Keratinocyte Transglutaminase Membrane Anchorage: Analysis of Site-Directed Mutants†

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ABSTRACT: Keratinocyte transglutaminase is anchored on the cytosolic side of the plasma membrane by fatty acid thioesterification near the amino terminus, a process which is seen to occur within 30 min of synthesis. The importance of a cluster of five cysteines (residues 47, 48, 50, 51, and 53) where acylation was presumed to occur is now demonstrated by site-directed mutagenesis. Transglutaminase mutants in which the cluster is deleted or the cysteines are all converted to alanine or serine are cytosolic. Partial replacement of the cluster, leaving two contiguous cysteines, is sufficient to confer membrane anchorage, while a single cysteine is only partially effective. As demonstrated with a soluble transglutaminase mutant, membrane anchorage confers susceptibility of the amino-terminal region to phorbol ester-stimulated phosphorylation. Attachment of 105 residues from the transglutaminase amino terminus to involucrin, a highly soluble protein, results in membrane anchorage of the hybrid protein. Attachment of the cysteine cluster alone does not result in membrane attachment of involucrin, but a 32-residue segment containing this cluster is sufficient. Stable transfectants of the human transglutaminase in mouse 3T3 cells are membrane-bound, indicating the fatty acid transacylation is not keratinocyte-specific.

A specific feature of keratinocyte differentiation in vivo and in culture is the formation of a protein envelope beneath the plasma membrane which contains a high level of ϵ-(γ-glutamyl)lysine cross-linking [cf. Green (1979)]. Keratinocytes in the stratified squamous epithelia express a plasma membrane-bound keratinocyte transglutaminase (TG_K)[1] which is capable of cross-linking these envelopes and can account for their peripheral localization in the cell (Lichti et al., 1985; Simon & Green, 1985; Thacher & Rice, 1985; Parenteau et al., 1986). TG_K is anchored in the cell membrane by acylated fatty acid in a neutral hydroxylamine-sensitive linkage (Chakravarty & Rice, 1989). Membrane anchorage evidently occurs near the amino terminus of the enzyme, since solubilization of the protein by mild trypsinization results in loss of some amino- but not carboxy-terminal sequence (Rice et al., 1992). Further elucidation of the mechanism of membrane anchorage may provide valuable perspective on its regulation and the evolution of isopeptide cross-linking at the cell periphery as a specific feature of mature cells in the stratified squamous epithelia.

Molecular cloning of TG_K has suggested a likely site of acylation near the amino terminus, a cluster of five cysteine residues conserved in the human and rat sequences (Phillips et al., 1990; Kim et al., 1991). Indeed, the major structural difference between tissue transglutaminase (80 kDa), a soluble enzyme, and TG_K (90 kDa) is the amino-terminal 10 kDa of the latter. The nearest relative of TG_K is the proenzyme blood clotting factor XIII, the activation peptide of which also constitutes an amino-terminal extension. Comparison among the several members of the transglutaminase superfamily that have been molecularly cloned (including erythrocyte band 4.2) has suggested that the amino-terminal regions of TG_K and factor XIII, recruited independently after gene duplication, are recent additions to a common ancestral mammalian gene and have been retained for the specialized functions they confer. This hypothesis is compatible with the finding that the N-terminal extension of TG_K is encoded by a separate exon in the genomic sequence (Phillips et al., 1992). A recently described membrane-bound insect transglutaminase also exhibits an N-terminal extension with a putative thioesterification site (Singer et al., 1992).

The distinctive N-terminal extension of TG_K has several interesting features which could contribute to the specialized function of the enzyme in keratinocytes (Phillips et al., 1990). First, despite its highly hydrophilic amino acid composition, this extension mediates membrane anchorage through fatty acid thioesterification. However, protein modification by palmitoylation appears reversible, permitting modulation of protein–membrane interaction (Staufenbiel & Lazarides, 1986) and alteration of the degree of membrane anchorage in response to physiological conditions (Huang, 1989; James & Olson, 1989; Jochen et al., 1991; Mundy & Warren, 1992). Earlier evidence that TG_K palmitoylation is reversible (i.e., detected in the absence of protein synthesis through a fatty acyl exchange reaction) suggested this enzyme might cycle on and off the membrane (Chakravarty & Rice, 1989). The N-terminal extension also contains a region of serine- and arginine-containing repeats near the cysteine cluster acylation site. This region likely is responsible for an observed hypersensitivity to release by proteolysis, which could lead to action of the released enzyme in the cytoplasm as well as the cell periphery (Rice et al., 1990). In addition, this region also accounts for the susceptibility of TG_K to TPA-stimulated phosphorylation, possibly modulating substrate specificity (Chakravarty et al., 1990). Present experiments explore the influence of palmitoylation on the regulation of phosphorylation. This type of interaction is illustrated by recent findings with the β_2-adrenergic receptor, where an observed agonist-induced increase in acylation (Mouillac et al., 1992) appears to reduce its phosphorylation and consequently desensitization (Moffett et al., 1993).

