The $\eta$ Isoform of Protein Kinase C Mediates Transcriptional Activation of the Human Transglutaminase 1 Gene*

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Transglutaminase 1 (TGase 1) is expressed during the terminal differentiation of keratinized squamous epithelium to form cornified cell envelope in differentiated keratinocytes by the $\epsilon$-(gamma-glutamyl) cross-linking reaction. The gene for human TGase 1 is responsible for autosomal recessive lamellar ichthyosis, a severe hereditary keratinizing disorder of the skin. We examined the transcriptional activity of the gene in FRSK, rat keratinocyctic cells, transfected with the luciferase reporter gene under control of the 5' upstream region of human TGase 1 gene. Transfection of the reporter gene with an expression vector for the $\eta$ isoform of novel protein kinase C (nPKC$_\eta$), as well as exposure to 12-O-tetradecanoylphorbol-13-acetate, markedly increased the luciferase activity in FRSK, but not in HT-1080 fibrosarcoma cells, although exogenous nPKC$_\eta$ was expressed in both. The induction was suppressed by deleting the TGase 1 upstream sequence from -95 to -67 and by deleting the kinase domain from exogenous nPKC$_\eta$. In comparison with other PKC isoforms, nPKC$_\eta$ most effectively induced the luciferase activity. We suggest that nPKC$_\eta$, an epithelium-specific isoform of PKC, mediates the activation of the TGase 1 transcription.

Terminal differentiation is a highly organized biological process that involves the programmed death of cells (1). The epidermis covering the most outer layer of the skin is maintained by a dynamic equilibrium of renewal with terminal differentiation of keratinocytes, the major cell lineage of the epidermis covering the most outer layer of the skin is maintained by a dynamic equilibrium of renewal with terminal differentiation of keratinocytes, the major cell lineage of the skin. This process, keratinization, proceeds with the differentiation of keratinocytes, the major cell lineage of the skin. The importance of this enzyme in the morphogenesis and barrier function of the skin has been shown also by discovery of its mutation in autosomal recessive lamellar ichthyosis (8-10), which is a hereditary keratinization disorder characterized by colloidon baby at birth and later, severe ichthyosis with large brownish plate-like desquamations and epidermal hyperkeratosis, ectropion, eczabium, and scarling alopecia. The gene for human TGase 1 is located on 1q411.2 (11, 12) and encodes a 92-kDa protein consisting of 816 amino acid residues (13-15). In the epidermis of the skin, TGase 1 gene is expressed mainly in the subcorneal granular layers (16-18). The enzyme activity of transglutaminase is enhanced by treating primary mouse epidermal cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (19). In cultured epithelial keratinocytes and tracheal epithelial cells, the level of TGase 1 mRNA is increased also by TPA (20, 21) as well as Ca$^{2+}$ (22-24), ganglioside GQ$_{1\beta}$ (24), and interferon-$\gamma$ (25). Besides the effect of TPA, the critical function of PKC in the regulation of TGase 1 gene expression has been suggested by studies using several PKC inhibitors (20, 26). However, there is no direct evidence indicating that PKC mediates the signal for the transcriptional activation of TGase 1.

