A Novel Tumor Suppressor Protein Promotes Keratinocyte Terminal Differentiation via Activation of Type I Transglutaminase*

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Tazarotene-induced protein 3 (TIG3) is a recently discovered regulatory protein that is expressed in the suprabasal epidermis. In the present study, we show that TIG3 regulates keratinocyte viability and proliferation. TIG3-dependent reduction in keratinocyte viability is accompanied by a substantial increase in the number of sub-G1 cells, nuclear shrinkage, and increased formation of cornified envelope-like structures. TIG3 localizes to the membrane fraction, and TIG3-dependent differentiation is associated with increased type I transglutaminase activity. Microscopic localization and isopeptide cross-linking studies suggest that TIG3 and type I transglutaminase co-localize in membranes. Markers of apoptosis, including caspases and poly(ADP-ribose) polymerase, are not activated by TIG3, and caspase inhibitors do not stop the TIG3-dependent reduction in cell viability. Truncation of the carboxyl-terminal membrane-anchoring domain results in a complete loss of TIG3 activity. The morphology of the TIG3-positive cells and the effects on cornified envelope formation suggest that TIG3 is an activator of terminal keratinocyte differentiation. Our studies suggest that TIG3 facilitates the terminal stages in keratinocyte differentiation via activation of type I transglutaminase.

TIG3 is a novel growth regulatory protein that is a member of the H-rev107 protein family (1). This family includes H-rev107 (2), RIG1/TIG3 (1, 3), H-rev107-1 (4), H-rev107-2 (4), and A-C1 (5). Immunostaining of epidermis reveals that TIG3 is expressed in the non-proliferating, suprabasal differentiated epidermal layers, but not in the proliferative basal cells (6), suggesting that TIG3 may play an important regulatory role during keratinocyte differentiation. TIG3 levels are reduced in tumor cells, consistent with the idea that the loss of TIG3 function may contribute to disease progression in cancer (1). However, the amino acid sequence of TIG3 does not reveal any particular motif that predicts a function, and no information is available regarding the TIG3 mechanism of action. Thus, identifying the mechanism of TIG3 action is an important goal that requires knowledge regarding the effects of TIG3 on cell regulatory mechanisms. In the present study, we show that TIG3 expression in cultured keratinocytes causes a cessation of cell proliferation and enhanced cornified envelope formation. Our results suggest that TIG3 associates with membranes and that the mechanism leading to reduced cell viability involves activation of type I transglutaminase.

MATERIALS AND METHODS

Adenovirus Infection—The tetracycline-regulated recombinant adenovirus, Ad5-TIG31–164, was constructed by cloning the TIG31–164 coding sequence, linked to an SV40 transcription terminator, into Ad5. This adenoviral vector contains a tetracycline-responsive element that includes the cytomegalovirus promoter and tetracycline operator. Activation of this promoter requires the presence of the tetracycline transactivator (TA) protein that is provided by co-infection with a second adenovirus, Ad5-TA. The ability of the TA protein to activate via the tetracycline operator can be reduced by addition of tetracycline. An empty recombinant adenovirus Ad5-EV was constructed for use as a control. A green fluorescent protein (GFP)-encoding adenovirus, Ad5-GFP, was used to determine keratinocyte infection efficiency. At greater than 10 plaque-forming units/cell, virtually 100% of the cells are infected (not shown). Human foreskin keratinocytes were cultured in keratinocyte serum-free media (KSF M, Invitrogen). Third passage cultures were plated at 15,000–20,000 cells/cm². After 24 h, the cells were infected with adenovirus for 12 h in KSF M containing 2.5 ng of polybrene/ml. The cells were then washed and incubated in fresh virus-free KSF M for varying times prior to harvest.