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[1] Abbreviations: TG_K, keratinocyte transglutaminase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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EXPERIMENTAL PROCEDURES

DNA Constructs. The promoter, the multiple cloning site, and the SV40 3′ processing signals of the expression vector pECE (Ellis et al., 1986) were excised and subcloned into pUC19 (pECE/pUC), which had been cut with *Eco*RI and *Hin*dIII to remove the polylinker region. In some cases, the Moloney MuLV LTR was substituted for the SV40 promoter. Both promoters worked efficiently in SCC4 cells. hTG19 (Phillips et al., 1990), which contains the entire coding region for human TG_K, was subcloned into the *Eco*RI site of the pECE polylinker (pTG), and the the proper orientation was determined by restriction digestion. TG_K deletion or substitution mutants were prepared using an Amersham kit for site-directed mutagenesis. hTG19 cloned into M13 was annealed to mutant oligonucleotides prepared using an Applied Biosystems oligonucleotide synthesizer and purified with OPC cartridges (Applied Biosystems). Following mutagenesis reactions, performed according to the manufacturer's protocol, the DNA was transformed into competent XL1 Blue (Stratagene), and single-stranded DNA was prepared for sequencing. The entire sequences of the mutants were verified using a Sequenase kit (United States Biochemicals). Mutant DNA was subcloned into pECE/pUC for transfection. TG_K mutants are designated by the identity and position of the substituted amino acid; e.g., TGAla(48,51) indicates that Cys residues 48 and 51 of TG_K were substituted by Ala, and TGΔ(47–54) indicates that amino acids 47–54 were deleted. The first Met in the open translation reading frame is designated as position 1. The Cys residues under investigation are at positions 47, 48, 50, 51, and 53.

An involucrin expression plasmid, pINV, was prepared by subcloning a PCR product into pECE/pUC. The template used was pλI-3H6B (Eckert & Green, 1986), and primers were designed to amplify most of the coding region (amino acids 8–585) in addition to 78 bp of 3′-noncoding sequence. Restriction sites and an in-frame initiation codon were incorporated into the primers. Fusion genes are identified according to which TG_K amino acids they contain with the first Met in TG_K designated as position 1. pTG(1–106)INV and pTG(32–63)INV were constructed by PCR amplification of the appropriate region of hTG19 using primers with suitable 5′-restriction sites for subcloning into pINV, such that TG_K sequences were fused in-frame to the amino-terminal side of amino acid 8 of involucrin. Hybrid vectors containing smaller regions of TG_K were prepared by insertion mutagenesis of the involucrin coding region subcloned into M13. Mutagenesis was performed as described above, and mutant DNA was subcloned back into the expression vector pECE/pUC. A small region of TG_K encoding residues 46–54 (the cysteine cluster) was inserted at (i) an amino-terminal site, after amino acid 29 of involucrin, pTG(46–54)N-INV; and (ii) a carboxy-terminal site, after amino acid 579, pTG(46–54)C-INV. pTG-(46–54)N-INV was further mutagenized by insertion of sequences 5′ or 3′ of the cysteine region to construct pTG-(32–54)INV and pTG(46–63)INV. Sequence verification of fusion vectors was only partial (TG_K sequences and immediately surrounding involucrin sequence) since the repetitive nature of involucrin makes routine sequence determination cumbersome. However, rare base changes in the involucrin coding region, if they occurred, should not affect the cellular distribution of the protein.

Cell Culture and Transfections. SCC-4 cells, cloned from a lingual squamous cell carcinoma (Rheinwald & Beckett, 1981), and keratinocytes derived from normal human epidermis were grown in Dulbecco–Vogt Eagle's medium supplemented with 5% fetal bovine serum and 10^{-6} M hydrocortisone using a feeder layer of lethally irradiated 3T3 cells (Rheinwald & Green, 1975). Transient transfections were performed using calcium phosphate precipitation (Gorman, 1985). SCC-4 cells (6×10^5) were plated in 10-cm dishes, and DNA–calcium phosphate coprecipitates were added the same day. Generally, 20 μg of expression vector (twice purified by CsCl gradient centrifugation) was used. Smaller amounts of DNA (down to 2 μg) resulted in lower amounts of TG_K expression, but did not alter its distribution in soluble and particulate fractions. The medium was changed the following day, and cells were harvested 3 days later by scraping them from the dish and stored frozen.

