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Extracellularly Extruded Syntaxin-4 Is a Potent Cornification Regulator of Epidermal Keratinocytes

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In the skin epidermis, keratinocytes undergo anchorage-dependent cornification, which gives rise to stratified multilayers, each with a distinct differentiation feature. The active formation of the cornified cell envelope (CCE), an important element in the skin barrier, occurs in keratinocytes of the upper epidermal layers and impacts their terminal differentiation. In the present study, we identified the extracellularly extruded syntaxin-4 as a potent differentiation regulator of epidermal keratinocytes. We found that differentiation stimuli led to the acceleration of syntaxin-4 exposure at the keratinocyte cell surface and that the artificial control of extracellular syntaxin-4, either by the forced expression of several syntaxin-4 mutants with structural alterations at the putative functional core site (AIEPQK), or by using antagonistic circular peptides containing this core sequence, dramatically influenced the CCE formation, with spatial misexpression of TGase1 and involucrin. We also found that the topical application of a peptide that exerted the most prominent antagonistic activity for syntaxin-4, named ST4n1, evidently prevented the formation of the hyperplastic and hyperkeratotic epidermis generated by physical irritation in HR-1 mice skin. Collectively, these results demonstrate that extracellularly extruded syntaxin-4 is a potent regulator of CCE differentiation, and that ST4n1 has potential as a clinically applicable reagent for keratotic skin lesions.
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INTRODUCTION

The stratified epidermis is constructed through the outward proliferation of keratinocytes and their successive cornification. Epidermal homeostasis is maintained under the strict control of a position-dependent progression of cornification/differentiation and a certain type of apoptosis (anoikis), which leads to dramatic cell-shape changes along with the ultimate exfoliation of the fully cornified scurf at the outermost skin surface (1–3). Perturbation of the proper cornification program results in a disruption of epidermal homeostasis, causing the onset of skin diseases such as dyskeratosis, atopic dermatitis and psoriasis (2,4–6). The cornified cell envelope (CCE) is an important element of the skin barrier and a major cornification/differentiation indicator in the middle and upper epidermal layers which is formed as a result of the exclusion of the plasma membrane by lamellar bodies containing abundant ceramide, fatty acids and cholesterol (7). CCE formation is preceded by a steep upregulation and accumulation of CCE structural proteins, such as involucrin, loricrin and envoplakin in the proximal membrane region (7,8). These CCE components and certain keratins are then cross-linked by transglutaminases (TGases) so as to reinforce the CCE structure (7,9,10). Given that epidermal keratinocytes lose the intact plasma membrane structure in accord with this cornification process, the cytoplasmic components anchored to the plasma membrane should be included as candidate elements for the differentiation control in the adjacent cells.

Previously, we showed that the keratinocyte cell line HaCaT extruded a small subpopulation of syntaxin-4 on the cell surface and its forcible expression accelerated CCE formation (11). In the same study, we showed that the circular peptide ST4n1 antagonized the effect of exogenous extracellular syntaxin-4. Syntaxin-4 belongs to the t-SNARE protein family, which mediates the fusion of intracellular vesicles with cell membranes and is abundantly expressed in the epithelial compartment of various tissues including the skin (11–13). Extracellular localization of syntaxin-4 is similar to that found for the related protein, epimorphin (known also

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as syntaxin-2), which is produced mainly in the stromal compartment of tissues (14–17) and which can translocate across the membrane in response to external stimuli to exert its latent signaling functions (18–21). Similarly, syntaxin-3 effluent from dying keratinocytes was recently found to mediate its extracellular functions (22). Although syntaxin-4 and epimorphin share secondary and tertiary structures (13,23,24), the effects of these two molecules on CCE formation are apparently the opposite in HaCaT keratinocytes: syntaxin-4 induces, while epimorphin inhibits CCE formation (11). However, the elucidation of their biological relevance and the molecular insights still remain unknown.

In the present study, an effort was made to clarify the extracellular role of extruded syntaxin-4 on epidermal differentiation using both normal human keratinocytes and dyskeratotic mice models. Based on the results, insight was obtained into the biological relevance of extracellularly extruded syntaxin-4 and the possible clinical applications of its antagonistic peptide ST4n1.

MATERIALS AND METHODS

Cells

Normal human epidermal keratinocytes (NHEK) were maintained in Keratinocyte Basal medium2 (KGM2) supplemented with SingleQuots (Lonza, Auckland, New Zealand), as per the manufacturer's protocol. To induce keratinocyte differentiation/cornification, the cells were incubated in KGM2 added with $CaCl_2$ (1 mmol/L) and JNK inhibitor SP600125 (1 μmol/L) for two days, or in KGM2 with the calcium ionophore A23187 (20 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 5 h, as described previously (25,26). Functionally and phenotypically normal HaCaT keratinocytes (a gift from M Manabe of Akita University) and 3T3-derived PT67 packaging cells (Clontech, Mountain View, CA, USA), as well as their derivatives, were maintained in DMEM/HamF12 medium (Wako Chemicals, Osaka, Japan) supplemented with 10% FCS along with penicillin and streptomycin (DH10). The human fetal lung fibroblast cell line MRC-5 was maintained in MEM alpha medium (Wako Chemicals) supplemented with 10% FCS. In some wells of NHEK cells, 50 μg/mL of soluble recombinant syntaxin-4 or GFP (11) (recombinant syntaxin-4 is also commercially available from R&D Systems [Minneapolis, MN, USA]) was added to the cells and cultured for 3 d (for the induction of CCE formation), 1 wk (for quantitative real-time PCR analyses) or 2 wks (for the Western blot analyses of TGase1 and involucrin).

Assessment for Cornified Cell Envelope (CCE) Formation Activity

To induce CCE formation in the cultured keratinocytes, NHEK or HaCaT cells suspended in serum-free medium (200,000 cells/mL) were treated with the calcium ionophore A23187 (20 μg/mL) for 5 h at 37°C, washed with PBS, resuspended in PBS containing 2% SDS and 20 mmol/L DTT and boiled for 10 min as reported previously (11,27,28). The number of the remaining hard-shelled cells due to the abundant CCE was counted and the relative value compared with the control was defined as the CCE formation index.