Cell Viability and Proliferation—Trypan blue exclusion and colorimetric XTT-based assays were used to measure cell viability and proliferation. For dye exclusion, cells, suspended in 200 μl of divalent cation-free phosphate-buffered saline (PBS), were added to 500 μl of 0.4% (w/v) trypan blue solution (Sigma catalog numberT8154) and maintained for 5 min at 37 °C prior to counting. Alternatively, cell viability and proliferation were measured using an assay based on the cleavage of the tetrazolium salt XTT. In this assay, only viable cells are able to convert the XTT to an orange product. Cells growing in 96-well plates are incubated with the XTT salt for 4–24 h, and the orange cleavage product is detected using an enzyme-linked immunosorbent assay reader.

Fluorescence Microscopy—Keratinocytes growing on coverslips were fixed in 2% paraformaldehyde, permeabilized with methanol, and incubated with rabbit anti-TIG3 (1:100) (7) followed by CY3-conjugated goat anti-rabbit IgG (1:1000, Sigma). After washing, cells were treated with...
1 μg/ml Hoechst 33258 to stain nuclei. The cells were washed, placed onto slides, and sealed with N-propyl galate for microscopic visualization by epifluorescence.

**Immunoblot Methods**—Equivalent quantities of protein were electrophoresed on 10% acrylamide gels, and separated proteins were transferred to a nitrocellulose membrane for immunoblot as described previously (7). The blots were then incubated overnight at 4 °C with primary antibody. Antibody binding was detected by incubation for 1 h with horseradish peroxidase-conjugated secondary antibody, and the antibody complex was visualized using chemiluminescence detection reagents.

**Flow Cytometry**—Keratinocytes were trypsinized, washed twice with cold PBS, and resuspended at 2 × 10^6 cells/ml of PBS containing 0.4% Paraformaldehyde (37 °C for 10 min). The cells were permeabilized by addition of 450 μl of methanol and stored at −20 °C. After methanol treatment, cells were incubated for 10 min at −20 °C in 500 μl of PBS containing 50 μg/ml RNase followed by incubation for 30 min in 500 μl of PBS containing 100 μg/ml propidium iodide. The cells were then sorted.

**Fluorescent Detection of Transglutaminase Substrate Incorporation in Situ**—Human keratinocytes, growing on coverslips, were treated with either 20 m.o.i. TA5–TIG3-164 or 20 m.o.i. TA5–EV with 5 m.o.i. Ad5-TA in KSF containing 2.5 μg/ml polybrene for 12 h followed by addition of fresh virus-free medium. At 48 h after infection, fresh KSF containing 100 μM fluorescein cadaverine (PC) (catalog number A-1030, Molecular Probes) was added for 24 h. The cells were then washed with PBS and fixed with 100% methanol at −20 °C, washed twice with cold methanol, and washed twice more with PBS prior to placing the samples onto slides using N-propyl galate for visualization by fluorescent microscopy using a Nikon Optiphot fluorescence microscope.

**Cell Fractionation and Transglutaminase Activity Assay**—Cells (1 × 50 cm² dish) were washed with PBS, collected by scraping, and homogenized in 1 ml of lytic buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride). The extract was centrifuged at 100,000 × g for 30 min to yield the supernatant fraction (cytosol) containing soluble type II transglutaminase. The pellet (membrane fraction) containing membrane-associated type I transglutaminase was assayed by liquid scintillation counting.

**Caspase 3 Activity Assay**—Human keratinocytes (one 10-cm² dish/polystyrene was washed twice with cold PBS and lysed in 50 μl of caspase buffer (25 mM HEPES, pH 7.4, 10% sucrose, 0.1% CHAPS, 2 mM EDTA, 5 mM dithiothreitol) (8). Cell lysates were centrifuged for 3 min at 15,000 × g, and the supernatant was saved for analysis. Supernatant (15 μg of protein in 50 μl of lysis buffer) was added to 50 μl of 2× assay buffer (25 mM HEPES, pH 7.4, 5 mM dithiothreitol, 100 μM Ac-DEVD-AFC) and incubated at 37 °C for 3 h. The caspase 3 substrate, Ac-DEVD-AFC, was obtained from Enzyme Systems. Caspase 3-dependent release of fluorescent AFC product was monitored at 360-nm excitation and 530-nm emission using a Shimadzu RF-5301 PC spectrofluorophotometer. Values were corrected for background and expressed as pmols of converted substrate/mg of protein/h.

