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Acylation of Keratinocyte Transglutaminase by Palmitic and Myristic Acids in the Membrane Anchorage Region*

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The membrane-bound form of keratinocyte transglutaminase was found to be labeled by addition of [3H]acetic, [3H]myristic, or [3H]palmitic acids to the culture medium of human epidermal cells. Acid methanolysis and high performance liquid chromatography analysis of [3H]myristate-labeled protein yielded approximately 40% of palmitate-labeled transglutaminase yielded only methyl palmitate. In contrast, analysis of the myristate-labeled protein yielded approximately 40% methyl myristate and 60% methyl palmitate. Incorporation of neither label was significantly affected by cycloheximide inhibition of protein synthesis. The importance of the fatty acid moiety for membrane anchorage was demonstrated in three ways. First, the enzyme was solubilized from the particulate fraction of cell extracts by treatment with neutral 1 M hydroxylamine, which was sufficient to release the fatty acid label. Second, solubilization of active enzyme from the particulate fraction upon mild trypsin treatment resulted in a reduction in size by approximately 10 kDa and removal of the fatty acid radiolabels. Third, the small fraction of soluble transglutaminase in cell extracts was found almost completely to lack fatty acid labeling. Keratinocyte transglutaminase translated from poly(A)* RNA in a reticulocyte cell-free system was indistinguishable in size from the native enzyme, suggesting anchorage requires only minor post-translational modification. Thus, the data are highly compatible with membrane anchorage by means of fatty acid acylation within 10 kDa of the NH2 or COOH terminus.

Keratinocyte transglutaminase participates in the remarkable maturation process of cells in the epidermis and related epithelia. Activated by calcium flux into the cytoplasm, this enzyme cross-links substrates by isopeptide bonding into envelopes that contribute to the cohesion of the terminally differentiated cells (Green, 1979). Primarily membrane-bound (Lichti et al., 1985; Simon and Green, 1985; Thacher and Rice, 1985), the enzyme is known to cross-link the soluble protein involucrin and several membrane proteins into particulate structures (Rice and Green, 1979; Simon and Green, 1984, 1985). The processes by which these structures are assembled and become esterified with ω-hydroxyacylphosphatidylglycerol (Swartzendruber et al., 1987) remain to be elucidated. Moreover, participating substrates (Michel et al., 1987; Nagae et al., 1987) and the resulting ultrastructure (Warhol et al., 1985) reportedly vary with the physiological state or derivation of the cell and the method or speed of inducing cross-linking. To clarify the mechanism of envelope formation, knowledge of critical structural features of the enzyme interactions with substrates and the membrane will be essential. As a first step in this direction, the present work addresses the biochemical basis of the enzyme anchorage in the membrane.

Generally, proteins are anchored in membranes by stretches of hydrophobic amino acids, by inositol phospholipid glycan moieties, or by fatty acids acylated directly to amino acid residues (Selton and Boss, 1987). In the last category, proteins modified by myristate may be found in cytosolic as well as membrane compartments (Olson et al., 1985) or even encapsidated in nonenveloped viruses (Streuli and Griffin, 1987; Chow et al., 1987; Paul et al., 1987). In contrast, palmitate labeling occurs on proteins which are found almost exclusively in membranes, but exceptions include the secreted apolipoprotein A-I (Hoeg et al., 1986), which binds lipid, and actin from Dictyostelium discoideum (Stadler et al., 1985). Keratinocyte transglutaminase is found not only in the particulate fraction of cultured human epidermal cell extracts, but also to a small extent in the soluble fraction, and can be released from membranes by mild trypsin treatment (Thacher and Rice, 1985). The present work reveals that the membrane-bound form incorporates both palmitate and myristate, whereas the native and trypsin-released soluble forms show little if any fatty acid labeling.