3T3 cells stably expressing TG_K were transfected by the same method using 20 μg of TG19ECE and 1 μg of pSV2neo (Gorman, 1985), both of which had been linearized by restriction enzyme digestion. Two days after transfection, each 10-cm dish was trypsinized and replated into 8–10 dishes. Cells were selected with 0.5 mg/mL G418 (Gibco-BRL) for 2 weeks, at which time resistant colonies were pooled and cultured thereafter without G418.

Labeling of Cultures with Radioactive Precursors. For pulse chase experiments with [^{35}S]methionine and cysteine (Translabel, ICN), 10-cm cultures of epidermal cells, confluent for 1 week, were rinsed in serum-free medium deficient in methionine and cysteine and then incubated in this medium for 15 min to deplete intracellular stores. Finally, cells were incubated in the deficient medium with 50 μCi of [^{35}S]amino acids for 30 min. At the end of the labeling period, cultures were rinsed with complete medium containing methionine, cysteine, and serum at the usual concentrations, harvested after further incubation for varying lengths of time, and frozen.

Transfected SCC-4 cells were labeled with fatty acid 3 days after addition of DNA. Two 10-cm cultures were rinsed with serum-free medium and then incubated with [^{3}H]-palmitate (1 mCi/dish) in medium containing 5% delipidized serum (Rothblat et al., 1976) and 5 mM sodium pyruvate for 6 h. At the end of the labeling, the cultures were rinsed, scraped from the dish, and frozen. Since the observed thioesterification of myristate in TG_K (Chakravarty & Rice, 1989) may reflect limited specificity of the responsible transacylation reactions (Riendeau & Guertin, 1986), the present investigation has focused on palmitate.

To monitor TG_K phosphorylation, 3 days after transfection, 10-cm cultures were prelabeled for 4 h in 5 mL of phosphate-free Dulbecco–Vogt Eagle's medium supplemented with 5% fetal bovine serum and 1 mCi of [^{32}P]P_i. Labeling was continued another 4 h after addition of TPA to 1 μM from a 1 mM stock in DMSO. The cultures were then rinsed twice in phosphate-buffered saline, and TG_K was isolated by immunoprecipitation with B.C1 monoclonal antibody and protein A–Sepharose (Chakravarty et al., 1990). Immunoprecipitates were electrophoresed directly or after mild trypsin treatment [15 min at 37 °C in 20 μL of 20 mM Tris (pH 8.0)–1 mM EDTA].

Cell Fractionation and Detection of TG_K and Involucrin. Frozen cells were thawed and sonicated after addition of 0.5 mL of hypotonic buffer (10 mM Tris, pH 7.5, and 1 mM EDTA). The sonicate was centrifuged at 100000*g* for 45 min at 4 °C to separate soluble from particulate-associated proteins. To the supernatant, Emulgen nonionic detergent was added to a final concentration of 1%. The pellet was resuspended and sonicated in the Tris–EDTA buffer containing 1% Emulgen and then recentrifuged at 100000*g*. This second supernatant contained proteins solubilized from the membrane fraction; TG_K was not detectable in the pellet by immunoblotting.

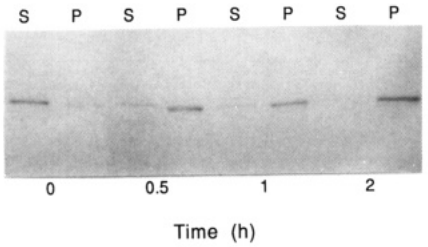

FIGURE 1: Pulse chase labeling of TG_K. Epidermal cells were labeled with a mixture of [^{35}S]Met and Cys and then chased for the indicated times. Extracts were separated into soluble (S) and particulate (P) fractions before immunoprecipitation, electrophoresis, and fluorography.

In some experiments, TG_K was detected by immunoblotting using a rabbit polyclonal antiserum raised to a C-terminal peptide of the enzyme (Rice et al., 1992). Alternatively, TG_K was immunoprecipitated from each fraction of extracts by addition of B.C1 monoclonal antibody, and antigen–antibody complexes were isolated using protein A–Sepharose (Chakravarty et al., 1990). The monoclonal was raised to the 80-kDa trypsin-released enzyme (Thacher & Rice, 1985) and hence was insensitive to mutations in the N-terminal extension. This was demonstrated by immunoblotting of the soluble TGAla-(47,48,50,51,53) and the mostly particulate TGLeu,Ala(47,-48,51,53) with or without initial B.C1 immunoprecipitation. TG_K was solubilized from immunoprecipitates by boiling in SDS sample buffer and resolved by electrophoresis in 7.5% polyacrylamide gels (Laemmli, 1970). Gels from cultures labeled with ^{35}S or ^{3}H were stained with Coomassie Blue and impregnated with Enhance (New England Nuclear), and TG_K was detected by fluorography at –80 °C. The fraction of ^{35}S-labeled TG_K that was membrane-bound was then estimated by laser densitometry.