**UVB Treatment**—Near confluent (70%) keratinocytes (one 50-cm² dish/polystyrene was exposed to 60 mJ/cm² of Kodacel-filtered UVB light and harvested at 20 h after treatment.

**Inhibition of Caspase Activity Using Z-VD-FMK**—Keratinocytes were infected with 5 m.o.i. of AD5-TA and 20 m.o.i. of either TA5–EV or TA5–TIG3-164. At 5 h after infection, fresh KSF was added containing 50 μM of the pan-caspase inhibitor Z-VD-FMK. The medium was changed with fresh medium and fresh 50 μM Z-VD-FMK. Apoptotic cell viability was assessed by trypan blue exclusion. Parallel dishes were treated with UVB (60 mJ/cm²) in the presence or absence of 50 μM Z-VD-FMK and then harvested and assayed for viability at 48 h.

**Antibodies**—Rabbit anti-caspase 3 (AHZ0052, 1:2000) was obtained from Biosource International. Goat anti-caspase 8 (sc-6355, 1:2000) and rabbit anti-caspase 9 (sc-6355, 1:2000) were obtained from Santa Cruz Biotechnology. Mouse anti-poly(ADP-ribose) polymerase (PARP) (55494, 1:1000) was obtained from Pharmingen. Mouse anti-human β-actin (A5441, 1:10,000) was obtained from Sigma. Rabbit anti-human involucrin (1:2000), rabbit preimmune serum, and rabbit anti-TIG3 (1:2000) were produced in our laboratory (7). Mouse anti-transglutaminase type 1 was obtained from Biomedical Technologies, Inc. (BT-621, 1:50). Alexa Fluor 488-conjugated goat anti-mouse IgG (A11001, 1:1000) was from Molecular Probes. Cy3-conjugated sheep anti-rabbit IgG (c-2306, 1:1000) was obtained from Sigma. Peroxidase-conjugated donkey anti-rabbit IgG (NA934, 1:8000) and peroxidase-conjugated sheep anti-mouse IgG (NA931, diluted 1:8000) were purchased from Amersham Biosciences.

**RESULTS**

**TIG3 Reduces Keratinocyte Cell Number**—To study the effects of TIG3 on keratinocyte function, we infected cultured normal human keratinocytes with an empty adenovirus, TA5–EV, or an adenovirus encoding full-length TIG3, TA5–TIG3-164. As shown in Fig. 1A, TIG3-164 expression can be detected as early as 12 h after adenovirus infection, and expression is maximal between 24 and 36 h. The appearance of TIG3 is associated with a reduction in keratinocyte cell number beginning at 24 h. At 60 h, the viable cell number is reduced to 10% of the initial density, suggesting that TIG3-164 promotes cell death. To assess the effects of TIG3-164 level on cell viability, cells were infected with TA5–EV or TA5–TIG3-164 in the presence of increasing concentrations of tetracycline. TIG3 expression is reduced with increasing tetracycline. As shown in Fig. 1B, reduced keratinocyte cell number is correlated with increased TIG3-164 expression. To characterize cell status, we sorted cells at various times after TA5–EV or TA5–TIG3–164 infection. As shown in Fig. 1C, at 24 h after infection, the sub-G1 cell population, i.e., cells with less than a full complement of DNA due to partial apoptotic degradation, is 2.4% in TA5–EV-infected cells. This number does not increase out to 72 h (not shown). In contrast, TIG3-164 expression increases the sub-G1 cell population from 1.4% at 24 h to 7.2% at 48 h and 21% at 72 h. As a control, we show that UVB treatment of keratinocytes, which promotes apoptosis and accumulation of cells in sub-G1, increases the sub-G1 population to 6.2%.