Immunodetection

Western blotting, immunohistochemistry and immunocytochemistry were performed according to standard protocols. The primary antibodies include those against TGase1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, for Western blotting, and Biomedical Technologies, Heysham, UK, for immunochemistry), involucrin (Santa Cruz Biotechnology), lamin A/C (Cell Signaling Technology, Tokyo, Japan, and Genetex, Irvine, CA, USA), β-actin (Sigma-Aldrich) and syntaxin-4 (11). Western blotting was carried out with appropriate HRP-labeled second antibodies (GE Healthcare, Pittsburgh, PA, USA) and ECL reagent (Invitrogen [Thermo Fisher Scientific Inc., Waltham, MA, USA]). To selectively detect the cell surface subpopulation of syntaxin-4, a medium of nonpermeabilized living keratinocytes on a chamber slide was added with affinity purified anti-syntaxin-4 antibodies (1/100) (11). After incubation for 1 h, the cells were washed twice with Tris-buffered saline (TBS), fixed with –20°C methanol for 10 min and treated with the secondary second antibodies. As the control, a mouse antibody against lamin A/C was also added to the medium. For immunocytochemistry and immunohistochemistry, the Cy3- or FITC-labeled second antibodies (GE Healthcare) were used with the nuclei counterstained with DAPI (Sigma-Aldrich). The cells were analyzed using the A1 confocal microscope system (Nikon, Tokyo, Japan) or the AXIOSHOP fluorescence microscope (Zeiss Japan, Osaka, Japan) with the VB-7010 CCD camera (Keyence, Osaka, Japan).

Plasmids and Transfection

Expression plasmids for the syntaxin-4 mutants StxΔ4, StxG4 and Stx(2)4, which harbor structural alternations at the putative functional core domain (amino acid number from 103 to 108 [aa 103-108]), were generated using extracellular syntaxin-4 (11) as a template. The cDNAs for the N-terminal interleukin-2 (IL-2) signal peptide, followed by the N-terminal portion of syntaxin-4 with its C-terminal modifications (aa 1-102 for StxΔ4, aa 1-108 plus 4× glycine for StxG4 and aa 1-102 plus epimorphin's functional core SIEQSC for Stx(2)4), were generated by PCR using a common forward primer containing an *Eco*RI restriction site and specific reverse primers. The common forward primer was 5′-AAAGAATTCA TGTACAGGATGCAG-3′ and the specific reverse primers were 5′-TTTTAGCTGC GCCCGGACC-3′ (for *STΔ4*), 5′-TCCTC CTCCTCCTTTTAGCTGCGCCCGGAC CTC-3′ (for *StxG4*) and 5′-ACAGCTCTGC TCAATAGATTTTAGCTGCGCCCGGA CC-3′ (for *Stx(2)4*). The cDNA for the

C-terminal portion of syntaxin-4 (aa 109-298) was prepared separately with an *Eco*RI restriction site at the C-terminus by PCR with the primer pair 5′-GCCATAGAGCCCCAGAAG-3′ and 5′-TTTGAATTCTTATCCAACGGTTAT-3′. The cDNAs for the N- and C-terminal portions were blunt ended, phosphorylated at their 5′ ends and ligated using a BRL kit (Takara, Kusatsu, Japan). The full-length cDNA for each syntaxin-4 derivative was then generated by PCR using the primer pair 5′-AAAGAATTCA TGTACAGGATGCAG-3′ and 5′-TTTGA ATTCTTATCCAACGGTTAT-3′, treated with *Eco*RI and cloned into the retroviral expression vector pQCXIN (Invitrogen [Thermo Fisher Scientific]). PT67 packaging cells were transfected with each plasmid using lipofectamine2000 (Invitrogen [Thermo Fisher Scientific]) and subsequently selected for G418 resistance, as described previously (28). To obtain HaCaT cells that stably express each syntaxin-4 derivative, cells were treated with retroviral particles in the supernatant of the PT67 transfectants and grown in the presence of G418 (500 μg/mL).

Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted from NHEK cells cultured with r-GFP or r-Stx4 (50 μg/mL) for 1 wk using an RNeasy mini kit (Qiagen, Tokyo, Japan) and was reverse transcribed with ReverTra Ace (TOYOBO, Osaka, Japan). qRT-PCR was performed using Fast-Start Essential DNA Green Master on LightCycler Nano system (Roche, Basel, Switzerland) according to the manufacturer's protocol. The primer pairs used in this study were 5′-CCAACCGCGA GAAGATGA-3′ and 5′-CCAGAGGCGT ACAGGGATAG-3′ (for *β-actin*), 5′-ATCAATCTCGGTTGGATTCG-3′ and 5′-TCCGCTTGTTGATTTCATCC-3′ (for *keratin1*), 5′-CAGCAGCTGGAGTTT GCTAGA-3′ and 5′-GTCCTTCGGA TGTCCTCACT-3′ (for *envoplakin*), and 5′-CAGACAAGATGTCTTATCAG AAAAAGC-3′ and 5′-GAGGTCTTCA CGCAGTCCA-3′ (for *loricrin*). The expression of the mRNA was normalized to that of β-actin and the relative amount to that from GFP-treated cells was measured for each category. The qRT-PCR analyses were performed three times.

3D Skin-Equivalent Model

The three dimensional (3D) skin-equivalent model was prepared as described previously (28) with a slight modification. In brief, type I collagen solution (Type I-AC, Nitta gelatin) containing MRC-5 cells (68,000 cells) was put into a cell-culture insert of 24 well culture plate (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA). After incubation at 37°C for 1 h, the upper surface of the resultant collagen gel was coated with 50 ng/mL fibronectin (BD), then seeded with HaCaT cells suspended in DH10 medium (10,000 cells/well). This prepared culture assembly was set in a well of 24-well plate containing DH10 medium and incubated for 1 d. The culture medium was then changed to DH10 medium containing hydrocortisone (0.4 μg/mL) (Sigma-Aldrich), gentamycin (100 μg/mL) (Gibco [Thermo Fisher Scientific]), insulin (5 μg/mL) (Sigma-Aldrich) and ascorbic acid (50 μg/mL) (Sigma-Aldrich). After 3 d, the medium in the upper insert was completely removed so that the apical surface of HaCaT cells was exposed directly to the air. In this culture system, HaCaT cells grew upwardly with successive differentiation within 2 wks, which gave rise to the stratified epidermal tissue on the dermis-like collagen gel.

