Up-regulation of Loricrin Expression by Cell Adhesion Molecule Nectin-1 through Rap1-ERK Signaling in Keratinocytes*

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Nectin is an immunoglobulin-like cell-cell adhesion molecule, which plays essential roles in the initial step of formation of adherens junctions and tight junctions. We demonstrate here the role of nectin-1 in the epidermis using nectin-1-/- mice. Newborn nectin-1-/- mice showed shiny and slightly reddish skin; the amount of loricrin, one of the differentiation markers and also a major component of cornified cell envelopes, was markedly reduced in the epidermis of nectin-1-/- mice. The amounts of repetin and SPRRP, other components of cornified cell envelopes, were markedly elevated probably due to a compensatory mechanism to overcome the impaired expression of loricrin. However, cornified cells from nectin-1-/- mice were sensitive to mechanical stress. Moreover, Ca2+-induced activation of ERK through Rap1 and expression of loricrin were sensitive to mechanical stress. Moreover, Ca2+-induced activation of ERK through Rap1 and expression of loricrin were reduced in primary cultured nectin-1-/- keratinocytes; in turn, the inhibition of ERK activation reduced the amount of loricrin in wild-type keratinocytes. These results indicate that nectin-1 plays a key role in the expression of loricrin in the epidermis.

The mammalian epidermis, the outermost surface of the body, consists of a multilayered epithelium (the interfollicular epidermis) and appendages including hair follicles and sebaceous and sweat glands (1). The epidermis comprises four stratified layers, including basal, spinous, granular, and cornified layers, and is a continuously renewing tissue composed of keratinocytes at diverse stages of differentiation (2). The cells in the basal layer of the epidermis proceed to terminal differentiation upon leaving the basal layer. Some members of the integrin family, such as integrin α6, have been reported to be involved in this terminal differentiation (3), but the molecular mechanism of terminal differentiation is largely unknown.

The main functions of the epidermis, including acting as a barrier against the external environment and preventing water loss from the inside body, are considerably dependent on the existence of adherens junctions (AJ)s2 and tight junctions (TJs) in the epidermis (4, 5). The functions of AJs and TJs have been well characterized in single layered epithelial cells. AJs serve as a mechanically adhesive apparatus between neighboring cells, whereas TJs act as barriers to prevent leakage of molecules through the gaps between cells and also function as fences to keep the cell surface molecules at the basolateral region separate from those at the apical region (4, 5). In epithelial cells, the formation of TJs always occurs at the apical side of AJs and mainly depends on the formation of AJs. The major cell-cell adhesion molecules (CAMs) at AJs are cadherin and nectin (6, 7), and those at TJs are claudin, occludin, and junctional adhesion molecule (JAM) (4, 5). It has been believed for a long time that only fragmented strands of TJs exist in a part of the granular layer and that they do not contribute to the epidermal barrier function (8). The results obtained with claudin-1-/- mice have revealed that there are continuous claudin-based TJ strands in the granular layer, which are indispensable for the barrier function of the mammalian skin (9). Moreover, recent studies on the epidermis-specific loss of E-cadherin, one of CAMs at AJs, have displayed the epidermal abnormalities in the conditional knock-out mice (10, 11). Thus, the components of AJs and TJs may play important roles in the maintenance of epidermal functions.

Nectin is an immunoglobulin-like CAM that comprises a family of four members: nectin-1, nectin-2, nectin-3, and nectin-4 (7, 12–14). Each member has one extracellular region with three immunoglobulin-like loops, one transmembrane region, and one intracellular region. Each nectin forms homo- or hetero-dimers and then homo- or hetero-trans-dimers (trans-interactions) in a Ca2+-independent manner to achieve cell-cell adhesion. At its cytoplasmic tail, nectin binds afadin, which connects nectin to the actin cytoskeleton. Recent evidence has shown that nectin first trans-interacts with each other and then recruits cadherin to the nectin-based cell-cell contact sites.

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‡ The abbreviations used are: AJ, adherens junction; Ab, antibody; CAM, cell-cell adhesion molecule; JAM, junctional adhesion molecule; mAb, monoclonal antibody; pAb, polyclonal antibody; SPRRP, small proline-rich protein; TJ, tight junction; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
leading to the formation of AJs (7, 12–14). Moreover, nectin recruits first JAM and then claudin and occludin to the apical side of AJs, resulting in the formation of TJ (15–17). Physiological roles of nectin have been reported in a variety of tissues, such as the brain, the testis, and the eye (18–24).