**TIG3 Regulates Keratinocyte Cell Morphology**—TIG3 expression is also associated with altered cell morphology. The photographs, taken 48 h after infection with empty or TIG3-encoding vector (Fig. 2A), indicate that TIG3-164-expressing cells display a cornified envelope-like morphology and retention of cell nuclei (arrows). To determine whether these structures display cornified envelope-like properties, we assessed their ability to survive boiling in detergent and reducing agent. As shown in Fig. 2B, TIG3-164 expression reduces cell number and markedly increases the percentage of cells, from 3.2 to 33%, that are resistant to boiling in detergent and reducing agent, a characteristic of cornified envelopes. To correlate time of TIG3-164 expression with morphological change, cells were infected with TA5–TIG3-164 and harvested at various times after infection. As shown in Fig. 2C, limited morphology change is observed at 24, 30, and 40 h after infection, but marked changes are observed at 48 h. Nuclear staining (blue) reveals that TIG3 expression (red) is associated with nuclear shrinkage (arrows).

**TIG3 Regulates Transglutaminase Activity**—Transglutaminases (TGs) are a family of enzymes that assemble differentiation- and cell death-associated structures, including the cornified envelope (9–14). To determine whether TIG3 influences TG activity, cells were treated with TA5–EV or TA5–TIG3-164 for 48 h. During the last 4 h of treatment, the medium was supplemented with 100 μM FC, a cell-permeable fluorescent transglutaminase substrate. Fig. 3A shows that FC incorporation (green) is greatly increased in TIG3-expressing (red) cells. As a second method of detecting TIG3-dependent transglutaminase activity, Fig. 3B shows that total transglutaminase activity, measured by monitoring [3H]putrescine incorporation, is increased 3-fold by TIG3-164. Separation of lysate into soluble and particulate fractions reveals that most of the increase in...
tranglutaminase activity is observed in the particulate (membrane) fraction, suggesting that membrane-associated type I transglutaminase is activated. The increase in type I transglutaminase (TG1) activity could be due to increased expression of TG1. The immunoblot of total extract prepared from tAd5-EV and tAd5-TIG31–164-infected cells (inset) shows that TG1 levels are not visibly altered by TIG3 treatment. Consistent with activating a membrane-associated target, the cell fractionation study shown in Fig. 3 indicates that TIG3 is also associated with the membrane (Part, particulate) fraction. In these studies, cells were infected with TIG3 and then fractionated into cytosol and particulate fractions. As a control, we demonstrate that involucrin, a cytosolic protein, is retained in the soluble fraction.

**Fig. 3. **TIG3-mediated cell death is time and dosage dependent. As shown in A, keratinocytes were infected with 5 m.o.i. of Ad5-TA and 20 m.o.i. of tAd5-TIG3 or empty vector (tAd5-EV) for 12 h. Cells were harvested at 0, 12, 24, 36, 48, and 60 h after the start of infection, and viability was evaluated using trypan blue exclusion. Immunoblot analysis (inset) shows the TIG31-164 expression level at each time point. KER, keratinocytes. B, TIG31-164 dose response. Cells were infected with Ad5-TA and tAd5-TIG31-164 as above, except that the level of tetracycline in the medium was varied from 0–125 ng/ml. After 48 h, the cells were harvested and assayed for the ability to exclude trypan blue. TIG31-164 level was monitored by immunoblot (inset). As shown in C, cells were infected with TIG31-164-encoding virus or empty virus. At the indicated times after infection the cells were stained with propidium iodide and analyzed by flow cytometry. As a positive control for activation of apoptosis, one group of cells was treated with 50 mJ of UVB/cm² and sorted 16 h later. The percentage of sub-G₁ cells is indicated in each panel.