PKC was initially characterized as a serine/threonine-type protein kinase, the activity of which is dependent on Ca$^{2+}$, phospholipids, and diacylglycerol (27-29). Subsequently, various isoforms of the enzyme have been identified (30). PKC is now classified into three major subgroups, Ca$^{2+}$-, phospholipid-, and diacylglycerol-dependent classical PKCs (cPKC) ($\alpha$, $\beta$, $\beta$II, and $\gamma$), Ca$^{2+}$-independent novel PKCs (nPKC) ($\delta$, $\epsilon$, $\eta$, and $\theta$) and Ca$^{2+}$- and diacylglycerol-independent atypical PKC (aPKC) ($\zeta$ and $\lambda$). In addition, the $\mu$ isoform, a membrane-bound PKC, has been identified (31). They differ in terms of tissue distribution and are involved in a variety of specialized functions of the tissues. One nPKC, called nPKC$_\eta$ (PKC-$\eta$), is epithelium-specific (32, 33). Although there are also other PKC isoforms, such as cPKC$\alpha$ and nPKC$_\alpha$, in the epidermis of the skin (34), the localization of nPKC$_\eta$ in the subcorneal granular layer is quite similar to that of TGase 1 mRNA (16, 17), suggesting that it mediates the activation signal for TGase 1 gene during the terminal differentiation of epithelial keratinocytes. Understanding the unique signal transduction for TGase 1 gene regulation should provide new insight into the molecular mechanism involved in the complex biological process of keratinization. Therefore, we analyzed the role of nPKC$_\eta$ in the...
transcriptional activation of TGase 1 gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials—**FRSK cells (35, 36) and HT-1080 fibrosarcoma cells (37) were supplied by the Japanese Cancer Research Resources Bank (Foundation for Promotion of Cancer Research, Tokyo, Japan). The luciferase reporter plasmids PGV-B and PGV-C were purchased from Toyo Ink Manufacturing Co., Ltd. and 12-O-tetradecanoylphorbol-13-acetate was obtained from LC Service Corp. Oligonucleotides were synthesized by Genset, France. All other reagents and chemicals were purchased from commercial sources and were either of reagent grade or the highest purity available.

**Cell Culture—**FRSK cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 2 mM glutamine in air. Cells were subcultured every 3–4 days until they reached 80% confluence.

**Construction of Reporter Plasmids—**Deletions of the 5'-flanking region of human TGase 1 gene from −819 to −49 were generated by the polymerase chain reaction (PCR) using a plasmid pdH1 as the template (38). These fragments were amplified using U1Tma DNA polymerase (Perkin-Elmer) and synthetic oligonucleotide primers, according to the manufacturer's instructions. The PCR products and the synthesized oligonucleotides for −20 deletion were inserted into the Smal site of PGV-B, a luciferase reporter plasmid containing neither enhancer nor promoter sequences. These reporter plasmids are summarized in Fig. 1. PGV-C is a constitutive expression plasmid for luciferase containing the SV40 enhancer.

**Cells and reagents—**PGV-B and PGV-C are negative and positive control reporter plasmids, respectively.

**Construction of SRD—**The kinase domain of mouse nPKC δ, which is encoded by a 865-bp sequence from nucleotides 1041–1905 of the cDNA (33), SRDγ, an expression plasmid containing the complete coding region of mouse nPKC δ, was co-transfected with the reporter genes into FRSK cells; 2 days later, the luciferase activity was determined. Data represent the means of duplicate experiments normalized to β-galactosidase activity, PGV-B and PGV-C are negative and positive control reporter plasmids, respectively. **RESULTS**

The Regulation of Transglutaminase 1 Gene by nPKC δ

**Fig. 1. The TGase 1-luciferase reporter genes.** Deletions of the human TGase 1 5'-flanking region from −819 to −49 were generated by PCR and inserted into the Smal site of PGV-B, a basic luciferase reporter vector. PGV-C is a constitutive expression vector for luciferase driven by SV40 promoter and enhancer. Arrows indicate PCR primers used to synthesize the deletion mutants. Upper primers are as follows: pd-819, aattcaggtgagagaaaggtg-95, cccatccccccagacggccpd-66, cccatccccccagacggccpd-49, gcagtttggcccctccctccc. The lower primer is tcattccagctctctcc. This primer was also used to prepare the deletion fragment pd-20, by annealing it with its sense oligonucleotide, E, EcoR I; P, Pvu I; B, Bam H I; H, Hind III; Luc, luciferase gene; SV40 box, SV40 enhancer; P box, SV40 promoter.

**Fig. 2. The transcriptional activation of the human TGase 1 gene by TPA and nPKC δ.** A, the effect of TPA on the transcriptional activity of human TGase 1. The TGase 1-luciferase reporter genes were transfected into FRSK, cultured rat keratinocytes. After a 2-day incubation, the cells were exposed to 10 nM TPA for 4 h, and then the luciferase activity was determined, as described under “Experimental Procedures.” B, the effect nPKC δ on the transcriptional activity of human TGase 1. SRDγ, an expression plasmid for nPKC δ, was co-transfected with the reporter genes into FRSK cells; 2 days later, the luciferase activity was determined. Data represent the means of duplicate experiments normalized to β-galactosidase activity, PGV-B and PGV-C are negative and positive control reporter plasmids, respectively.