Peptides

The circular peptides for the potential antagonists of extracellular syntaxin-4 were generated by the KNC Laboratories (Kobe, Japan). The putative functional core of syntaxin-4 (AIEPQK) was connected with cysteine (ST4n0), cysteine–glycine (ST4n1) or GABA-cysteine (ST4gaba) at the N-terminus and with cysteine at the C-terminus, followed by the introduction of a disulfide bridge between the N- and C-termini. The purity of all of the peptides was more than 97%, as judged by reverse-phase chromatography, and each peptide was added to the culture at a concentration of 1 μg/mL.

Model Mice and the Topical Administration of ST4n1

To prepare the physical irritation-triggered hyperplastic skin, the outer epidermal layers in the dorsal skin of HR-1 female mice (7-wk-old, Japan SLC, Hamamatsu, Japan) were removed by repeated tape stripping (15 times), as described previously (29,30), and the injured skin regenerated a complete, but hyperplastic, epidermis in 5 d. To test the effect of ST4n1, 100 μL of the peptide (10 μg/mL in 50% ethanol) or placebo (50% ethanol) was applied daily onto five or four mice, respectively, for 5 d. After measurement of the moisture of the skin surface with a skin moisture checker (MY-808S, Scalar Corp., Chigasaki, Japan), all the mice were euthanized and the transverse sections of the skin stained with hematoxylin to measure the thickness of the total epidermis as well as the denucleated horny layer. The transverse skin sections were stained with hematoxylin or DAPI. To prepare the dry skin model, six male HR-1 mice (4 wks old) were fed a low-magnesium diet (Hoshino Laboratory Animals, Bando, Japan) as described previously (31). All of the experimental procedures using mice were approved by the Animal Care Committee of Kwansei Gakuin University.

Statistical Analyses

Results are expressed as the mean ± standard deviation (s.d.) of three independent experiments. Data were analyzed using the t test, and p values <0.05 were considered statistically significant.

All supplementary materials are available online at www.molmed.org.

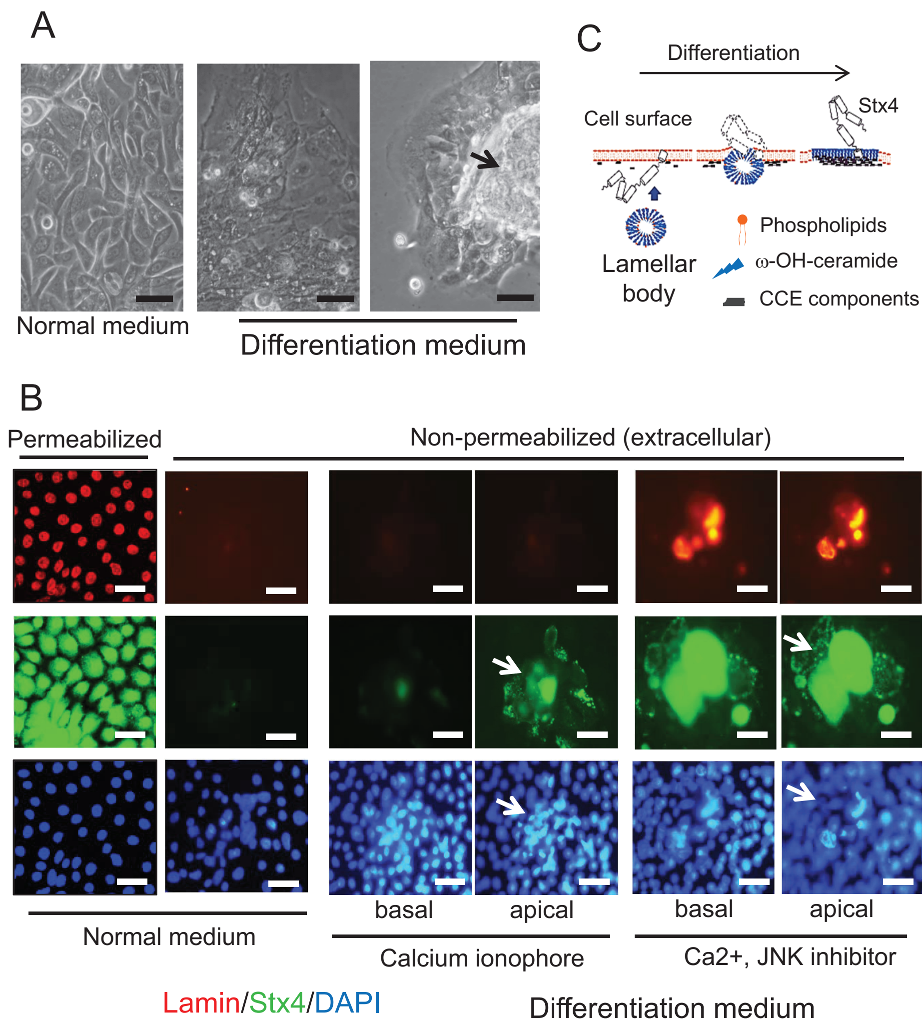

Figure 1. Extracellular presentation of syntaxin-4 in consequence of keratinocyte differentiation. (A) Morphology of NHEK cells in response to the differentiation stimuli. In the presence of the calcium ionophore or $CaCl_2$/JNK inhibitor, NHEK cells exhibited flattened morphology (middle) or underwent stratification (arrow in the right panel). Left, morphology of NHEK cells in the normal medium. Scale bars, 30 μm. (B) Extracellular extrusion of endogenous syntaxin-4. The expression of total (permeabilized) and extracellularly presented (nonpermeabilized) syntaxin-4 (green) was detected in NHEK cells cultured with (differentiation medium) and without (normal medium) differentiation stimuli. As the differentiation medium, the calcium ionophore A23187 or $CaCl_2$/JNK inhibitor SP600125 was added to the medium. A structural protein lamin that underlies nuclear envelope was used for the internal control in the same area (red, upper panels). The nuclei were counterstained with DAPI (lower panels). Arrowheads, stratified cells undergoing terminal differentiation with the disruption of the nuclear structure. The apical and basal planes are shown for stratified cells in the differentiation medium. Scale bars, 30 μm. The signal for syntaxin-4 (Stx4, green), but not for lamin (red), in nonpermeabilized living cells of stratified populations (arrows) was increased upon differentiation induction. (C) A conceptualization of the extrusion of syntaxin-4, depicted along with the well-accepted CCE formation process (7).