In nonradioactive experiments, the distribution of native and mutant TG_K in soluble and particulate fractions was estimated by visual inspection of immunoblots. Similarly, the distribution of native and chimeric involucrins in the soluble and particulate fractions was estimated visually after gel electrophoresis and immunoblotting. Approximately 1% of each cell extract, without prior immunoprecipitation, was applied to the gel, and involucrin was detected after blotting to nitrocellulose using a rabbit polyclonal antibody (Rice & Green, 1979).

Transglutaminase Assay. Cultures were harvested and fractionated as described above. Aliquots of soluble proteins and of proteins Emulgen-solubilized from 100000*g* pellets were incubated for 20 min at 37 °C in 0.5-mL reaction volumes containing 1 mg of dimethylcasein, 0.1 M Tris (pH 8.2), 4 mM $CaCl_2$, 5 mM DTT, and 8 nmol (0.5 μCi) of [^{3}H]-putrescine (Thacher et al., 1985). Trichloroacetic acid was added to 10%, and proteins were collected on glass fiber filters, washed, and counted after addition of scintillation fluid. Increasing amounts of extracts were used to verify the linearity of the assay.

RESULTS

Kinetics of TG_K Membrane Localization. Human epidermal cells were labeled for 30 min with [^{35}S]Met and Cys and then harvested at various times after incubation with unlabeled amino acids. From the soluble and particulate cell extracts, TG_K was immunoprecipitated, electrophoresed, and detected by autoradiography. At the end of the labeling period, most of the TG_K was in the soluble fraction (78% at 0 time in Figure 1), but quickly became membrane-associated (77% by 0.5 h and 91% by 2 h). Thus, TG_K is synthesized in the cytoplasm, and soon afterward fatty acid is attached, leading to membrane anchorage. This behavior resembles that reported for a neural growth cone-associated protein, neuromodulin (GAP-43), which is initially soluble but quickly becomes anchored in the membrane by palmitoylation at a cluster of two cysteines near the amino terminus (Virag & Skene, 1989).

Role of the Cysteine Cluster in Membrane Anchorage. While fatty acid thioesterification near the amino terminus was shown previously to mediate TG_K membrane anchorage (Chakravarty & Rice, 1989), the importance of the cysteines clustered at residues 47, 48, 50, 51, and 53 has not been demonstrated directly. To this end, deletion or substitution mutants in the cysteine cluster region were produced by standard site-directed mutagenesis, sequenced completely to verify genotype, and then subcloned into the eukaryotic expression vector pECE (Ellis et al., 1986). The mutant proteins were analyzed for cellular location by transfection and cell fractionation. Both soluble TG_K and TG_K solubilized from particulate fractions were electrophoresed and detected by immunoblotting. The host chosen for transfection was a squamous cell carcinoma line (SCC-4) (Rheinwald & Beckett, 1981) which expresses very low levels of TG_K, alleviating the problem of distinguishing endogenous from transfected enzyme while allowing use of the appropriate cell type (keratinocyte).

Mock-transfected cells exhibited no detectable TG_K in either the soluble or the particulate fraction (Figure 2A, lanes 1–2), while cells transfected with native (nonmutant) TG_K expressed the vast majority of the protein in the particulate fraction, estimated >90% by visual inspection of immunoblots (Figure 2A, lanes 3–4). A similar intracellular distribution is evident in cultured normal human epidermal cells, where TG_K is expressed from the endogenous gene (Thacher & Rice, 1985). In contrast, transfection of mutants in which the cysteine cluster region (normal sequence CCGCCSC) was deleted or the cysteines were all replaced by serine or alanine (mutant sequences SSGSSSS or AAGAASA, respectively) resulted in expression only of soluble TG_K (Figure 2A, lanes 5–6 and Table I). Although hydroxy amino acids, particularly serine, have been proposed as esterification sites for palmitate or other fatty acids (Alvarez et al., 1990; Jing & Trowbridge, 1990; Kent & Fleming, 1990), the serine-enriched mutant showed no propensity to be membrane-bound. Similar results were obtained when transglutaminase cross-linking activity was measured in both cell fractions (Table II). These results also demonstrate that the soluble TG_K mutants are enzymatically active and thus not grossly deranged in conformation.