We have shown that normal human epidermis expresses nectin-1 at the cell-cell junctions and that nectin-1 colocalizes with E-cadherin there (25). The expression of nectin-1 at cell-cell junctions was reduced in human epithelial cancer cells located at the advancing border of the tumor, losing the cell-cell junctions and facilitating the invasion of cancer cells into the neighboring tissue. Mutations in human nectin-1 are also responsible for cleft lip/palate, and, in some cases, mental retardation, clinically characterized by unusual faces, dental anomalies, hypotrichosis, palmoplantar hyperkeratosis and onychodysplasia, syndactyly, cleft lip/palate, and, in some cases, mental retardation. These results strongly suggest that nectin-1 plays important physiological and pathological roles in the formation and maintenance of cell-cell junctions in the epidermis.

To further explore the role of nectin-1 in morphology of the epidermis, we first examined the phenotypes of the epidermis in wild-type and nectin-1−/− mice and then analyzed the difference of their phenotypes on the molecular level using keratinocytes prepared from both types of mice.

**EXPERIMENTAL PROCEDURES**

**Generation of Nectin-1−/− Mice—**Nectin-1−/− mice were generated as described previously (22). In brief, nectin-1+/− mice with mixed genetic background were back-crossed at least six times onto the C57BL/6 strain and were then intercrossed to obtain nectin-1−/− mice. Experiments were performed on these nectin-1−/− mice and littermate nectin-1+/+ (wild-type) mice.

The animals and procedures used in this study were in accordance with the animal care guidelines of and approved by the Osaka University Medical School Animal Care and Use Committee.

**Antibodies—**A rabbit anti-nectin-1 polyclonal antibody (pAb), which recognizes the intracellular region of both mouse and human nectin-1, was prepared as described (25, 29). Rat anti-nectin-1 and rat anti-nectin-2 monoclonal antibodies (mAbs), both of which recognize the extracellular regions of nectin-1 and nectin-2, respectively, were prepared as described (18, 29). A rat anti-E-cadherin (ECCD2) mAb was kindly supplied by Dr. M. Takeichi (RIKEN, Kobe, Japan). All of the following Abs were purchased from commercial sources: a rabbit anti-mouse keratin 5 pAb (Covance), a rabbit anti-mouse keratin 10 pAb (Covance), a rabbit anti-filaggrin pAb (Covance), a rabbit anti-involucrin pAb (Covance), a rabbit anti-laminin pAb (Sigma), a rabbit anti-nectin pAb (Covance), a mouse anti-Ki-67 mAb (Novocastra), a mouse anti-phospho-ERK1/2 mAb (Cell Signaling Technology and Sigma), a mouse anti-ERK1/2 mAb (Zymed Laboratories Inc.), a mouse anti-phospho-p38 mitogen-activated protein kinase (MAPK) mAb (Cell Signaling Technology), a rabbit anti-p38 MAPK pAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a rabbit anti-JNK pAb (Cell Signaling Technology), a mouse anti-phospho-JNK mAb (Cell Signaling Technology), a rabbit anti-Rap1 pAb (Santa Cruz Biotechnology), and a mouse anti-Ras mAb (Upstate Biotechnology).

**Immunoblot Analysis—**As described previously (30), the epidermis was homogenized in a lysis buffer (0.1 M Tris hydroxymethyl aminomethan-HCl (pH 9.0), 8 M urea, 1% 2-mercaptoethanol, 1% SDS, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged (15,000 × g for 15 min). The supernatant was used for immunoblotting. Primary keratinocytes were collected by scraping into Buffer A (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride). The cells were lysed by sonication on ice and centrifuged (15,000 × g for 15 min). The supernatant was used for immunoblotting. Equal amounts of protein in each sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were immunoblotted with the primary Ab, followed by the horseradish peroxidase-conjugated secondary Ab (Amersham Biosciences). Blots were developed using the ECL kit (Amersham Biosciences). Densitometric analysis was performed using Image software (National Institutes of Health), and paired Student’s t tests were performed for statistical analysis.

**Sonication Experiment, Barrier Function, and Water Loss Assays—**For the sonication experiment, cornified cells were prepared as described (31). Isolated cornified cells were suspended in 2% SDS solution and sonicated with a TAITEC sonicator for 15 min. The skin barrier assay was performed as previously described (31, 32). Briefly, after the backs of newborn pups were soaked in 1 mM lucifer yellow solution for 1 h, the pups were killed, and frozen back skin sections were prepared as described above. The penetration of the dye was analyzed by immunofluorescence microscopy. For the water loss assay, the weight of newborn pups was monitored at the indicated time points for 24 h without feeding the pups. Data are presented as the percentage of initial body weights.