RESULTS

Differentiation-Triggered Extrusion of Syntaxin-4 from NHEK Cells

Our previous study identified positive correlation between exogenous extracellular syntaxin-4 and CCE formation in HaCaT keratinocytes (11). To define the extracellular presentation of endogenous syntaxin-4 upon keratinocyte differentiation, NHEK cells were treated with a calcium ionophore or $CaCl_2$/JNK inhibitor, which are known as stimulants of epidermal differentiation/CCE formation (27,28). In response to these differentiation stimuli, NHEK cells became dramatically flattened and partly stratified, confirming the progression of keratinocyte differentiation in culture (Figure 1A). We found that the cell surface presentation of endogenous syntaxin-4 was increased dramatically upon differentiation: extracellularly added antibodies against syntaxin-4 clearly bound to the stratified populations of nonpermeabilized keratinocytes, whereas antibodies against lamin did not bind nuclear component unless cells were permeabilized (Figure 1B). Although antilamin antibodies often labeled the central portion of these piled up cell clusters, the nuclear structure of the cells was almost destroyed, suggesting that these cells were undergoing terminal differentiation (Figure 1B, right panels). Such differentiation-triggered extrusion of syntaxin-4 was confirmed with the anti-T7 monoclonal antibody in HaCaT keratinocytes introduced with syntaxin-4 containing an N-terminal fusion of T7 peptide. Given that ω-OH-ceramides, fatty acids and cholesterol on the lamellae fuse to and push aside the phospholipid cell membrane upon epidermal differentiation/cornification (6,7,32), cytoplasmically oriented syntaxin-4 may become effectively delivered extracellularly in this process so as to be accessible to the adjacent cells (Figure 1C).

Effect of Extracellular Syntaxin-4 on CCE Formation of NHEK Cells

Then, we investigated the effect of extracellularly supplied syntaxin-4 on the

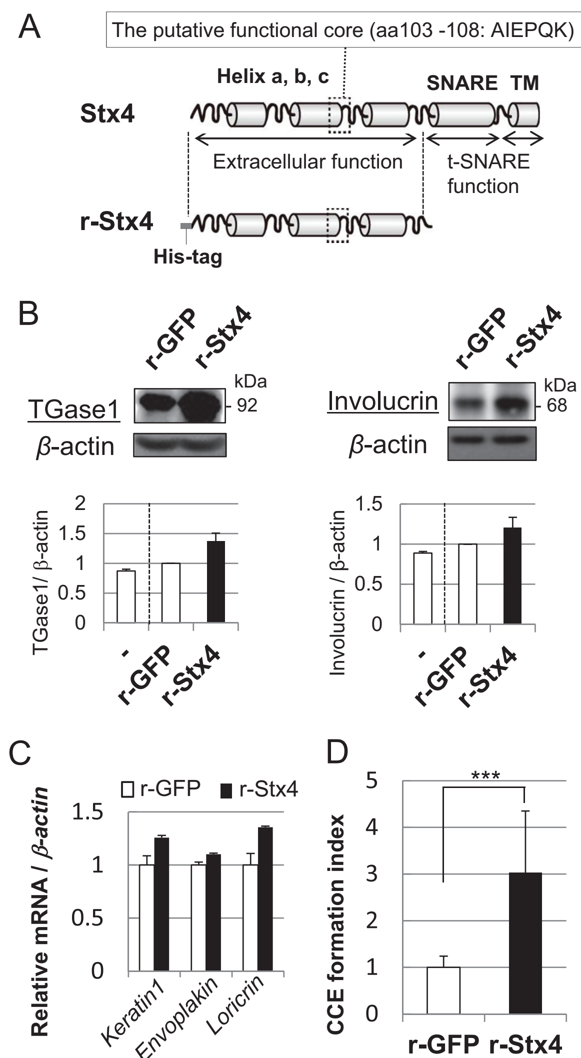

Figure 2. Effect of an extracellular supply of recombinant syntaxin-4 on CCE formation in NHEK cells. (A) Schematic diagrams of syntaxin-4 (Stx4) and its soluble recombinant protein (r-Stx4). Syntaxin-4 consists of an N-terminal unstructured domain, triple helices (helix a, b and c) and SNARE/transmembrane (TM) domain. r-Stx4 has an N-terminal His-tag and lacks the SNARE/TM domain that is required for cytoplasmic t-SNARE function but not the extracellular one. The putative functional core site (AIEPQK) lies in aa 103-108. (B) Expression of TGase1 (left) and involucrin (right) in NHEK cells cultured in the presence or absence of r-Stx4 or recombinant GFP (r-GFP) for two weeks. TGase1 was detected as 92 kDa zymogen complex. Lower, the signal intensity of TGase1 and involucrin relative to that of β-actin revealed the inductive effect of extracellular syntaxin-4. N = 3. (C) Quantitative RT–PCR (qRT–PCR) analyses of mRNA expression of keratin1, envoplakin and loricrin in NHEK cells incubated with r-GFP or r-Stx4 for one week. (D) CCE formation was evidently accelerated in NHEK cells treated with r-Stx4.

differentiation of NHEK keratinocytes. Full-length syntaxin-4 showed poor solubility, likely because of its C-terminal transmembrane and coiled-coil SNARE domains, which have been shown to be dispensable for the extracellular function (11,12). Thus, we prepared a soluble form of recombinant syntaxin-4 (r-Stx4) that lacks these C-terminal domains (Figure 2A). We found that NHEK cells stimulated by r-Stx4 upregulated the expression of involucrin, a major element of CCE, and of transglutaminase1 (TGase1), a key cross-linker protein responsible for the rigid assembly of CCE (Figure 2B). Similarly, we detected upregulation of other CCE components, such as keratin1, envoplakin and loricrin (Figure 2C). Consistent with this notion, stimulation with r-Stx4 facilitated CCE formation in response to the artificial calcium influx (Figure 2D). Such an acceleration of CCE formation by syntaxin-4 also was observed in the cultured embryonic skin, where the nascent epidermal basal layer undergoes successive growth/differentiation so as to give rise to stratified epidermis within a week: the denucleated horny layer became hyperplastic when r-Stx4 was supplied in the extracellular medium. By contrast, the epidermal thickness often became thinner and the formation of the horny layer was severely impaired in the presence of anti-syntaxin-4 antibodies (Supplementary Figure S1).