A series of key alanine substitution mutants containing one or more cysteines were constructed to determine which of these residues are necessary for efficient membrane localization (see complete listing in Table I). Mutant proteins with cysteines in three of the five usual positions, such as CCGAASC (Figure 2A, lanes 7–8) and CAGCASC, were as efficiently bound to the membrane as the native enzyme. Mutants with two cysteines (ACGACSA illustrated in Figure 2A, lanes 11–12) were also effectively anchored with one exception. The mutant sequence CAGAASC (Figure 2A, lanes 9–10), where three cysteine residues were replaced, leaving two with maximal separation, resulted in half or slightly more of the enzyme being soluble. A single cysteine residue in the mutant sequences AAGCASA (Figure 2A, lanes 13–14), AAGAASC, CAGAASA, and ACGAASA was sufficient to anchor only a small proportion of TG_K in the membrane, estimated <5% by visual inspection of immunoblots.

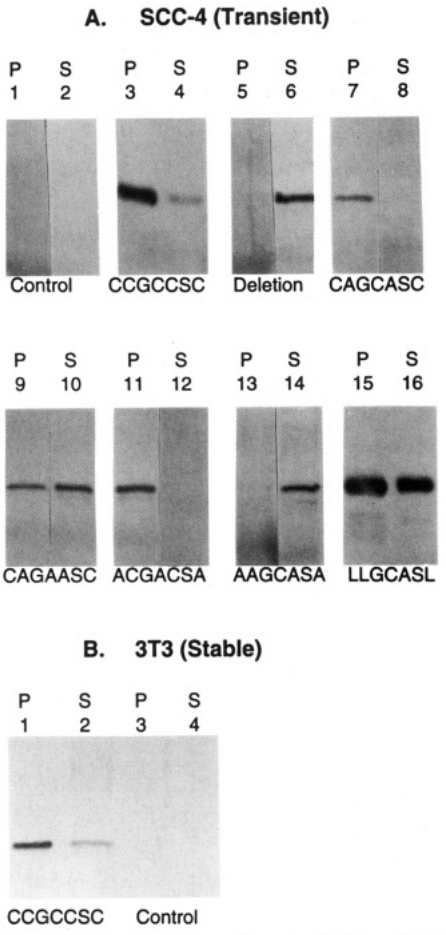

FIGURE 2: Localization of native and mutant TG_Ks. SCC-4 (A) or 3T3 cells (B) were transfected with expression vector constructs, and TG_K was identified in soluble (S) and particulate (P) fractions by immunoblotting of immunoprecipitates. Amino acid sequences in the cysteine cluster region, where the vectors differ, are indicated. Equivalent amounts of total extract are shown for each construct. Nontransfected (control) and mock-transfected SCC-4 or 3T3 cells had no detectable TG_K in either fraction.

Table I: Localization of TG_K Mutants

form of TG_K tested[a]	sequence[b]	particulate[c]
TG	CCGCCSC	+
TGAla(48,51)	CAGCASC	+
TGAla(50,51)	CCGAASC	+
TGAla(47,50,53)	ACGACSA	+
TGAla(47,48,53)	AAGCCSA	+
TGAla(48,50,51)	CAGAASC	±
TGLeu,Ala(47,48,51,53)	LLGCASL	±
TGAla(47,48,51,53)	AAGCASA	–
TGAla(47,48,50,51)	AAGAASC	–
TGAla(48,50,51,53)	CAGAASA	–
TGAla(47,50,51,53)	ACGAASA	–
TGSer(47,48,50,51,53)	SSGSSSS	–
TGAla(47,48,50,51,53)	AAGAASA	–
TGΔ(46–54)	(deleted)	–

[a] Mutations are designated by the amino acid replacement of cysteine residues (positions in parentheses) as described under Experimental Procedures. [b] The sequence of the cysteine cluster is shown with Cys residues in boldface type (**C**). [c] The intracellular distribution of the mutant protein is indicated: +, large majority particulate; ±, substantial amounts in both particulate and soluble fractions; –, large majority soluble.

Three findings described above suggest that the efficiency of acylation is influenced by the hydrophobicity of the cysteine cluster region: (i) a single cysteine is insufficient for membrane localization; (ii) two cysteines are insufficient if widely spaced; and (iii) serine does not substitute for cysteine. To test this inference, a mutant protein containing a single cysteine, one alanine replacement, and three leucine replacements (LLGCASL) was constructed. Unlike the less hydrophobic mutant containing only alanine replacements, approximately half (or more) of this mutant protein was anchored in the membrane (Figure 2A, lanes 15–16).

