Identification of phosphorylation sites in keratinocyte transglutaminase

Robert H. RICE*§, Majid MEHRPOUYAN*‡, Qin QIN*, Marjorie A. PHILLIPS* and Young M. LEE†

*Department of Environmental Toxicology and †Protein Structure Laboratory, University of California, Davis, CA 95616-8588, U.S.A.

Phosphorylation of keratinocyte transglutaminase occurs in its N-terminal extension and is stimulated several-fold by the protein kinase C agonist phorbol myristate acetate. In the present work, this stimulation was prevented by simultaneous treatment of the cells with the protein kinase C-selective inhibitor GF109203X. In contrast, phosphorylation occurring in the absence of phorbol ester was essentially unaffected by GF109203X, although it was decreased dramatically by the non-selective kinase inhibitor staurosporine. Four serine residues that are subject to phosphorylation have been identified by sequencing of radio-

active tryptic peptides. Serines at positions 24 and 92, each containing 2–8% of the total radioactivity with or without phorbol ester stimulation, were minor sites of phosphorylation. Serine-82 was by far the dominant site of phosphorylation in the absence of phorbol ester treatment, and was also the major site in its presence. Serine-85 was phosphorylated to a high degree in the presence but not in the absence of phorbol ester stimulation. Thus the data indicate the influence of at least two different kinase activities in transglutaminase phosphorylation.

INTRODUCTION

The terminal differentiation programme of epidermal keratinocytes results in the formation of corneocytes with a cross-linked protein envelope [1,2]. This envelope consists of a number of proteins joined covalently by ϵ -(γ -glutamyl)lysine isopeptide bonds through transglutaminase (TG) action [3,4]. Considerable progress in estimating the contributions of different protein components has been made [5], and the relative importance of the various TGs expressed in keratinocytes is under continuing scrutiny. Epidermal keratinocytes are capable of expressing three types of TG, designated keratinocyte (TG_K or TG1), tissue (TG_C or TG2) and epidermal (TG_E or TG3) forms. Expression of TG_K appears to be restricted largely to differentiating keratinocytes, while TG_C is expressed in many cell types [6]. By virtue of its membrane localization, TG_K is anticipated to be important in the initial stages of envelope formation, while TG_E (a soluble enzyme) could be important in the later stages. Little evidence points to TG_C having an important role in this process.

Several reports indicate that TG_K is indeed critical for proper envelope formation and thus proper function of the epidermis. Autosomal recessive lamellar ichthyosis has been associated with a lack of TG_K [7] and with mutations in the structural gene [8–10], although not all cases involve TG_K [10,11]. These observations raise the possibility that more subtle changes in the properties of the enzyme could result in impaired epidermal function. Thus continued study of this enzyme, including post-translational modifications that affect its function, is warranted.

Three types of post-translational modification are evident in the N-terminal 10 kDa of the protein, an extension not found in other mammalian TGs that confers specific functions on TG_K [12]. First, TG_K is synthesized as a soluble 90 kDa protein but becomes rapidly membrane-anchored by fatty acylation in a cluster of cysteine residues near the N-terminus [13]. In serum-containing epidermal cell cultures, typically 90% or more of the TG_K activity in cultured normal keratinocytes is recovered in the membrane fraction of extracts [8,14], and this is also true for rabbit tracheal epithelial cells with squamous differentiation [15].

In serum-free cultures, efficient anchorage reportedly depends upon confluence and high medium calcium concentrations [16]. Secondly, a small proportion of TG_K can be released as a soluble 80 kDa fragment by calcium-dependent endogenous proteolysis, a phenomenon that can also be mediated by plasmin [17] which gains entry to cells undergoing terminal stages of differentiation [18]. The possibility of even smaller proteolytic fragments being formed and their potential importance for cross-linking has been noted [19]. Intact TG_K is also subject to phosphorylation in the N-terminal extension, stimulated by the tumour promoter phorbol 12-myristate 13-acetate (PMA) [20]. PMA stimulates total TG_K activity [21] and mRNA levels [22,23]. While phosphorylation has not been shown to affect the intrinsic enzyme activity using casein as a substrate, it is possible that this modification affects interactions of TG_K with endogenous substrates at the membrane.