Conformation of the Putative Functional Core is Important for CCE Formation Activity

Our previous study implicated that the inductive activity necessary for CCE formation depends on the tertiary structure of the putative functional core site (AIEPQK) in syntaxin-4 (11,12). To verify this, we generated keratinocytes stably expressing several different syntaxin-4 mutants at the cell surface. The mutations include the removal of the functional core (StxΔ4), the insertion of 4× glycine residues between the core site and the flanking C-terminal domain (StxG4), and the replacement of the core site with that of epimorphin (SIEQSC) (Stx(2)4). All of these syntaxin-4 mutants, as well as intact syntaxin-4, were connected with a signal peptide (14) for the effective delivery to the cell surface through the ER/Golgi secretory pathway (Figure 3A). Since the isolation of primary keratinocytes with the stable expression of transgenes was not feasible, we used immortal HaCaT keratinocytes, which have been shown to possess normal differentiation potential

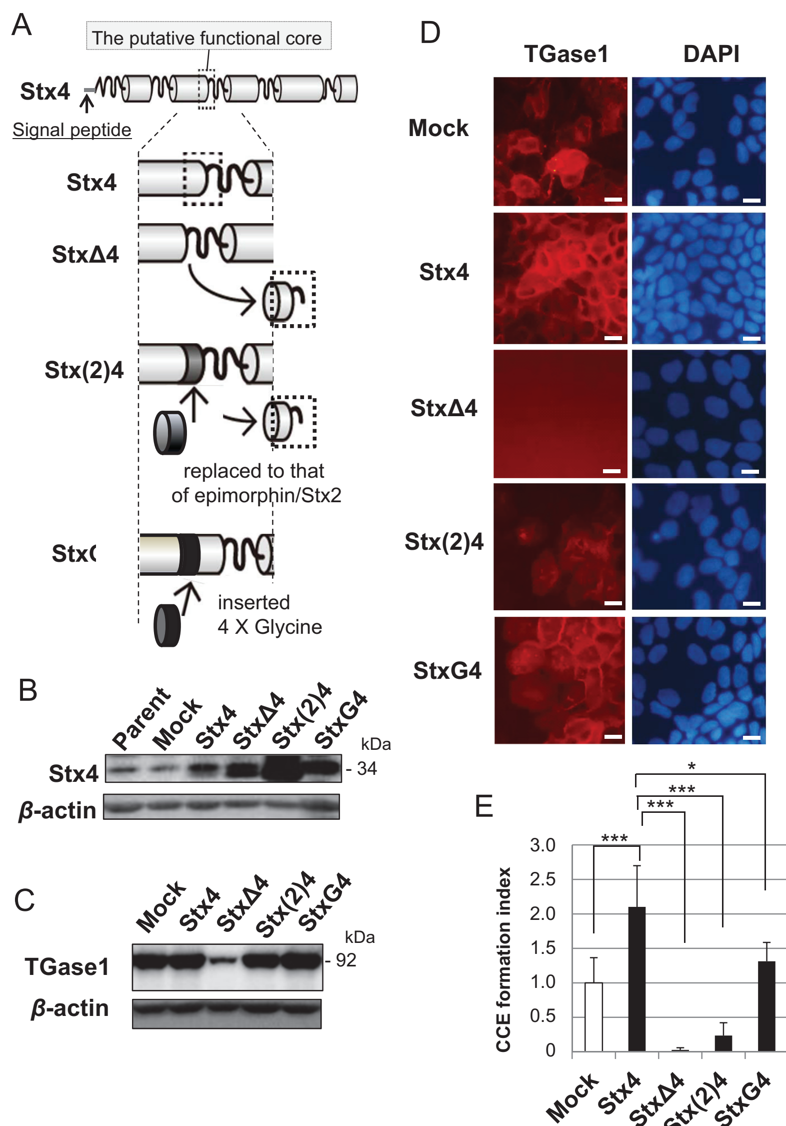

Figure 3. Cell surface expression of syntaxin-4 mutants impacted CCE formation in HaCaT keratinocytes. (A) Schematic diagram of the syntaxin-4 mutants for the expression on the outer surface of keratinocytes. StxΔ4, removal of the putative functional core site (AIEPQK); StxG4, insertion of four glycine residues at the C-terminus of the functional core; Stx(2)4, replacement of the functional core with that of epimorphin/syntaxin-2 (SIEQSC). The signal peptide of mouse IL-2 was connected to all of these syntaxin-4 mutants, as well as the intact form of syntaxin-4, for the effective delivery to the cell surface and the sensitive detection, respectively, as described (14). (B) Expression of the syntaxin-4 derivatives (34 kDa) in HaCaT cells. All the syntaxin-4 derivatives were detected as a 34 kDa protein band with anti-syntaxin-4 antibodies. Parent, untransfected HaCaT cells. Mock, HaCaT cells transfected with an empty vector. β-actin, loading control. (C) Expression of TGAse1 in HaCaT cells expressing the transgenes. β-actin, loading control. (D) Expression pattern of TGAse1 (red in the left panels) in HaCaT cells with the syntaxin-4 derivatives. The nuclei were counterstained with DAPI (right panels). Scale bar, 10 μm. Accumulation of TGase1 at the membrane-proximal site was apparent in cells with the forcible expression of extracellular syntaxin-4, but not with syntaxin-4 mutants. (E) Quantitative analyses of CCE formation activity in cells expressing the syntaxin-4 derivatives in response to calcium influx. Forcible expression of extracellular syntaxin-4 accelerated CCE formation, while this activity was abrogated when the putative functional core site was mutated. **, $p < 0.05$, N = 5.