MATERIALS AND METHODS

Cell Culture—Keratinocytes from normal human epidermis were cultured in 10-cm dishes with support from feeder layers of lethally irradiated 3T3 cells according to standard methods (Allen-Hoffman and Rheinwald, 1984) in a 3:1 mixture of Dulbecco-Vogt Eagle's and Ham's F-12 media supplemented with fetal bovine serum (5%), hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), adenine (0.18 mM), insulin (5 μg/ml), transferrin (5 μg/ml), triiodothyronine (20 μM), and antibiotics. The cells were inoculated in the presence of cholera toxin (10 ng/ml) and held at confluence for 3-5 days prior to harvesting for isolation of poly(A)* RNA or up to 10 days for protein isolation.

Cell-free Translation—Typically, 10 cultures were dissolved with homogenization in buffered 6 M guanidine thiocyanate, and the RNA was pelleted through CsCl by standard procedures (Chirgwin et al., 1979). Poly(A)* RNA was then isolated on oligo(dT)-cellulose (type III, Collaborative Research) essentially as described (Aviv and Leder, 1972), precipitated with ethanol, and used to program rabbit reticulocyte extracts (Promega Biotech). Translations were performed in final volumes of 50 μl containing up to 9 μg of poly(A)* RNA (sufficient for maximal incorporation), 40 μl of reticulocyte extract preheated with micrococcal nuclease, and 6 units of RNasin (Promega Biotech). The relatively low level of radioactivity incorporated into transglutaminase was not increased by addition of a mixture of required amino acids lacking methionine or by use of [35S]cysteine or [3H]leucine.

Fatty Acid Labeling and Immunoprecipitation—Cultures were incubated for 4.5 h with 1 μCi of [3H]palmitic, [3H]myristic, or [3H]acetic acid in medium supplemented with 10% delipidized (Rothblat et al., 1976) fetal bovine serum and 5 mM sodium pyruvate (Olson et

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Transglutaminase Fatty Acid Acylation

al., 1985). (To maximize incorporation of acetate label into fatty acids, cultures were preincubated overnight in this medium prior to addition of [3H]acetic acid.) Each culture was then rinsed several times with isotonic neutral saline, scraped from the dish, stored frozen overnight, and homogenized in 6 ml of 50 mM Tris-Cl (pH 8.0), 1 mM EDTA. The particulate material was isolated by high-speed centrifugation (100,000 × g for 1 h) and resuspended in 6 ml of 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.3% Emulgen 911 nonionic detergent and stirred at 4°C for 2 h. (Alternatively, the transglutaminase was solubilized from the isolated particulate material by suspension for 5 min in 2 ml of 20 mM Tris-Cl (pH 8.0) containing 50 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin ( Worthington, followed by addition of 150 pg of soybean trypsin inhibitor.) The solubilized material was clarified by high-speed centrifugation and to the supernatant were added first 2 μg of B.C1 monoclonal antibody (Thacher and Rice, 1985) and NaCl to 0.2 M, and then (after 1 h at 4°C), 4 μg of rabbit anti-mouse IgG ( Cappell) and 2 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,1 electrophoresed in 10% polyacrylamide gels (Laemmli, 1970), stained with Coomassie Blue, treated with EN'HANCE ( New England Nuclear), and submitted to autoradiography.

Analysis of Incorporated Fatty Acids by High Performance Liquid Chromatography—Immunoprecipitates of labeled protein were extracted several times with chloroform-methanol until no further radioactivity was obtained in the washes. The dry residue was then incubated in 1 ml of 2 N HCl, 83% methanol at 95°C for up to 60 h in vacuo and extracted with petroleum ether. The ether extract was dried under nitrogen gas, dissolved in 80% acetonitrile, and submitted to reverse-phase chromatography in this solvent ( Olson et al., 1985) using C8 columns from Waters.

Transglutaminase Assay—Enzymatic activity was measured by incorporation of [3H]putrescine (15 μM, 125 Ci/mol) into 2 mg/ml reductively methylated (Means and Feeney, 1968) α-casein (Worthington) in 100 mM Tris-Cl (pH 8.3), 4 mM CaCl2, 0.4 mM EDTA, 5 mM dithioerythritol. Samples (typically 10–20 μl containing approximately 4 μg of protein) were incubated in final volumes of 0.26 ml at 35°C for 30 min, in the linear range of the assay (Thacher and Rice, 1985).