**Immunoblotting Analysis—**Cells were immersed in 10% trichloroacetic acid at 4°C for 15 min, then scraped off the dishes. After centrifugation at 10,000 × g for 5 min, the trichloroacetic acid was removed and the pellet was homogenized in 80 μl of Urea-TX (9 M urea, 2% Triton X-100, 5% 2-mercaptoethanol) by sonication on ice. To this, 20 μl of 10% lithium dodecyl sulfate and 1 μl of 2 M Tris were added, then the proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (41). The separated proteins were electrophoretically transferred onto a Clearblot-P membrane (Atto Corp.). The blot was probed with anti-PKCM δ antibody (Life Technologies, Inc.), anti-PKCM δ antibody (Santa Cruz Biotechnology, Inc.), and anti-nPKC δ antibody (42). The protein was detected using secondary anti-rabbit IgG antibody conjugated with alkaline phosphatase (Bio-Rad).

**RESULTS**

The Transcriptional Activity of the 5'-Upstream Region of Human TGase 1 Gene—FRSK is a keratinocytic cell line de-
The Regulation of Transglutaminase 1 Gene by nPKC_\eta

Ref: "The Regulation of Transglutaminase 1 Gene by nPKC_\eta"

Fig. 3. The comparison of the effects of nPKC_\eta on the transcriptional activation of human TGase 1 gene between FRSK cells and HT-1080 cells. A reporter gene, pd-95, was co-transfected with the indicated amounts of SRD_\eta into FRSK cells (A) or into HT-1080 fibrosarcoma cells (B), after which the luciferase activity was determined, as described under "Experimental Procedures." PL27 is an expression vector for human nPKC_\eta (PKC-L). Data represent the means of duplicate experiments normalized to \beta-galactosidase activity.

Fig. 4. The effect of mutant SRD_\eta on the human TGase 1 transcriptional activity in FRSK cells. A reporter gene pd-95 was co-transfected into FRSK cells with 12.5 fmol of either SRD_\eta or SRD_\etaRD, a mutant SRD_\eta with deletion in the sequence encoding the kinase domain of nPKC_\eta, as described under "Experimental Procedures." After 2 days of incubation, the cells were incubated with or without 1 nM TPA for 4 h, and then luciferase activity was determined. Data represent the means of duplicate experiments normalized to \beta-galactosidase activity.

derived from fetal rat skin (35, 36). FRSK cells, like HaCaT cells derived from a human epidermoid carcinoma, are grown in medium with a normal Ca^{2+} concentration. We have studied the transcriptional regulation of human TGase 1 gene by means of electroporation (38). In this study, we adopted lipofection using DOTAP, because the transfection efficiency of this procedure was 10-fold higher than that of electroporation. There was practically no difference in the transcriptional activities between the 2.5-kb and 819-bp upstream regions of TGase 1 gene (38). Therefore, we examined the activity of the region up to –819 of the gene. When the luciferase reporter plasmid pd-819 containing the 819-bp upstream sequence of human TGase 1 gene was transfected into FRSK cells by lipofection, there was substantial basal luciferase activity (Fig. 2A). The activity was potentiated by 1 nM TPA, which induces the expression of TGase 1 mRNA in keratinocytes (20). To assess the sequence required for the transcriptional activation of the gene, we prepared deletion mutants of its 5’ upstream region from –819 to –20 and luciferase reporter plasmids containing these sequences were transfected into FRSK cells. As shown in Fig. 2A, the luciferase activity remained even when the sequence from –819 to –96 was deleted from the upstream region. However, the major loss of TPA-stimulated luciferase activity was seen when the sequence between –95 and –67 was deleted. A deletion from –66 to –50 substantially reduced the basal luciferase activity. These results suggest that the induction of TGase 1 gene expression by TPA is regulated at the transcriptional level and that this 29-bp 5’ upstream sequence is required for the signaling.