**TIG3 Regulation of Apoptosis—**The above results suggest that TIG3 regulates apoptosis and keratinocyte differentiation. To determine whether TIG3 also regulates apoptotic processes, untreated cells (control) and cells treated for 48 h with empty vector (EV) or TIG31-164-encoding vector were harvested and assayed for caspase 3 activity using the caspase 3-specific substrate, Ac-DEVD-AFC. Uniformly low caspase ac-
activity is observed in control, EV- or TIG3-treated cells (Fig. 4A). As a positive control, we show that UVB treatment, a treatment that causes extensive cell apoptosis and caspase activation, markedly increases apoptosis-associated caspase 3 activity. Caspase 3 has been shown to be activated by UVB treatment of keratinocytes (8, 18–21). Caspases are expressed as pro-forms that require cleavage for activation (22). To confirm the caspase assay result, we assayed procaspase 3 protein level by immunoblot. Fig. 4B shows that procaspase 3 is not degraded by TIG3, suggesting that it is not activated. However, levels are markedly reduced by UVB treatment, consistent with the known ability of UVB light to promote caspase-associated cell death (8, 19, 20). The middle panel confirms that TIG3 is expressed in tAd5-TIG3 vector-infected cells, and the β-actin blot confirms appropriate protein loading. We next determined whether activity of the initiator caspases (caspase 8 and 9) and PARP are increased by TIG3. These enzymes are also expressed as pro-forms that require cleavage for activation (22). As shown in Fig. 4C and D, expression of TIG3 in tAd5-TIG3 vector-infected cells, and the β-actin blot confirms appropriate protein loading. We next determined whether the pan-caspase inhibitor, Z-VAD-FMK, halts the reduction in TIG3-associated cell number. As shown in Fig. 5, TIG3 reduces viable cell number to 30%, but the reduction is not reversed by addition of 50 μM Z-VAD-FMK. In contrast, the UVB-associated reduction in viable cell number is substantially reversed by the inhibitor.

TIG3 Mutant Lacking Carboxyl Hydrophobic Domain Is Inactive—To begin identifying TIG3 functional domains and to show that the cell response is not an artifact of protein overexpression, we tested the ability of TIG3 to reduce cell viability. The immunoblot shown in Fig. 6A confirms that TIG3 and TIG3Δ134 are expressed at comparable levels. The β-actin blot confirms that protein loading is appropriately normalized. Fig. 6B compares TIG3 and TIG3Δ134 subcellular localization. The left panel confirms that TIG3Δ134 is preferentially distributed in the particulate fraction and that a fraction of the particulate-localized TIG3 is present in a high molecular weight, presumably cross-linked form. In contrast, unlike TIG3, most TIG3Δ134 immunoreactivity distributes in the cytosol (right panel).

We next determined whether TIG3Δ134 reduces cell viability. Cells were infected with TIG3Δ134 or TIG3Δ134-encoding virus. After 48 h, cells were harvested and counted. As shown in Fig. 6C, TIG3Δ134 does not reduce keratinocyte viability. The microscopy data presented in Fig. 3E indicate that TIG3Δ134 is preferentially associated with the particulates. We therefore used microscopic methods to monitor TIG3Δ134 subcellular localization. Cells were infected with TIG3Δ134 and then stained with anti-TIG3 (red) and anti-TG1 (green). As shown in Fig. 6D, in contrast to TIG3Δ134, TIG3Δ134 is diffusely distributed throughout the cell. In addition, the combined image shows only a partial and weak co-localization of TIG3Δ134 and TG1.

TIG3Δ134 Does Not Activate Type I Transglutaminase—To evaluate whether the TIG3 truncation mutant can regulate biochemical responses, we expressed TIG3Δ134 in keratinocytes and then determined whether the cells incorporate FC in situ. The immunohistology (anti-TIG3) shown in Fig. 7 indicates that the cells produce TIG3Δ134 and TIG3Δ134 does not reduce cell viability. The green fluorescence, which indicates the transglutaminase-dependent incorporation of FC substrate, is absent in cells expressing TIG3Δ134. Thus, TIG3Δ134 does not cause transglutaminase activation.