RESULTS

Under the extraction conditions used in the present work, an improvement over the more rapid method previously employed (Thacher and Rice, 1985), approximately 90% of the transglutaminase activity in keratinocyte particulate material typically was solubilized with the nonionic detergent Emulgen 911. Initial experiments showed that the enzyme was solubilized nearly as well by treatment of particulate material with 1 M hydroxyamine at neutral pH for 2 h at room temperature.

In these experiments, efficacy was judged not only by transglutaminase assays after removal of hydroxyamine (showing the enzyme retains activity), but also by the amount of Coomassie Blue-stained enzyme visible upon electrophoresis of immunoprecipitates (Fig. 1, e and f). As illustrated, the apparent molecular weights of the enzyme solubilized by hydroxyamine or detergent were indistinguishable.

The efficacy of hydroxyamine solubilization suggested that the enzyme was anchored by esterified or thioesterified fatty acids. To confirm this interpretation, keratinocyte cultures were incubated in the presence of [3H]acetic acid in the medium for 4.5 h ( Towler and Glaser, 1986), after which the transglutaminase was immunoprecipitated and examined by autoradiography. A low degree of labeling was observed, as shown in Fig. 2a. Labeling under similar conditions with [3H]myristate or [3H]palmitate was considerably more effective (lanes b and e), with the latter giving the most incorporation of radioactivity. As illustrated in Fig. 3, most of the radiolabel was lost (typically 80%) upon treatment of the palmitate (compare lanes a and b) or myristate-labeled (lanes c and d) transglutaminase with hydroxyamine after immunoprecipitation and gel electrophoresis. In addition, when the myristate-labeled cell particulates were treated with 1 M hydroxyamine for 2 h and the released enzyme was then immunoprecipitated and examined by SDS gel electrophoresis, virtually no radioactivity was detected upon autoradiography (Fig. 4d).

Keratinocyte transglutaminase can also be solubilized from the particulate fraction of cell extracts by mild trypsin digestion (Thacher and Rice, 1985). This treatment results in a reduction in size from approximately 92 to 80 kDa (Fig. 1, lanes a and b). When palmitate- or myristate-labeled enzyme was released from the membrane by trypsin and immunoprecipitated, no radioactivity was evident in the autoradiograms (Fig. 2, c and d). In such experiments, similar amounts of Coomassie Blue-stained protein (quantitated by laser densitometry) were electrophoresed as in the parallel samples which were solubilized with nonionic detergent and which

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
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Fig. 4. Fatty acid acid labeling of soluble transglutaminase. Similar amounts of particulate (a, d, e) and soluble (b, c) forms of transglutaminase from cells labeled with [3H]palmitate (a, b) or [3H]myristate (c, d, e) were applied to the gels. In d, the myristate-labeled enzyme was solubilized from the particulate fraction by treatment with 1 M hydroxylamine and immunoprecipitated.

Fig. 5. Analysis of methyl esterified fatty acids released from radiolabeled transglutaminase. The cultures were labeled with palmitic acid (a) or myristic acid in the absence (b) or presence (c) of cycloheximide (10 μg/ml). The elution positions of methyl myristate (M) and methyl palmitate (P) were determined using [3H]myristic and [3H]palmitic acids esterified and chromatographed in parallel. The elution of methyl palmitate in a differs from that in b, and c, since the former and latter chromatograms were obtained from different columns.

gave substantial labeling (lanes b and c). Thus, unless fatty acids were removed from both ends of the protein, the labeling was restricted to an anchorage region at one terminus.