in terms of the cornification/stratification (11,33). We found that HaCaT cells stably introduced with these transgenes successfully expressed these syntaxin-4 derivatives (Figure 3B) and that StxΔ4 dramatically downregulated TGase1, whereas the other syntaxin-4 derivatives had much less effect on the production of this CCE-cross-linker as compared with intact syntaxin-4 (Figure 3C). However, while the forced expression of intact syntaxin-4 induced the accumulation of TGase1 at cell membrane-proximal sites, its spatial expression pattern was severely perturbed to a cytoplasmically diffuse pattern in cells treated with Stx(2)4 (Figure 3D). Consistently, the overexpression of extracellular syntaxin-4 dramatically increased CCE formation in response to the calcium influx, whereas all the other syntaxin-4 mutants lost this activity (Figure 3E). Of note, the CCE formation was severely suppressed by StxΔ4 and Stx(2)4 as compared with mock-transfected cells, suggesting that syntaxin-4 with an abnormal functional core hinders the function of the endogenous syntaxin-4 extruded at the cell surface. Indeed, these two syntaxin-4 mutants appeared to adhere to keratinocytes with a relatively high affinity (Supplementary Figure 2).

Circular Peptides Generated from the Functional Core of Syntaxin-4 as Potential Antagonists of Extracellular Syntaxin-4

Given that the tertiary structure of the functional core appears critical for the exertion of syntaxin-4 activity, we next tested the antagonistic potential of several AIEPQK-containing circular peptides with different diameters (Figure 4A). We confirmed that one of these peptides, ST4n1, significantly decreased the CCE formation in HaCaT cells expressing extracellular syntaxin-4, as has been shown previously (11). On the other hand, ST4 n0, the peptide with the sharp curve, appeared to produce a weaker response, and ST4gaba that possesses a gentler curve had no impact (Figures 4B, C). Importantly, such a curvature-dependent antagonistic effect was also reproducibly ob-

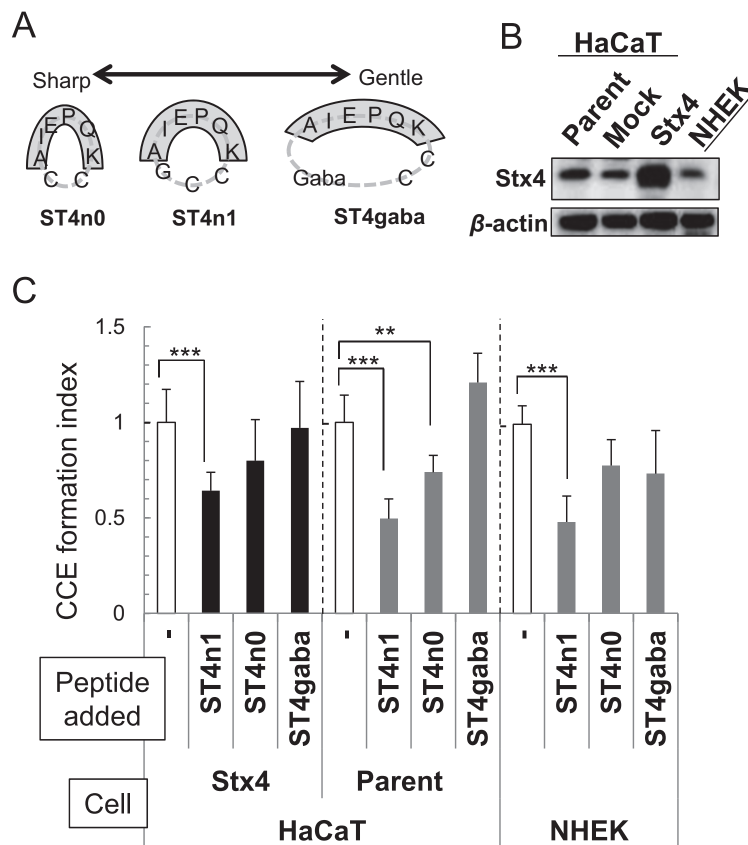

Figure 4. Effect of peptide antagonists on syntaxin-4 dependent CCE formation. (A) Circular peptides used in this study. Each peptide was generated with cysteine (ST4n0), cysteine-glycine (ST4n1) or cysteine-gaba (ST4gaba) at the N-terminus of AIEPQKC sequence, followed by the introduction of an intramolecular disulfide bridge. (B) Expression of syntaxin-4 in HaCaT cells with an empty vector (mock), the syntaxin-4 expression plasmid (Stx4), the parental HaCaT (parent) or primary cultured keratinocytes (NHEK). β-actin, loading control. Both NHEK and HaCaT cells expressed endogenous syntaxin-4. (C) Effect of peptides on the CCE formation in NHEK and HaCaT cells with (Stx4) and without (parent) forcible expression of extracellular syntaxin-4. The CCE formation index was normalized for each cell type. The antagonistic effect of ST4n1 was statistically significant. ***, $p < 0.01$, **, $p < 0.02$, N = 5. A strong inhibitory effect of ST4n1 on endogenous syntaxin-4 was also evident in NHEK and parental HaCaT cells.

served in NHEK keratinocytes as well as untransfected HaCaT cells, demonstrating that ST4n1 antagonizes the endogenous syntaxin-4 extruded by human epidermal keratinocytes, as well (see Figure 4C).