DISCUSSION

TIG3 is a member of a recently discovered H-rev family of proteins (1, 2, 4). These proteins are important because they function to inhibit cell proliferation (2, 3, 5, 23). Moreover, their
expression is reduced in cells that have escaped growth regulation (1, 4). Each family member displays a distinct pattern of tissue-specific expression. For example, RIG1 is expressed in human gastric cancer cells, and expression is increased by treatment with retinoic acid (3). H-REV107-2, a 216-amino-acid protein, is highly expressed in leukocytes and in the thymus (4). TIG3 is expressed in several cell types, including keratinocytes (1, 6), and TIG3 levels are increased by agents that inhibit cell proliferation (1, 3). A common feature shared by all of these proteins is a carboxyl-terminal hydrophobic domain. Studies with H-rev107 (2) and TIG3 (7) suggest that this domain may function as a membrane anchor. However, apart from these observations, very little information is available regarding their mechanism of action. The present studies were designed to gain new insight regarding the mechanism whereby TIG3 regulates keratinocyte function.

TIG3 halts keratinocyte proliferation and enhances differentiation—TIG3 is expressed in the differentiated, suprabasal epidermal layers in vivo (6). Consistent with the expression in epidermis, we also detected TIG3 mRNA in cultured keratinocytes. However, we could not detect TIG3 protein, an observation consistent with the finding that H-rev family members are generally expressed at barely detectable levels. To study TIG3 effects on keratinocyte proliferation, apoptosis, and differentiation, we expressed exogenous TIG3 using adenovirus. Our studies show that TIG3 expression markedly reduces keratinocyte proliferation/survival and that this is associated with accumulation of cells in the sub-G_1 growth phase. The reduction in viable cell number is associated with a 10-fold increase in accumulation of structures resembling cornified envelopes.

TIG3 promotes cell death by enhancing transglutaminase activity—There is virtually no information available regarding how members of the H-rev family cause cell death. Our studies, however, indicate that TIG3-dependent cell death is associated with cornified envelope formation. Cornified envelopes are cross-linked arrays of protein that are assembled during the terminal stages of normal keratinocyte differentiation (24). These structures are assembled via formation of covalent ε-(γ-
Since cornified envelopes are assembled via action of transglutaminases, we explored the possibility that TIG3 may activate these enzymes. Transglutaminases exist in multiple forms, are expressed in a tissue-specific manner, and are involved in formation of differentiated structures (14, 26, 27). They are also involved in assembly of apoptosis-associated structures (9, 11, 28–30). Keratinocytes produce several types of transglutaminase (31–34). However, the major type involved in corneocyte assembly is TG1. TG1 is anchored to the plasma membrane via a palmitate or myristate linkage (31, 35). Biochemical and in situ substrate incorporation assays reveal a substantial increase in membrane-associated transglutaminase activity in TIG3-expressing keratinocytes, suggesting that TIG3 acts to increase TG1 activity. Type 2 transglutaminase (TG2) is also expressed at low levels in keratinocytes and other surface epithelial cells and can be induced under specific conditions (32, 33). It is possible that TIG3 could induce TG2, as TG2 activity is known to facilitate apoptosis. TG2 activity is assayed based on changes in cross-linking activity in the soluble phase. As shown in Fig. 3B, TIG3 expression does not alter TG activity in this compartment. These results confirm that TG2 is not involved in the TIG3-dependent response. Moreover, we suspect that the soluble TG activity may actually be TG1 that is contaminating the soluble phase. Additional results further support the idea that TIG3 activates TG1. First, TIG3 and TG1 are located in the same subcellular location, in membranes. Second, as determined by monitoring of sites of incorporation of TG fluorescent substrate, the intracellular sites of TG1 activity correspond with site of TIG3 localization. Finally, a fraction of TIG3 is cross-linked to form high molecular weight structures. The final point suggests a close juxtaposition, as TG1 and TIG3 must actually make close contact for TIG3 to serve as a TG substrate. The covalent cross-linking of TG-associated proteins, either by design or incidentally, is known to occur. Thus, it is not surprising that a regulatory protein such as TIG3 can become cross-linked by TG1. Overall, these results strongly suggest that TG3 activation is required for the cell to manifest the TIG3-associated effects.

