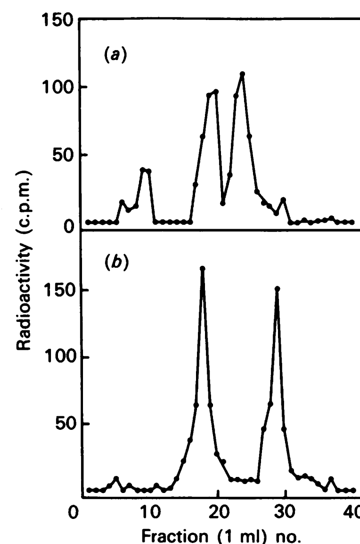

Fig. 4. H.p.l.c. separation of tryptic peptides

The elution patterns of peptides generated by digestion for 15 min (*a*) and 5 h (*b*) are shown. In the 5 h digest (*b*), the peaks were eluted at salt concentrations of 0.05 M- (18 ml) and 0.2 M-NaCl (29 ml). The radioactivity refers to that in the labelled peptides.

Table 1. Effect of PMA exposure on transglutaminase activity

Cultures (6 cm) were grown to confluence, treated with 1 μM-PMA for the times indicated and stored frozen until assayed for incorporation of [^{3}H]putrescine into dimethylcasein (nmol/h per mg of protein) as previously described (Thacher *et al.*, 1985). Activities were normalized to cell protein (Smith *et al.*, 1985). (*a*) Duplicate cultures grown in 1.6 mM-Ca^{2+} were homogenized and separated into high-speed supernatant and pellet fractions. The pelleted material was solubilized in Emulgen-containing buffer before assay. (*b*) Quadruplicate (0, 1.5 and 8 h) or duplicate cultures grown in 25 μM-Ca^{2+} were briefly sonicated in 1 ml of 10 mM-Tris/HCl/1 mM-EDTA/0.3% Emulgen and assayed.

(*a*) 1.6 mM-Ca^{2+}

Time (h)	Activity (nmol/h per mg of protein)	
	Pellet	Supernatant
0	76±1.8	1.2±0.2
4	110±7.0	1.8±0.2

(*b*) 25 μM-Ca^{2+}

Time (h)	[Cycloheximide] (μM)...	Activity (nmol/h per mg of protein) 0	10
0		9.5±1.4	
1.5		10.2±1.8	10.8±1.3
4		14.3±3.1	
8		18.9±2.2	9.4±0.1
26		9.9±0.1	

the state of differentiation, including the content of particulate transglutaminase (Rubin & Rice, 1986, 1988), is greatly reduced [although the proportion of cells expressing involucrin appears unchanged (Watt & Green, 1982)], similar to findings with mouse epidermal cells (Hennings *et al.*, 1980). In the present experiments (Table 1), PMA treatment induced a 50% increase in activity after 4 h and nearly doubled transglutaminase activity after 8 h, despite evident cell death (detachment from the culture dish) after 1 day. However, this increase did not occur when protein synthesis was inhibited by cycloheximide (which does not prevent phosphorylation). Hence, the increase in activity was similar in low- and high-Ca^{2+} medium and was not attributable to PMA-mediated phosphorylation.

DISCUSSION

In addition to post-translational fatty acid acylation (Chakravarty & Rice, 1989), keratinocyte transglutaminase evidently is subject to phosphorylation, a dynamic process also observed for keratins (Steinert, 1988). It is clear that membrane anchorage (Thacher, 1989) and proteinase release (Rice *et al.*, 1990) and now PMA-stimulated phosphorylation observed in culture occur in the epidermis as well. Loss of the majority of phosphate label from the active enzyme released from membrane fragments of cultured cell homogenates by mild trypsin digestion indicates the proximity of the serine residues accepting the phosphate label to the fatty acid membrane anchor. It is evident that the phosphate and fatty acid moieties are separated by at least one trypsin-cleavage site. This site may also be sensitive to the exogenous (plasmin) or endogenous proteinase activities capable of releasing the 80 kDa form and to which the enzyme is likely to be exposed during terminal differentiation (Rice *et al.*, 1990). In any case, the specificities of the various known proteinases that are effective suggest that the cleavage producing the 80 kDa form of the enzyme occurs at an arginine residue.

Crude extracts of cultured keratinocytes exhibit a substantial fraction of protein kinase C activity in a membrane-bound state (Choi & Toscano, 1988; Isseroff *et al.*, 1989). Since the kinase is likely to be active independent of added Ca^{2+} and not removed by altering the ionic strength (Palfrey & Waseem, 1985), present results are highly compatible with direct phosphorylation of the transglutaminase in the membrane anchorage region by protein kinase C. However, the participation of other kinases cannot be ruled out for two reasons. First, as in the case of calcineurin (Hashimoto & Soderling, 1989), a given serine residue can be subject to phosphorylation by a second kinase in addition to protein kinase C. Moreover, stimulation of the latter enzyme with PMA can lead to increased cyclic AMP accumulation in several systems (Gordeladze *et al.*, 1989), including epidermis (Iizuka *et al.*, 1989), thus increasing cyclic AMP-dependent phosphorylation. Preliminary experiments testing whether increased cyclic AMP in cultured keratinocytes leads to stimulation of transglutaminase phosphorylation have given equivocal results (X. Rong, R. Chakravarty & R. H. Rice, unpublished work).

Upon phosphorylation by protein kinase C, enzymes and structural proteins are often modified in activity or with respect to their interactions with other proteins. For example, PMA stimulation of phosphorylation of receptors for insulin and several other growth factors near their membrane anchorage regions has been observed and has functional consequences (cf. Takayama *et al.*, 1988). In the case of keratinocyte transglutaminase, phosphorylated serine residues in the membrane anchorage region are likely to be a considerable distance from the catalytic active site, judging from the primary structures of this and related transglutaminases (Floyd & Jetten, 1989). Although treatment of keratinocyte cultures with PMA increased the total enzyme activity toward the artificial substrate casein, the increase could not be attributed to phosphorylation. However, the absolute change in phosphate stoichiometry or even the degree to which PMA treatment stimulates phosphate turnover are unknown. If, as in the case of the keratinocyte protein profilaggrin (Resing *et al.*, 1985), the transglutaminase is ordinarily fully phosphorylated at the sites in question, alterations in the enzyme activity would not be observed as a result of acute treatment. If this phosphorylation increased the enzyme activity, then depletion of protein kinase C by chronic PMA treatment could lead to a reduction in activity, as observed in the malignant SCC-9 keratinocyte line (Rice *et al.*, 1988).

Phosphorylation even of a single residue can have dramatic effects on the secondary structure of proteins, as in the case of myelin basic protein (Ramwani *et al.*, 1989). By analogy, the PMA stimulation of phosphorylation may have little effect on the intrinsic catalytic ability of the enzyme, but it could alter substantially its interaction with specific substrate proteins, especially such a highly negatively charged one as involucrin (Rice & Green, 1979). For example, modulation of the keratinocyte protein kinase C activity by physiological conditions could alter the degree of transglutaminase phosphorylation and hence lead to the cross-linking of different substrate proteins. In addition, phosphorylation of the anchorage region (deleted in the monomeric trypsin-released form) could contribute to the observed dimerization of hydroxylamine-released transglutaminase (Rice *et al.*, 1990). Exploration of the various possible effects of the phosphate incorporation could be pursued by examining absolute changes in phosphorylation states and, more powerfully, by preparing transglutaminase variants by site-directed mutagenesis.

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