Altered Localization of TGase1 in the Stratified Keratinocytes Induced by Forcible Expression of Extracellular Syntaxin-4 is Reverted by ST4n1

We next analyzed the effect of the forcible expression of extracellular syntaxin-4 and the antagonistic effect of ST4n1 in the stratified epidermis. For this purpose, we prepared skin equivalent 3D models using immortal HaCaT keratinocytes with or without exogenous extracellular syntaxin-4 as an epidermal component. Transverse sections with hematoxylin staining indicated that the forcible expression of syntaxin-4 resulted in hyperplasia in the denucleated epidermal layer, which was prone to being antagonized by ST4n1 (Figure 5A). Consistent with this, the restricted expression pattern of TGase1 in the middle and upper layers expanded, even to the basal layer, when an excess amount of syntaxin-4 was expressed on all of the keratinocytes (Figures 5A, B). Intriguingly, such spatial misexpression of TGase1 was essentially normalized when ST4n1 was also present (Figures 5A, C), suggesting that hypercornification was induced by extracellular syntaxin-4 and that such a spatially disrupted differentiation was restored, at least in part, by ST4n1 in the stratified epidermis.

ST4n1 Remedies Hyperkeratosis in the Epidermis of HR-1 Mice

We finally tested the clinical application potential of ST4n1. In response to physical irritation of the epidermis by repeated tape stripping, HR-1 hairless mice regenerate a complete, but dramatically thickened epidermis in a period of several days with loss of skin moisture (Figures 6A, B). We found that the thickness of the hyperplastic epidermis became dramatically reduced with restoration of the skin moisture when the irritated area was topically applied with ST4n1 (Figures 6A–C). Consistent with this, the thickness of both the total epidermis and the horny layer became robustly normalized by the ST4n1 administration (Figure 6D). In addition, ST4n1 appeared to partly remedy the abnormal features that appeared in the dry skin of the epidermis generated with the low-Mg diet: the appearance of DAPI-positive/hematoxylin-negative cells in the lower epidermal layers was dramatically reduced by ST4n1 (Supplementary Figure S3). These results suggest that topical application of the ST4n1 peptide affords protection against keratotic lesions in the epidermis.

DISCUSSION

While the role of cytoplasmic syntaxin-4 in vesicular fusion has been investigated extensively, here, we studied its cell surface expression pattern and uncovered a critical role in epidermal differentiation. As in the case of other

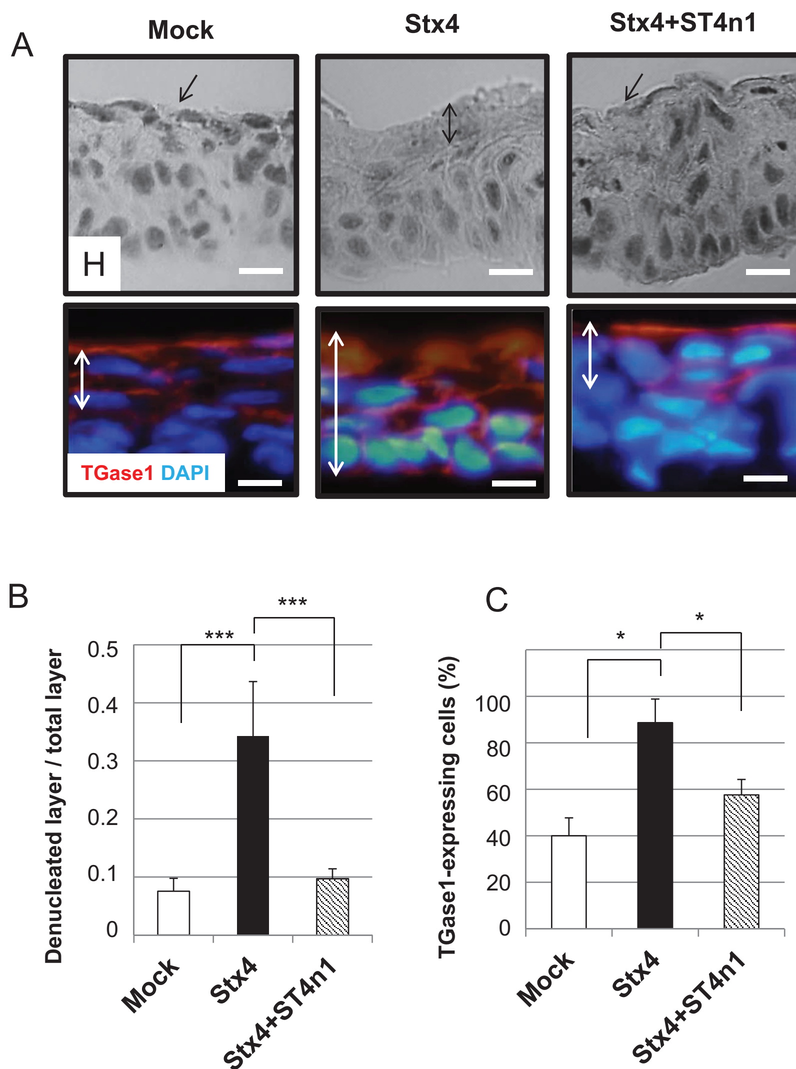

Figure 5. Effect of the extracellular syntaxin-4 and its peptide antagonist ST4n1. (A) Cryosections of 3D-skin equivalent model consisting HaCaT keratinocytes and fibroblastic MRC-5 cells were stained with hematoxylin (upper) or for TGase1 (lower). Overexpression of extracellular syntaxin-4 resulted in the increase of the number of denucleated (black arrow) and TGase1-expressing (white arrow) cells as compared with those with empty vector (Mock). The abnormal phenotype induced by syntaxin-4 was practically neutralized by the addition of ST4n1 (Stx4 + ST4n1). Scale bar, 10 μm. (B) Quantification of thickness of denucleated upper cell layer in the 3D model. Thickness of the denucleated and total cell layers was measured in three random areas and the relative values to Mock-control were calculated. ***; $p < 0.01$, N = 4. (C) Quantification and statistical analyses of TGase1-positive cell populations in the 3D-model. Percentage of TGase1-expressing cell number was shown for each category. *; $p < 0.05$, N = 4.

plasmalemmal syntaxins, epimorphin translocates across the plasma membrane via a nonclassical secretion pathway that is utilized by other leaderless proteins, such as FGF or IL1β (20). We also detected extracellularly effluent syntaxin-3 from keratinocytes undergoing necrotic/apoptotic cell death (22). Although these syntaxins may execute distinctive functions after being exposed extracellularly, they have been commonly shown to elicit survival activity from adjacent cells (11,12,22,34). This function may account for the thickened lower epidermal layers in organ culture and the mice model, the cells of which otherwise progress with an active anoikis program, which may be often driven by the extracellular expression of syntaxin-4. In the later differentiation stages, the keratinocyte cell membrane becomes gradually replaced by a lining of CCE (7), which may provide a means for the active extrusion of membrane-tethered cytoplasmic syntaxin-4. In accord with this, the induction of CCE-formation led to an increased exposure of syntaxin-4 at the cell surface, which in turn appears to support CCE formation in adjacent cells, thereby regulating epidermal cornification.