**TIG3 Does Not Activate Killer Caspases**—Keratinocyte cell

![Fig. 4. TIG3 regulation of cell death markers.](image)

Keratinocytes were mock-infected (Control) or infected with 5 m.o.i. of Ad5-TA with 20 m.o.i. tAd5-EV or 20 m.o.i. tAd5-TIG3 for 12 h and harvested at 48 h after infection. Parallel cultures were treated with UVB (50 mJ/square cm) and harvested at 16 h after treatment. As shown in A, cell extracts were prepared and assayed for the ability to cleave a fluorescent caspase 3 substrate, Ac-DEVD-AFC. Caspase activity is expressed as nmoles of substrate cleaved/mg protein/h. Control cells were not treated. As shown in B, TIG3 does not regulate caspase 3 activation. Extracts from the experiment shown in panel A were electrophoresed, and procaspase 3, TIG3, and β-actin levels were assayed by immunoblot. As shown in C and D, TIG3 does not activate procaspase 8, procaspase 9, or PARP. Extracts were prepared as outlined above, and the level of each protein was measured by immunoblot. The position of known molecular mass markers are indicated in each panel.

![Fig. 5. Inhibition of caspase activity does not prevent TIG3-mediated responses.](image)

Cells were infected with 5 m.o.i. of Ad5-TA with 20 m.o.i. of tAd5-EV or tAd5-TIG3 for 12 h. Cells were grown during and after infection in the presence or absence of 50 μM Z-VAD-FMK. Parallel cultures were treated with 50 mJ of UVB in the presence or absence of 50 μM Z-VAD-FMK. After 48 h, the cells were harvested, and viable cell number was assayed by trypan blue exclusion. The means ± S.E. of mean is presented as n = 3.
death can occur via at least two distinct pathways: normal differentiation and activation of apoptotic processes (37, 38). These processes are distinguished by the differential activation of killer caspases. Ultraviolet light treatment of epidermis and cultured keratinocytes activates caspases, and the cells undergo apoptosis (8, 21). This is in contrast to normal differentiation, which does not involve killer caspase activity (39, 40). Thus, in contrast to normal differentiation, caspase-involved apoptosis is usually a response to trauma. Our present studies show that the killer caspases (caspases 3, 8, and 9) are not activated as part of the cell death program in TIG3-positive keratinocytes. PARP is a nuclear enzyme that protects against genome damage and increases during apoptosis (41, 42). TIG3 expression does not induce PARP cleavage. In addition, TIG3-dependent cell death is not inhibited by the pan-caspase inhibitor, Z-VAD-FMK. These observations, together with the finding that TIG3 enhances differentiation-associated responses, increased TG1 activity, and cornified envelope formation, suggest that TIG3 is primarily an activator of keratinocyte differentiation.

**TIG3 Activity Requires the Carboxyl-terminal Hydrophobic Domain**

Our previous report identified homologous domains shared between TIG3 and other H-rev family proteins (7). This comparison revealed the presence of a conserved 30-amino-acid hydrophobic carboxyl-terminal domain. It is hypothesized that this domain may be required for membrane localization (7). To test this hypothesis, we expressed the carboxyl-terminal truncation mutant. To examine whether the TIG3 carboxyl-terminal domain is necessary for function, we determined whether the truncation mutant has activity. Our studies show that TIG31-134 does not inhibit cell proliferation, reduce cell viabil-
ity, or promote cornified envelope formation. This loss of activity is associated with a distribution in the soluble phase, reduced localization with TG1, and a loss of the ability to activate transglutaminase. These findings strongly suggest that membrane localization, and perhaps localization with or near TG1, is required for TIG3-dependent cell differentiation. Future studies, using additional mutants, will be necessary to identify additional functional domains.

Mechanism of TIG3 Action—Our studies have identified a new regulator and a new mechanism regulating keratinocyte differentiation. We hypothesize that TIG3 promotes keratinocyte differentiation via activation of membrane-associated transglutaminase. TIG3 is produced and inserted into various membranous structures where it functions to activate type I transglutaminase. Activation may be via direct interaction with TG1 or by facilitating the local release of calcium that, in turn, activates the calcium-dependent enzyme. Additional studies are underway to test these hypotheses.

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