EXPERIMENTAL

Cell culture

Normal human epidermal cells and the near-normal SIK line [24] were grown with 3T3 feeder layer support in a 3:1 (v/v) mixture of Dulbecco–Vogt Eagle's and Ham's F-12 media supplemented with fetal bovine serum (5%), cortisol (0.4 μ g/ml), adenine (0.18 mM), insulin (5 μ g/ml), transferrin (5 μ g/ml), tri-iodothyronine (20 pM) and antibiotics [25]. The cells were cultivated in the presence of epidermal growth factor and cholera toxin (each 10 ng/ml) until confluence but not afterwards.

Inhibitor studies

Confluent SIK cultures (10 cm) were incubated in phosphate-free medium with or without the kinase inhibitors staurosporine or GF109203X for 30 min, and then [³²P]P_i and PMA were added for 4 h. Cultures were rinsed, scraped from the dishes in 10 mM Tris buffer (pH 7.8) and 1 mM EDTA, and stored frozen. They were subsequently homogenized, and TG_K was isolated by

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TG, transglutaminase; TG_K, TG_C and TG_E, keratinocyte, tissue and epidermal TG respectively.

‡ Present address: Becton Dickinson, San Jose, CA 95131-1807, U.S.A.

§ To whom correspondence should be addressed.

immunoprecipitation with B.C1 monoclonal antibody [20], immunoprecipitated, electrophoresed in 7.5% polyacrylamide gels, transferred to nitrocellulose membranes and autoradiographed.

Isolation of phosphate-labelled TG_K for sequencing

At 2 weeks after confluence, 10 normal epidermal cultures were each preincubated for 15 min in 5 ml of phosphate-free medium (supplemented with 5% serum dialysed against 0.15 M NaCl to remove phosphate), followed by the addition of [³²P]P_i (0.5 mCi/10 cm culture) and, 15 min later, 1 μM PMA (diluted from a 1 mM stock in DMSO). In parallel, 10–30 normal cultures were treated with PMA but not [³²P]P_i. In some instances, PMA treatment was omitted. After 4 h of labelling, the cells were scraped from the dishes in 10 mM Tris buffer (pH 7.8) and 1 mM EDTA, and stored frozen overnight. Radioactive and non-radioactive cultures were subsequently homogenized and pooled. TG_K was isolated by scaling up the immunoprecipitation procedure, separated from antibody subunits by electrophoresis in 10% polyacrylamide SDS gels [26], and the Coomassie Blue-stained band was excised from the gel with a razor blade.

Proteolytic digestion of TG_K

The gel slice containing TG_K, typically 2–3 mm wide, 1.5 mm thick and 10 cm long, was diced into ~2 mm cubes, dehydrated in a SpeedVac, rehydrated in 0.3 ml of 0.1 M Tris buffer (pH 9)/0.05% SDS containing 2 μg of Lys-C endopeptidase (Promega or Wako), and incubated overnight at 30 °C. Peptides were eluted by extracting the gel twice for 2 h with water and twice for 2 h with 80% acetonitrile/0.075% trifluoroacetic acid (TFA). The pooled extracts were dried, dissolved in water, and the SDS was removed by extraction with heptane/3-methylbutanol-1-ol (4:1, v/v). The peptides (aqueous phase) were dried, dissolved in 8 M urea/0.4 M Tris buffer (pH 8.2), reduced with dithiothreitol and alkylated with iodoacetamide. The solution was then diluted with water to 2 M urea/0.1 M Tris buffer and digested overnight with 0.5 μg of Lys-C endopeptidase. The digest was microfuged for 5 min and the supernatant, exhibiting negligible radioactivity, was discarded. The pellet, containing the N-terminal extension of TG_K, was suspended in 0.1 M Tris buffer (pH 8.0) and digested overnight with 1 μg of modified (methylated and 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated) trypsin (Promega).

Fractionation and sequencing of TG_K tryptic peptides

Peptides were separated by HPLC (ABI 172 System) using a reverse-phase microbore C₁₈ column (1.0 mm × 10 cm). After injection of the sample, the column was washed with 92% solvent A (0.1% TFA in water)/8% solvent B (70% acetonitrile/0.075% TFA) for 5 min (0.1 ml/min flow rate) and then with a gradient of 8–18% solvent B for 10 min. Peptides adhering to the column were then eluted with a linear gradient of 18–60% B over 65 min. All fractions were monitored for radioactivity by scintillation counting. Of the radioactivity applied to the column, ~80% was recovered. Peptides were sequenced using an ABI 477A sequencer with on-line HPLC identification of phenylthiohydantoin (PTH)-amino acids. To identify phosphoserine, which ordinarily is not detected as a PTH derivative, in some cases these residues were converted into S-ethylcysteines [27] and detected as PTH-S-ethylcysteine, which was eluted just before diphenylthiourea.