In the keratinocyte model with HaCaT cells, expression of syntaxin-4 mutants with structural alterations at their functional core site showed loss of their original activity. However, one lacking the functional core (StxΔ4) was selectively downregulated, whereas another possessing with the epimorphin functional core (Stx(2)4) dramatically perturbed the localization of TGase1, both of which induced the severe defect in CCE formation as compared with mock control. The fact that extracellular epimorphin abolished CCE formation (27,35) may account only for the latter case: the functional core of epimorphin may confer the characteristic activity of epimorphin upon syntaxin-4. By contrast, the mechanism for impairment of CCE formation by the overexpression of StxΔ4 is not as clear, except for a dominant-negative effect on syntaxin-4. While it is possible that StxΔ4 associates with endogenous syntaxin-4 via the coiled-coil motifs so as to hinder its activity, or with epimorphin to potentiate its inhibitory effect on CCE formation, further investigation is obviously needed to clarify this issue.

ST4n1, but not ST4 gaba, antagonizes the function of endogenous syntaxin-4,

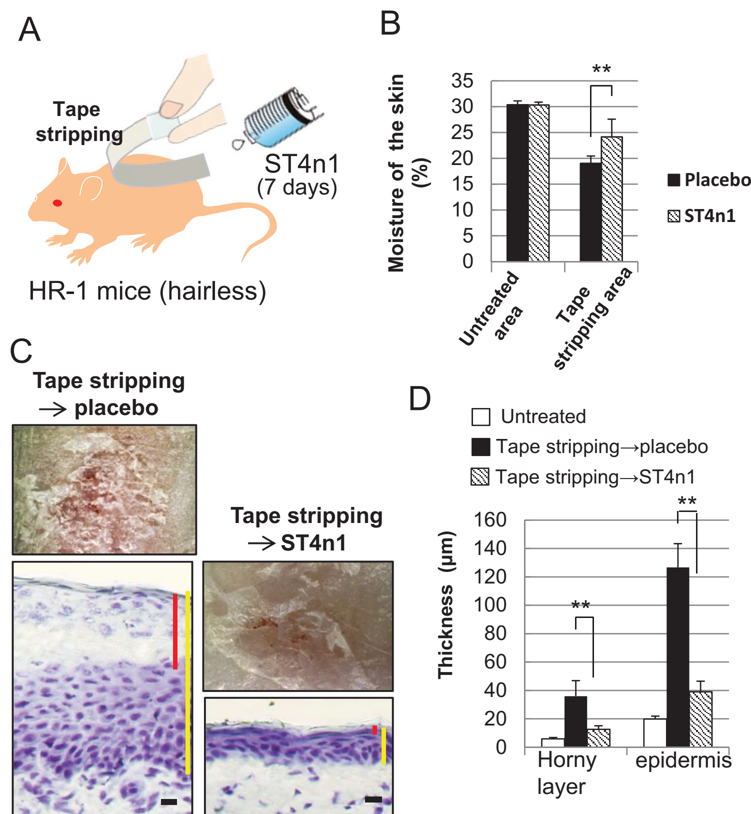

Figure 6. Effect of ST4n1 in epidermal keratosis generated with physical irritation in HR-1 mice. (A) Schematic diagram of the experimental procedure. After repeated tape-stripping, ST4n1 or Placebo sample was daily applied onto the irritated area. (B) The moisture of the skin from untreated or tape-stripped areas, latter of which were applied with ST4n1 or placebo, was measured after five days. Placebo, N = 4; ST4n1, N = 5. **, $p < 0.02$ ST4n1 prevented water loss from the skin that has been injured by tape stripping. (C) Typical skins (upper) and hematoxylin-stained transverse sections (lower) of the areas that have been physically irritated by tape-stripping, followed by topical application of St4n1 (right) and placebo (left). Yellow and red bars in lower images represent epidermis and its horny layer, respectively. Scale bar, 10 µm. (D) Thickness of the epidermis and the horny layer of the skin. Placebo, N = 4; ST4n1, N = 5; Untreated, those of untreated mice (N = 5). **, $p < 0.02$. ST4n1 normalized stratification and cornification of epidermis in the regenerated skin from the physical damage.

confirming the importance of three dimensional structure of the functional core site. The importance of the corresponding site has been revealed equiponderant also in epimorphin, albeit its effect in CCE formation may be completely opposite (11). Consistent with their complementary role, ST4n1 also exerts the opposite effect as EPn1, the antagonistic peptide generated from the functional core of epimorphin (27). Then how are these proteins from the same family, as well as their antagonistic peptides, able to have such different effects? ST4n1 and EPn1 are comprised of different numbers of distinct peptide motifs, suggesting the existence of a specific receptor for the extracellular forms of syntaxin-4 and epimorphin, each of which propagates a specific signaling pathway. On the other hand, antibodies against integrins blocked the keratinocyte adherence to both epimorphin (18–20) and syntaxin-4 (data not shown). Thus, it is conceivable that keratinocytes possess receptor complexes for these syntaxins, in which integrins mediate recognition of both syntaxins, while as-yet-unidentified molecules elicit distinct cellular responses.

CONCLUSION

This study shows that extracellularly extruded syntaxin-4 plays a causal role in CCE formation in keratinocytes and that the syntaxin-4 antagonist ST4n1 may have utility for certain keratotic skin lesions. Together with the previously reported finding that another family member, epimorphin, elicits the opposite cellular response in CCE formation, epidermal differentiation may be critically controlled by extracellularly extruded populations of syntaxin family proteins.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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