The degree of transglutaminase labeling by tritiated myristate or palmitate was not reduced (and may actually have been stimulated slightly) by including 10 μg/ml cycloheximide in the culture medium (Fig. 3, c–e), which inhibits protein synthesis by 96% in these cells (Rice and Green, 1978). To be sure this finding represented a lack of dependence on protein synthesis of the incorporation of each fatty acid, the identity of the protein-bound radioactivity was analyzed. Thus, the immunoprecipitated tritium-labeled protein was subjected to acid methanolysis in parallel with fatty acid standards and the products were analyzed by high performance liquid chromatography. The profile from labeling with either fatty acid was unchanged by cycloheximide treatment of the cells. When the protein was labeled with [3H]palmitic acid, methyl palmitate was obtained in high yield and was the only major product obtained (Fig. 5a). In contrast, when the protein was labeled with [3H]myristic acid, approximately 40% of the esterified fatty acid eluted from the column at the position of methyl myristate and 60% with methyl palmitate (Fig. 4b).

Fig. 6. Autoradiography of cell-free translation products. Immunoprecipitates of material synthesized in reticulocyte extracts programmed with keratinocyte poly(A+) RNA were prepared with (a) or without (b) B.CI antitransglutaminase monoclonal antibody. The arrowhead shows the migration of native transglutaminase electrophoresed in parallel and detected by Coomassie Blue staining.

A small fraction (typically 5%) of the keratinocyte transglutaminase in crude extracts is detected as a soluble protein of the same apparent molecular weight as the particulate form in SDS gels (Thacher and Rice, 1985). In the present experiments, the relative palmitic and myristic acid labeling of the two forms were compared. As shown in Fig. 4, the particulate form contained far greater (at least 30-fold more) labeling than the soluble form when the same amount of Coomassie Blue-stained transglutaminase was electrophoresed in adjacent lanes. The little radioactivity evident in the autoradiogram of the soluble enzyme in lane b may even exceed the actual amount, since a slight contamination with the particulate form is difficult to avoid.

Poly(A+) RNA isolated from cultured keratinocytes was translated in a rabbit reticulocyte cell-free system. As shown in Fig. 6a, polyacrylamide gel electrophoresis of the translation products with subsequent autoradiography led to the detection of a band of material specifically immunoprecipitated by antitransglutaminase monoclonal antibody. This band matched in mobility the native transglutaminase electrophoresed in the adjoining lane and detected by Coomassie Blue staining (indicated by the arrow). Thus, major post-translational modification of the translated form to yield the native protein, such as the proteolytic activation of the clotting factor XIII catalytic subunit (Schwartz et al., 1973), was not detectable in this fashion. Several other translation products, presumed to be primarily the insoluble keratins which are synthesized in abundance in keratinocytes, were observed to precipitate nonspecifically (shown in lane b), but they did not interfere with the interpretation. These results are consistent with the finding that post-translational fatty acid acylation suffices to anchor the enzyme in the membrane.

DISCUSSION

The observed post-translational addition of fatty acid provides a molecular basis for membrane anchorage of the majority of keratinocyte transglutaminase and is consistent with the minimal fatty acid labeling of the small fraction found in a soluble form. This enzyme, then, resembles the folate-binding protein in cultured KB cells, which is expressed in membrane-bound and soluble forms that differ detectably only in the lack of covalently bound fatty acid in the latter (Lohrs et al., 1987). In the present case, release of a soluble transglutaminase from cell particulates by neutral hydroxylamine strongly indicates fatty acid acylation is required for anchorage. If the soluble form does not differ from the acylated form in other respects, its relative amount could reflect the efficiency of acylation after translation. No information is available as to which amino acid residues are acylated and
whether single enzyme molecules contain both fatty acids. Isolation and sequencing of fatty acid-containing peptides from the myristate-labeled protein, for example, are anticipated to clarify this point. Conclusive evidence concerning a requirement for myristate or palmitate or both for anchorage may be generated by alteration of the appropriate acylated residue(s) by site-specific mutagenesis. This approach has shown, for example, that the ras-related yeast YPT1 protein has 2 COOH-terminal cysteines subject to palmitoylation (Molenar et al., 1988), whereas only 1 of 2 neighboring cysteines in the human transferrin receptor becomes palmitoylated (Jing and Trowbridge, 1987).