RESULTS

Basal and PMA-stimulated phosphorylation

Previous work has shown that TG_K phosphorylation is stimulated by PMA, a powerful protein kinase C (PKC) agonist [20]. To determine whether this TG is subject to modification by more than a single kinase, the extent of phosphorylation was examined in the presence of the non-selective kinase inhibitor staurosporine and the PKC-selective inhibitor GF109203X. As shown in Figure 1(a), PMA treatment of the cultures substantially stimulated the degree of TG_K phosphorylation. Concomitant treatment with GF109203X at concentrations that prevent the action of conventional PKCs in cells [28] essentially prevented stimulation by PMA, decreasing the phosphorylation to that seen in the absence of PMA. By contrast, as shown in Figure 1(b), this inhibitor at concentrations up to 20 μM had only a minor effect on the degree of labelling in the absence of PMA (≤10% decrease). Staurosporine, which is known to be a potent inhibitor of numerous protein kinases, decreased the extent of labelling by nearly 90% at 300 nM (Figure 1c). These findings indicate that basal and PMA-stimulated phosphorylation are mediated by distinct kinases.

Identification of labelled residues

Gel-purified TG_K was digested with Lys-C endopeptidase, which generated a fatty acylated (and therefore insoluble) 20 kDa fragment derived from the N-terminus, as predicted by its deduced amino acid sequence [12]. Consistent with earlier findings that the phosphorylation of TG_K occurs close to the N-terminus [20], negligible label was detected in the peptides solubilized by Lys-C endopeptidase. Digestion of the insoluble material with trypsin then solubilized the radioactivity, permitting isolation of labelled peptides by reverse-phase chromatography.

Figure 1 PMA-stimulated and basal phosphorylation

(a) Cultures incubated with [³²P]P_i were treated (+) or not (–) with 1 μM PMA and with the PKC-specific inhibitor GF109203X at the concentrations indicated. Immunoprecipitated TG_K was then electrophoresed, blotted and autoradiographed. (b, c) In the absence of PMA, cultures were incubated with [³²P]P_i and the kinase inhibitors GF109203X (b) or staurosporine (c) at the concentrations indicated. Immunoprecipitated TG_K was electrophoresed, blotted, autoradiographed (insets) and the film scanned by laser densitometry to yield the data in the graphs.

Figure 2 Reverse-phase separation of tryptic peptides

After Lys-C endopeptidase 'in gel' digestion of electrophoretically purified TG_K , the radioactive (insoluble) material was digested with trypsin and applied to a reverse-phase column. Peptides were detected by A_{210} and monitored for radioactivity by scintillation counting of 25 μ l aliquots of column fractions. Material in the radioactive peaks indicated by the arrows was then sequenced.

Figure 3 Phosphorylation sites in the TG_K N-terminal extension

The terminal 106 amino acids of TG_K are shown, with the N-terminus at the far left. Sequences obtained from radioactive peptides (identified by numbers underneath; see the text) are underlined. Residues identified as PTH-S-ethylcysteine are indicated (†). The cluster of cysteine residues where palmitoylation and membrane anchorage occur is also indicated (*).

Figure 2 shows the peptide profile obtained. Of the approx. 46 distinct peaks detected by A_{210} , three were radioactive. The two that were separated well (numbered 2 and 3 in Figure 2) were sequenced and found to span serine residues at positions 92 and 24 respectively in the sequence (Figure 3). In several experiments, these peaks contained only a minor fraction of the radioactivity. Peak 2 contained 7% of the total applied to the column when the cultures were treated with PMA and 2% when they were not. Peak 3 contained 7–8% of the radioactivity with or without PMA treatment. The large majority of the radioactivity, routinely 70%, was not retained but was eluted with the flow-through.