The Activation of Human TGase 1 Transcription by nPKC_\eta—Of the PKC family, the localization of nPKC_\eta is closely related to that of TGase 1 mRNA in human epidermis (16, 17, 34, 43), implying that it is involved in the induction of TGase 1 transcription. To investigate this notion, we co-transfected SRD_\eta (33), an expression plasmid for mouse nPKC_\eta, into FRSK cells with the same set of reporter constructs used for the experiment described above. As shown in Fig. 2B, the co-transfection of SRD_\eta with pd-819 increased the luciferase activity and the induction was comparable to that by TPA. The effect of the co-transfection was abolished by deleting the 5’ upstream sequence from –95 to –67, which was the same region required for the effect of TPA. These findings indicated that nPKC_\eta mediates the activation of TGase 1 transcription.

The Effect of nPKC_\eta on the TGase 1 Transcription in Different Cell Types—To study the dose-responsive effect of nPKC_\eta, various amounts of SRD_\eta were transfected into FRSK cells with the reporter plasmid pd-95, which contains the minimum proximal upstream sequence responsive to both TPA and nPKC_\eta. As shown in Fig. 3A, the increase in the luciferase activity was maximal at 12.5 and up to 500 fmol of SRD_\eta. The level of the induction by SRD_\eta was similar to that elicited by co-transfection with 500 fmol of PL27, an expression plasmid for human nPKC_\eta (44).

In contrast to FRSK cells, TPA fails to activate TGase 1 transcription in HT-1080, a human fibrosarcoma cell line, even when a reporter plasmid containing –819 upstream sequences is transfected (38). When pd-95 was co-transfected with SRD_\eta from 2.5 to 25 fmol into HT-1080 cells, the induction of luciferase activity remained at low levels (Fig. 3B), indicating that the machinery that transduces the signal elicited by nPKC_\eta is defective in HT-1080 cells.

Deletion of the Kinase Domain of nPKC_\eta Abolishes Its Effect on Human TGase 1 Gene—The enzyme nPKC_\eta is composed of a regulatory and a kinase domain. Unlike classical PKCs, the regulatory domain of nPKC_\eta has no Ca^{2+} binding subdomain, but its kinase domain is homologous to those of other PKCs. To study the role of the kinase domain of nPKC_\eta in the activation...
of TGase 1 transcription, we constructed the plasmid SRD
from SRD\(\eta\) by deleting the cDNA sequence encoding this
domain and co-transfected this plasmid with pd-95 into FRSK
cells. SRD\(\eta\)-RD did not induce luciferase activity over the basal
level (Fig. 4). TPA caused no additional increase in the lucifer-
ase activity in FRSK cells transfected with SRD\(\eta\)-RD as well as
SRD\(\eta\). Thus, nPKC\(\eta\) is essential for TGase 1 gene expression
and possibly the kinase domain of the enzyme is involved in the
signaling for the transcription.

Expression of PKC Isoforms by Transfection with Their Ex-
xpression Vectors—The effect of nPKC\(\eta\) expression on human
TGase 1 transcription was almost a plateau, when over 12.5
fmol of SRD\(\eta\) were transfected into FRSK cells (Fig. 3A). This
might be due to the limitation on the expression level of exog-
enous nPKC\(\eta\) in FRSK cells. A similar mechanism might lead
to low levels of activation of TGase 1 transcription in HT-1080
cells (Fig. 5A). Thus, nPKC\(\eta\) expression was almost a plateau,
when over 12.5 fmol of SRD\(\eta\) were transfected into FRSK cells.