Table II: Transglutaminase Activity in Extracts of Transfected Cells[a]

form of TG_K tested[b]	particulate	soluble
TG	1.10 ± 0.34	0.03 ± 0.03
TGΔ(46–54)	0	1.15 ± 0.31
TGSer(47,48,50,51,53)	0	1.43 ± 0.09
TGAla(47,48,50,51,53)	0	1.89 ± 0.28

[a] Activities measured in soluble and solubilized particulate fractions, normalized to 10-μL aliquots from extract volumes of 1 mL, are given in thousands of cpm as the mean and range of two determinations. Assay background (0.72×10^3 cpm), determined in the absence of added extract, has been subtracted. [b] Expression vectors differing by amino acid deletions or replacements in the cysteine cluster are the same as shown in Table I.

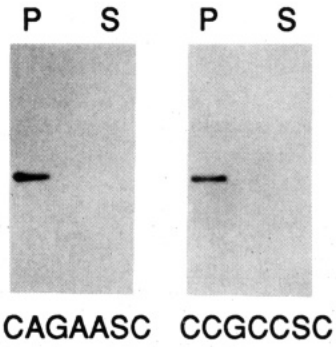

FIGURE 3: [^{3}H]Palmitate labeling. SCC-4 cells transfected with TG_K expression vectors were labeled with [^{3}H]palmitate and extracts separated into soluble (S) and particulate (P) fractions before immunoprecipitation, electrophoresis, and fluorography. The sequences of the vectors differed only in the Cys cluster region as indicated. For the mutant CAGAASC, approximately half of the protein was detected in each fraction, while a large majority of the native form (CCGCCSC) was observed in the particulate fraction similar to that shown in Figure 2A.

Cysteine-containing mutants which are partially soluble could be so because they do not contain enough fatty acid per protein molecule to be efficiently retained in the membrane fraction or because only some of the molecules are fatty acid acylated. These two possibilities were distinguished by determining whether soluble forms of TG_K are labeled with [^{3}H]palmitate. Cells were transfected with pTGAla(48,50,-51) or pTG (approximately half or 5–10% soluble, respectively) and labeled with [^{3}H]palmitate. After immunoprecipitation and electrophoresis, TG_K protein was stained with Coomassie Blue, verifying the distribution between soluble and particulate forms, and then processed for fluorography. Figure 3 shows that only the particulate form of either transglutaminase contains [^{3}H]palmitate despite a substantial amount of the mutant protein (approximately half) evident in the soluble fraction.

Cell Specificity of Membrane Anchorage. Many cell types are capable of fatty acid acylation. For a given protein, however, cell type-specific auxiliary proteins could be necessary for recognition by the fatty acid transferase or for efficient membrane localization. To test this possibility, the pTG expression vector was transfected into 3T3 cells and transglutaminase detected by immunoblotting in both soluble and particulate fractions. Because of the low transfection efficiency of this strain of 3T3 cells, stable transfectants were examined (Figure 2B, lanes 1–2). The large majority of the transglutaminase was membrane-associated, indicating this process must not rely exclusively on keratinocyte-specific

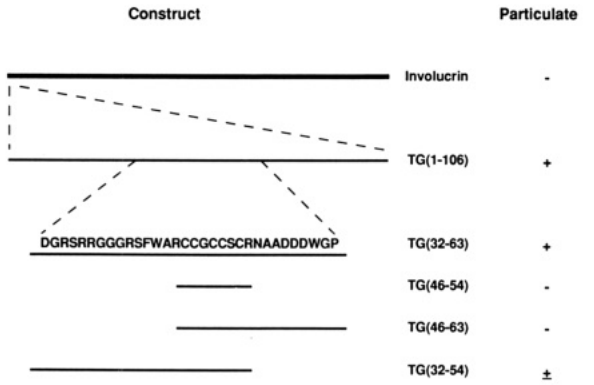

FIGURE 4: Structures of TG_K/involucrin fusions. The top line depicts involucrin (thick line) with TG_K sequences (thin lines) below. The latter (residues in parentheses) are fused to the involucrin amino terminus (left end). Below the N-terminal extension (residues 1–106) are expanded drawings to show shorter regions of TG_K fused. The sequence of the smallest region (residues 32–63) that results predominantly in membrane association of the hybrid protein is given. Below this sequence are bars showing smaller regions which were tested. Distributions of fusion protein are indicated as follows: +, large majority particulate; ±, substantial amounts in both particulate and soluble fractions; –, large majority soluble.

factors. As in the case of TG_K in normal epidermal cells (Chakravarty & Rice, 1989), the particulate form was largely solubilized by treatment with neutral 1 M hydroxylamine (not shown), consistent with anchorage by thioesterified fatty acid.

Minimum Determinants of Acylation Site. The preceding data demonstrate that the cluster of cysteines near the amino terminus of TG_K is important for membrane localization, with a minimum of two residues required for efficient anchorage. To determine whether this region is sufficient to confer membrane localization, we constructed fusion genes which encode different segments of TG_K attached near the amino terminus of an otherwise highly soluble protein, involucrin (Figure 4). These hybrid constructs were transfected into the same host cell (SCC-4), which contains vanishingly small amounts of endogenous involucrin, and the fusion proteins were detected on immunoblots using anti-involucrin antiserum.