**Reverse Transcription PCR—**Total mRNA was extracted from the epidermis of newborn mice using ISOGEN (Wako). The extracted total mRNA was treated with the RQ1 RNase-free DNase (Promega). For semiquantitative reverse transcription PCR, first strand cDNA synthesis was performed using the Ready To Go reverse transcription-PCR beads (Amersham Biosciences) with the pd(N)6 primer. Second strand cDNA synthesis and PCR were carried out using ExTaq polymerase (Takara). The loricin, SPRRP2A, SPRRP2D, repetin, and GAPDH primers were as follows: loricin, aacagctactgctgccagc (forward) and tctgacttgtctgcagag (reverse); SPRRP2A, cttggacctgaatctggtgta (forward) and acaagtcccagactcttcgag (reverse).
gatgcttactacc (forward) and ttttcttgaggagccatcaggc (reverse); SPRRP2D, acccgatctggaatcagcagc (forward) and tttgcttgagctgtcagagc (reverse); repetin, atctcctctgtcctgact (forward) and ttggcttgatatttcttgactttcctcata (reverse); GAPDH, ccatacctatctcagga (forward) and ttgtatcctcaggagata (reverse). PCR was performed with cycles at 96 °C for 15 s, 58 °C for 15 s, and 72 °C for 60 s. PCR products were collected every five cycles from 25 to 35 cycles and examined by electrophoresis on 2% agarose gels. For quantitative real time PCR, we used TaqMan probes and the Applied Biosystems Prism 7900HT Sequencing Detection System (Applied Biosystems). The repetin and GAPDH TaqMan probes, primer mix, and TaqMan Universal PCR master Mix were purchased from Applied Biosystems. PCR was performed with 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After 40 cycles, data were processed using the software accompanying the Applied Biosystems Prism 7900HT Sequencing Detection System. The threshold cycle was automatically determined by the software. The mRNA level of repetin was normalized to that of GAPDH.

**Primary Culture of Mouse Keratinocytes**—Mouse keratinocytes were cultured as described (33). Briefly, full-thickness skin taken from newborn mice was treated with 10 mg/ml dispase (Invitrogen) overnight at 4 °C. The epidermis was peeled off the dermis, followed by trypsinization to isolate mouse keratinocytes. Collected mouse primary keratinocytes were cultured in MCDB153HAA medium (Nihon Pharmaceutical Co.) supplemented with insulin (5 µg/ml), hydrocortisone (0.5 mM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), and bovine hypothalamic extract (150 µg/ml). After keratinocytes reached confluence, the Ca²⁺ concentration in the culture medium was increased to 2 mM to induce terminal differentiation. To inhibit phosphorylation of ERK, 10 µM U0126 (Sigma) was added to the high Ca²⁺ medium. Recombinant adenovirus encoding Rap1GAPII was obtained from S. Hattori (University of Tokyo). To examine the effect of Rap1 on the phosphorylation of ERK, keratinocytes were infected by adenoviruses encoding Rap1GAPII or β-galactosidase as a control at 24 h before the Ca²⁺ switch. For the rescue experiment, the plasmid of FLAG-tagged human nectin-1 (amino acids 27–518; pFLAG-CMV1-nectin-1) was transfected into nectin-1⁻/⁻ keratinocytes using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions.

**Pull-down Assay for Rap1 and Ras**—The pull-down assay was performed as described (34). In brief, keratinocytes, with or without the Ca²⁺ switch, were washed with 1 ml of ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, lysed in Buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate) containing 10 µg of GST fusion proteins, GST-RalGDS-RBD (the Ras binding domain of Ral GDP dissociation stimulator fused to GST) for Rap1, or GST-Raf-RBD+CRD (the Ras binding and cysteine-rich domains of Raf-1 fused to GST) for Ras, and incubated at 2 °C for 30 min. The cell extract was obtained by centrifugation at 20,000 × g at 0 °C for 5 min and incubated with 50 µl of glutathione-agarose beads (Amersham Biosciences) at 2 °C for 1 h. After the beads were washed with Buffer B, proteins bound to the beads were eluted with Laemmli buffer and subjected to SDS-PAGE, followed by Western blotting using the anti-Rap1 pAb or the anti-Ras mAb.