Comparison of the Effect of nPKC\(\eta\) with Other PKC Isoforms
on the Human TGase 1 Transcription—In epidermal kerato-
cytes, at least \(\alpha\)-PKC, nPKC\(\delta\), and nPKC\(\theta\) are expressed (34,
45). \(\alpha\)-PKC\(\beta\) is found in Langerhans cells of the skin as well as
other organs (46), and \(\gamma\)-PKC is predominantly expressed in
the brain. \(\zeta\)-isoform is expressed in a variety of tissues (47). In addition to nPKC\(\eta\), we examined the effects of other
PKC isoforms on TGase 1 transcription in FRSK cells. The
expression plasmids for \(\alpha\)-PKC, \(\beta\)-PKC, \(\gamma\)-PKC, nPKC\(\delta\),
nPKC\(\theta\), or \(\zeta\)-PKC were co-transfected with pd-95, and the luciferase activity was compared. As shown in Fig. 5E, nPKC\(\eta\) most efficiently induced luciferase activity. Next to nPKC\(\eta\), nPKC\(\delta\) was also effective (Fig. 5E). However, \(\alpha\)-PKC, \(\beta\)-PKC, \(\gamma\)-PKC, and \(\zeta\)-isoform hardly induced the luciferase activity (Fig. 5, A-D and G). Thus, among the PKC isoforms examined, nPKC\(\eta\) was the most efficient activator of human
tTGase 1 transcription.
Several reports have provided evidence that PKC regulates Tgase 1 gene expression. We found that TPA, an activator of PKC, increases Tgase 1 mRNA in cultured normal human epidermal keratinocytes and this effect is suppressed by the PKC inhibitors H-7 and staurosporine (20). Dlugosz and Yuspa (26) have also shown that the effect of TPA is blocked by the PKC inhibitors, GF-109203X and bryostatin 1, in cultured primary mouse epidermal keratinocytes. They also postulated that Tgase 1 mRNA expression is regulated at the transcriptional level, from the results of a nuclear run-on assay. The transcriptional regulation by TPA has been confirmed in rabbit and human Tgase 1 genes by means of a transient expression assay using the 5’ upstream region of the genes (21, 38). In this study, we show further direct evidence that nPKC η, an isofrom of PKC, regulates the transcriptional activation of the Tgase 1 gene. This mechanism may also be functional in the epidermis of the skin, because the localization of nPKC η is closely associated with that of Tgase 1 mRNA in normal and psoriatic epidermis (16, 17, 34, 43).

Deletion of the 5’ upstream region of the human Tgase 1 gene indicates that the sequence from −95 to −67 induces critical elements in response to both nPKC η and TPA. The overexpression of nPKC η with TPA induces no additional potentiation of its transcription in FRSK and nPKC η without the kinase domain cannot increase the transcriptional activity. Thus, it is likely that TPA activates nPKC η and the signal generated by phosphorylation is concentrated on the elements of the upstream region. Saunders et al. (21) have demonstrated the promoter activity of the proximal upstream region of rabbit Tgase 1 gene. Its activation requires the sequence from −95 to −50 of the gene, the position of which is similar to that of human Tgase 1 gene (12). However, the homology of their sequences was not so high, suggesting a difference in Tgase 1 gene regulation between the species.

As shown by immuno blotting, exogenous nPKC η is well expressed in FRSK as well as HT-1080 fibroblastic cells. An increase in the expression levels of nPKC η by exogenous introduction of an nPKC η expression plasmid activates the transcription of Tgase 1 in FRSK cells, but to a very limited extent in HT-1080 cells. Therefore, the signaling for Tgase 1 gene expression is probably mediated by nPKC η, but its presence is not necessarily sufficient to induce the transcriptional activation of the gene.

In terms of ability to induce Tgase 1 transcription, nPKC η was the most potent of all the PKC isozymes tested, cPKC ξ, cPKC β1, cPKC γ, nPKC δ, and aPKC ζ. This prominent efficiency of nPKC η seems to represent its selective importance in epidermal differentiation. It is localized in rough endoplasmic reticulum (48), and, unlike cPKC η, it cannot be translocated to the plasma membrane by activation (45). This unique feature of nPKC η may be related to the induction of Tgase 1 transcription. We also find that nPKC δ can induce Tgase 1 transcription to some extent, suggesting possible contribution of the gene expression, although the PKC isoform is expressed in various organs including epidermis (45, 47).

There is neither a conventional nor other TPA-responsive elements in the 5’ upstream sequence from −95 to −67, which is critical to Tgase 1 transcription (12). We pointed out two Sp1 motifs in the 5’ proximal upstream region of human Tgase 1 gene (12). Indeed, when a Sp1 expression plasmid was introduced into FRSK cells with a reporter plasmid pd-95, the luciferase activity increased (data not shown). Hence, we speculate that Sp1 or Sp1-related factors are involved in the transcriptional activation of Tgase 1 gene. Further studies on the characterization of the signaling system via nPKC η will provide new insights in the control of Tgase 1 gene expression, which will lead to the elucidation of the molecular mechanisms involved in epidermal terminal differentiation and the molecular pathogenesis of keratinizing disorders, including lamellar ichthyosis.
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