Fusion proteins containing either the first 106 amino acids of TG_K [called pTG(1–106)INV] or 32 residues centered on the cysteine cluster [pTG(32–63)INV] directed the large majority of the protein to the membrane (Figure 5, lanes 3–4 and 5–6, respectively). In contrast, a fusion protein containing only nine amino acids at the cysteine cluster [pTG(46–54)N-INV] was mostly soluble (lanes 7–8). A similarly soluble hybrid protein was observed when the nine amino acid segment was fused near the carboxyl terminus of the involucrin molecule [pTG(46–54)C-INV, not shown], indicating that the protein context did not inhibit fatty acid acylation. Fusion proteins containing the Cys cluster region and additional adjacent TG_K residues [TG(32–54)INV and TG(46–63)INV] were only partially successful at directing the protein to the membrane. Of these two regions, as shown in Figure 5, the residues N-terminal to the cysteine cluster region were more effective. Consistent with its importance for membrane association, a 30 amino acid region containing the cysteine cluster is well conserved between rat and human (93% identity) despite greater variability (≈75% identity) in the rest of the amino-terminal extension (Phillips et al., 1990).

Influence of Membrane Anchorage on Phosphorylation. The soluble TG_K mutant with alanine substitutions at each of five cysteines in the membrane attachment region was tested for phosphate incorporation after transfection into SCC-4

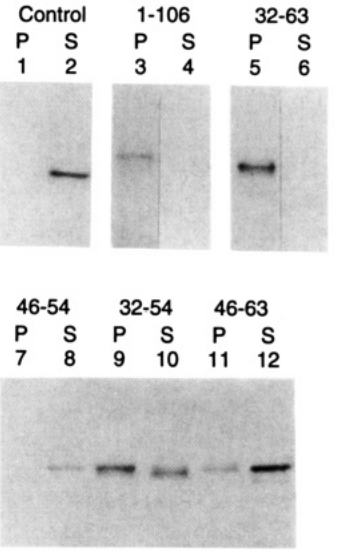

FIGURE 5: Localization of TG_K/involucrin fusions. The distribution of fusion protein in transfected SCC-4 cells, detected on Western blots with anti-involucrin antibody, is indicated by P (particulate) and S (soluble). The control is involucrin with no added TG_K sequence. Numbers indicate which TG_K amino acids are contained in the chimeric proteins.

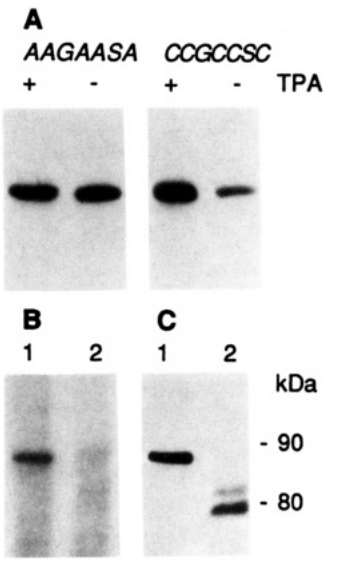

FIGURE 6: Phosphorylation of a soluble TG_K mutant. Transfected SCC-4 cells expressing either native TG_K or a mutant with alanines substituted for the cluster of five cysteines [TGAla(47,48,50,51,53)] were incubated with $[^{32}P]P_i$, solubilized, immunoprecipitated, and electrophoresed. (A) Extracts of cultures incubated with (+) or without (–) TPA were autoradiographed after immunoprecipitation and electrophoresis. (B) Immunoprecipitates of extracts of TPA-treated cells expressing the soluble TG_K mutant were treated with 0 (lane 1) or 0.01 μg (lane 2) of TPCK-treated trypsin before electrophoresis and autoradiography. (C) Immunoblot of panel B. Positions of intact (90 kDa) and trypsin-treated (80 kDa) TG_K are indicated.

cells. As shown in Figure 6A, the mutant was subject to phosphorylation nearly to the same extent as the transfected native protein in TPA-treated cells. In contrast to the latter, where TPA clearly stimulated the degree of labeling, the radioactivity of the mutant was not affected by exposure of the cells to TPA. When the immunoprecipitated mutant enzyme was subjected to mild trypsin digestion, thereby removing the 10-kDa N-terminal extension (Figure 6C), the resulting 80-kDa protein exhibited little radioactivity (Figure 6B). Thus, as previously shown for native TG_K with or without TPA treatment (Chakravarty et al., 1990), phosphorylation occurred primarily in the N-terminal extension.