Myristoyl linkages in proteins largely are resistant to hydroxylamine treatment and occur cotranslationally (Olson et al., 1986; Magee and Courtneidge, 1985; McIlhinney et al., 1985; Wilcox et al., 1987), leading to observation of N-myristoyl-glycine at the amino terminus as the predominant form of this modification (Paul et al., 1987). However, a clear example of post-translational N-myristoylation has now been reported in the B-lymphocyte membrane immunoglobulin heavy chain (Pillai and Baltz, 1985) containing both N- and S- (or O-) acyl linkages (Mytilinakis, 1985). Although this elongation did occur to a considerable extent in the present case, it is clear that myristate and palmitate were both incorporated in hydroxylamine-sensitive (ester or thioester) linkages. Several attempts to obtain amino acid sequence information from an estimated 20 nmol of the tryptic-released 80-kDa form electroeluted from gels or blotted onto Immobilon polyvinylidene difluoride membrane (Matsudaira, 1987) gave negative results, suggesting that its NH2 terminus is blocked. Although not unique, the most plausible interpretation of these results is that the protein is anchored by fatty acid at the carboxyl terminus and is blocked at the NH2 terminus but not by myristate. We note that the tissue transglutaminase also has a blocked NH2 terminus, which may be pyroglutamic acid (Connellan et al., 1971) or acetyllalaine (Ikura et al., 1988). Our current approach of localizing fatty acid acylation sites in peptides isolated from proteolytic digests, in concert with the primary structure deduced from the cDNA nucleotide sequence, should permit more definitive analysis of the orientation of the anchorage region in the protein.

Palmitate acylation has been found to occur in the absence of protein synthesis in general (Magee and Courtneidge, 1985; McIlhinney et al., 1985) and in the specific cases of ankyrin (Staufenbfiel and Lazareides, 1986) and N-ras (Magee et al., 1987) as well as in the present study. Although exogenous stimulation of hydroxylamine-resistant myristoylation in cells such as macrophages (Adarem et al., 1988) most likely becomes manifest during synthesis of the modified proteins, post-translational recycling of esterified fatty acids could serve a modulatory role during the functional life of the protein. Indeed, the effect of turnover at two sites might even be greater than for a single site. The solubility of the enzyme or its interaction with protein substrates could plausibly be altered in this fashion. Attempts to demonstrate endogenous solubilization of the enzyme from particulate extracts have been successful (Rice et al., 1988). This observation by itself does not reflect fatty acid recycling, however, since the solubilized enzyme exhibits a trypsin-like cleavage, which would in any case result in release from the membrane.

The incorporation of two different fatty acids into keratinocyte transglutaminase raises the interesting possibility that each has a distinct function. For example, one might be sufficient for anchorage, whereas the other interacts with substrates. The possibility that acylated fatty acids could have a structural function other than simply anchorage has been suggested by the finding that the transferrin receptor (Jing and Trowbridge, 1987) and the histocompatibility antigen HLA-D are palmitoylated post-translationally, despite their anchorage by hydrophobic amino acid segments. The fatty acid modification can have marked structural consequences, since, in the latter, it reportedly prevents disulfide bond formation (Koch and Hammerling, 1986). The apparent specificity of labeling of cellular proteins with palmitate and myristate (Olson et al., 1985; Magee and Courtneidge, 1985; McIlhinney et al., 1985) presumably reflects (i) the paucity of myristoyl transglutaminase, which is blocked at the amino terminus but not by myristate and (ii) the marked fatty acid specificities of the transacylases involved. In cell-free systems, the transacylases forming hydroxylamine-sensitive linkages may show a decided preference for incorporating palmitate, but myristate can be incorporated as well (Schmidt, 1984; Riendeau and Guertin, 1986). Thus, whether keratinocyte transglutaminase has more than a single acylation site remains to be established. In any case, the soluble guinea pig liver tissue transglutaminase has 17 free sulphydryls (Folk and Cole, 1966; Ikura et al., 1988); a similar number on the keratinocyte enzyme would provide more than enough sites for the observed fatty acid acylation. Indeed, comparison of the structures of the two enzymes expressed by a given cell type such as human keratinocytes (Thacher and Rice, 1985) might prove valuable for understanding the specificity of the acylating machinery leading to membrane anchorage only of one of them.

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