The flow-through material was recovered and re-chromatographed under isocratic conditions using 0.1% TFA in water as solvent. As illustrated in Figure 4(a), three radioactive peaks were obtained when the cells were treated with PMA. Peak 1-1 contained an estimated 57% of the radioactivity applied to the column and peak 1-3 contained 31%, while peak 1-2 comprised no more than 7%. This material was sequenced, yielding a peptide in each peak with the sequences shown in Figure 3. After modification with ethanethiol, serines 82 and 85 were detected as PTH-S-ethylcysteine derivatives and in this way confirmed as the major sites of phosphorylation in PMA-treated cells. The

Figure 4 Isocratic elution of flow-through material

Peptides from the initial reverse-phase separation flow-through were re-chromatographed. The peptide peaks detected by A_{210} were monitored for radioactivity, and the material in the radioactive peaks was submitted for sequencing. Cultures were treated (a) or not (b) with 1 μ M PMA.

resulting sequences indicated that peptide 1-1 was derived by partial cleavage of peptide 1-3, and peptide 1-2 was derived by partial cleavage of peptide 2. When this experiment was repeated using cells not treated with PMA (Figure 4b), the degree of labelling was greatly decreased, and virtually all of the radioactivity was eluted in peak 1-1; no more than traces of peptides 1-2 and 1-3 were detectable by sequencing.

DISCUSSION

The evidence obtained in this work indicates that TG_K is subject to phosphorylation by at least two kinase activities, one PMA-dependent and the other PMA-independent. The former recognizes Ser-82 and Ser-85 as substrates, while the latter recognizes Ser-24, Ser-82 and Ser-92. Confirming earlier findings, these sites are all serines located within the N-terminal 10 kDa of the protein, and they are in tryptic peptides separate from that containing the site of fatty acylation [20].

The two sites recognized by PMA-stimulated activity (Ser-82 and Ser-85) are in sequences well within the range of substrate specificity noted previously for PKC, namely a serine or threonine residue flanked closely by lysine or (preferably) arginine on either or (preferably) both sides [29]. Indeed, the TG_K N-terminal extension from residues 67 to 95 consists of a repetitive sequence rich in serine and arginine (consensus sequence Gly-Ser-Arg-Gly-Ser; see [12]) and hence contains 10 potential PKC phosphorylation sites. This region is similar in character, but is not highly conserved, in the rat (consensus Asp-Ser-Arg-Gly-Gly; see [12]) and particularly the rabbit [22] TG_K sequences. Nevertheless, numerous potential PKC-mediated phosphorylation sites are evident in the repeat region in the latter species, though it is not clear whether the function served by phosphorylation in the human cells is preserved. Perhaps in compensation for a truncation of this repeat region, the rabbit sequence contains a similar 30-residue repeat region rich in Arg and Ser residues (consensus Ser-Asp-Met-Gly-Arg; see [22]) very

close to the N-terminus that is lacking in TG_K from the other two species.

Keratinocytes are known to express at least five isoforms of PKC, namely α , δ , ϵ , ζ and η . The first four are stimulated by PMA, and all are translocated to a large extent to the membrane fraction in differentiating cells [30]. Although the α isoform has been suggested as playing a major role in the differentiation process [23], including TG_K induction, whether this isoform is responsible for TG_K phosphorylation remains uncertain. Future work using isoform-specific inhibitors [31] and cells manipulated to express given PKC isoforms may help to confirm the involvement of PKC, determine the isoenzyme responsible and ascertain whether it acts directly on TG_K.

The distinct activity phosphorylating serine residues at positions 24, 82 and 92 may not originate from a single kinase. Indeed, Ser-24 is located between two prolines and is followed by several acidic residues, a sequence resembling a substrate for a proline-directed kinase [32] or casein kinase II [29], while these features are not evident near Ser-82 or Ser-92. TG_K becomes considerably more highly phosphorylated in the N-terminal extension without PMA stimulation if membrane anchorage is prevented [13]. If phosphorylation at Ser-24 and/or -92 were augmented in constitutively soluble TG_K mutants, it may be that such phosphorylation ordinarily is limited by the rapid membrane anchorage of the native form. Alternatively, prevention of anchorage may allow phosphorylation to occur at additional sites. This phenomenon may contribute to the dysfunction of soluble mutants of TG_K observed in certain cases of recessive familial lamellar ichthyosis [8].

Since TG_K is necessary for the correct assembly of cornified envelopes, its structural features are anticipated to be important in carrying out its cross-linking function. The interactions of TG_K with neighbouring proteins at the membrane, particularly those that serve as substrates, may guide the assembly process. Phosphorylation of the enzyme thus may influence substrate recognition, perhaps at a critical initial stage of envelope formation. The stoichiometry of phosphorylation has not been measured, but partial modification at the sites identified, as seems likely, may even facilitate some diversity in substrate interactions. In any case, perturbation of phosphorylation may alter these interactions and thereby lead to compromised epidermal maturation and functional deficiency.

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