DISCUSSION

Observation of TG_K localization after pulse labeling has now shown that the initially soluble enzyme quickly becomes membrane-bound. The small fraction of the enzyme lacking palmitate labeling may be due to inefficiency of palmitoylation, though it could reflect some recycling on and off the membrane. The mutation studies show clearly the importance of the cysteine cluster region to membrane attachment. Multiple sites of potential thioesterification in this cluster could prevent inadvertent release or may simply increase the likelihood of at least one thiol being palmitoylated. Palmitoylation of tandem cysteine residues has been observed in a lung surfactant peptide (Curstedt et al., 1989) and could occur in TG_K also. The possibility that partially anchored mutants (e.g., those with the sequences CAGAASC and LLGCASL in place of CCGCCSC) could undergo cyclic release and reattachment has not been excluded. However, similar to previous results with the native TG_K in normal epidermal and SCC-9 cells (Rice et al., 1990), neither the former mutant nor the native enzyme appeared to be released during incubation of the particulate fraction of transfected SCC-4 cells when proteolysis was prevented by EDTA (Q. Qin and R. H. Rice, unpublished results).

Considerable attention has been focused on discerning short consensus amino acid sequence(s) responsible for protein palmitoylation. In contrast to farnesylation and geranylation (Glomset et al., 1990), comparison of sequences of known protein palmitoylation sites has not yet identified such consensus sequences. Enzymes capable of fatty acid transacylation have been detected in endoplasmic reticulum (Kasinathan et al., 1990; Gutierrez & Magee, 1991) but not plasma membrane, raising the possibility that palmitoylation of proteins bound to the plasma membrane could be nonenzymatic (Hartel-Schenk & Agre, 1992). If nonenzymatic (as well as enzymatic) palmitoylation actually occurs in cells, its prevalence could be enhanced by providing free cysteine residues through fatty acylesterase activity. The latter activity has been detected in microsomes (Berger & Schmidt, 1986), but its presence in the plasma membrane is also uncertain.

Difficulty in identifying a consensus amino acid sequence for palmitoylation is compatible with the existence of a family of transacylases with diverse specificities or with a need for docking of substrate proteins at the membrane prior to acylation. Efficient anchorage of human TG_K in mouse 3T3 fibroblasts suggests a substrate sequence from this protein may be recognized generally by transacylases, perhaps due to the high cysteine content, or that the docking process is not highly cell type-specific. Possible weak interaction of soluble (nonacylated) TG_K with the plasma membrane remains to be explored.

Nevertheless, specificity is demonstrable in the identification of amino acid sequences conferring susceptibility to palmitoylation. In the case of neuromodulin, for example, an N-terminal 10-residue segment transferred to chloramphenicol acetyltransferase sufficed to anchor the latter to cell membranes (Zuber et al., 1989). In the present case, further analysis of the 32-residue TG_K sequence which confers membrane anchorage of involucrin may yield better definition of a minimal anchorage region. In addition to the cysteine cluster, we speculate that hydrophobic and perhaps basic residues are particularly important. The ability of three leucines to stimulate anchorage at a single adjacent cysteine in the TG_K anchorage region suggests that the hydrophobic character assists recognition of an appropriate thioesterification site. By analogy, farnesylation of ras proteins precedes and may assist subsequent palmitoylation. However, the possibility exists that proper recognition involves secondary or tertiary structural information as well. Analogous to farnesylation reactions (Reiss et al., 1991), further studies may be assisted by use of model peptides in conjunction with site-directed mutagenesis.

Use of a soluble mutant has demonstrated the influence of membrane anchorage on TG_K phosphorylation. The finding that TPA does not stimulate the observed labeling of the mutant is consistent with direct phosphorylation of the native TG_K by a protein kinase C isozyme that is active only in close proximity to the plasma membrane, befitting models of the activation process (Bell, 1986). Although most protein kinase C substrates are membrane-bound, exceptions such as soluble mutants of the MARKS protein (Graff et al., 1989) and neuromodulin (Chapman et al., 1992) have been reported. However, with these mutants (unlike the soluble TG_K mutant), TPA stimulation of the labeling is observed. Thus, present observations suggest that the soluble TG_K is subject to phosphorylation by an activity distinct from protein kinase C. This possibility is supported by our more recent experiments showing that the phosphorylation occurs even in the presence of GF 109203X (Mehrpouyan & Rice, 1993), a specific inhibitor of protein kinase C (Toullec et al., 1991). Hence, a consequence of anchorage is control of phosphorylation by protein kinase C, stimulation of which by TPA or diacylglycerol induces terminal differentiation (Jeng et al., 1985). Identification of the modification sites is in progress.

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