**RESULTS**

**External Appearance in Nectin-1⁻/⁻ Mice**—The external appearance of newborn pups was different between wild-type and nectin-1⁻/⁻ mice. As shown previously (22), ~30% of nectin-1⁻/⁻ pups were born with one or both eyes open. In addition to this phenotype, nectin-1⁻/⁻ pups had shiny and slightly reddish skin compared with wild-type pups (Fig. 1, A and B). Although this difference in skin tone was apparent at birth, nectin-1⁻/⁻ mice at postnatal day 7 were indistinguishable from wild-type mice in terms of the external appearance with normal hair coat (data not shown), suggesting that some compensatory mechanisms may exist in the skin.

**Alternation in Terminal Differentiation of the Epidermis in Nectin-1⁻/⁻ Mice**—To clarify the cause of the skin phenotype of nectin-1⁻/⁻ newborn mice, epidermal differentiation markers were immunostained. Prior to this, we confirmed the expression pattern of nectin-1 in the epidermis. In wild-type mice, nectin-1 was expressed from the basal layer to the cornified layer (Fig. 2A), whereas in nectin-1⁻/⁻ mice, nectin-1 was not observed in the epidermis except for nonspecific staining in the cornified layer (Fig. 2A). The epidermis of the nectin-1⁻/⁻ mice showed lower expression of loricrin than that of wild-type mice (Fig. 2B), although there was no significant difference in the expression of other differentiation markers, such as keratin 5, keratin 10, involucrin, and filaggrin, between wild-type and...
nectin-1<sup>-/-</sup> mice (data not shown). We also determined the expression of each differentiation marker in the epidermis by Western blotting. Consistent with the results from immunofluorescence microscopy, the expression of loricrin was markedly reduced in nectin-1<sup>-/-</sup> mice (Fig. 2C), whereas those of keratin 5, keratin 10, involucrin, and filaggrin were indistinguishable between wild-type and nectin-1<sup>-/-</sup> mice (data not shown).

It has been shown that nectin is necessary for the recruitment of E-cadherin to the nectin-based cell-cell adhesion sites and is involved in the formation of E-cadherin-based AJs (7). Using immunofluorescence microscopy, we then examined whether the expression pattern of E-cadherin is affected in the epidermis of nectin-1<sup>-/-</sup> mice. The expression pattern of E-cadherin was not changed in the epidermis of nectin-1<sup>-/-</sup> mice (Fig. 3A).

This prompted us to assume that other nectin family members may compensate for the deficiency of nectin-1 in the formation of E-cadherin-based AJs. Therefore, we examined the expression of nectin-2, nectin-3, and nectin-4 in the epidermis. Although the expression of nectin-3 was not detected in wild-type or nectin-1<sup>-/-</sup> mice (data not shown), the epidermis of wild-type and nectin-1<sup>-/-</sup> mice expressed nectin-2 (Fig. 3B) and nectin-4 (data not shown) to a similar extent. These results may explain why the formation of E-cadherin-based AJs is not impaired in nectin-1<sup>-/-</sup> mice.

We also examined whether the lack of nectin-1 affects the proliferation ability of the epidermis. To assess this, the epidermis of wild-type and nectin-1<sup>-/-</sup> mice was stained with the anti-Ki-67 mAb. Ki-67 is a proliferative marker. Staining with the anti-Ki-67 mAb in the epidermis of nectin-1<sup>-/-</sup> mice was similar to that of wild-type mice (Fig. 3C). The basal layer of wild-type epidermis had 14% Ki-67-positive cells per basal cell, whereas the basal layer of nectin-1<sup>-/-</sup> epidermis had 16% Ki-67-positive cells. No suprabasal staining with the anti-Ki-67 mAb was observed in either strain of mice. Collectively, these results indicate that nectin-1 plays a role in the regulation of terminal differentiation but not proliferation.
Mechanical Property and Barrier Function of the Epidermis in Nectin-1<sup>-/-</sup> Mice—To test whether the reduced expression of loricrin, a major component of the epidermal cornified cell envelope, in the nectin-1<sup>-/-</sup> epidermis affects the resistance to mechanical stress, we isolated cornified cells from wild-type and nectin-1<sup>-/-</sup> mice and exposed them to ultrasound. The morphology of both types of cornified cells was similar before the ultrasound procedure, whereas the cells from nectin-1<sup>-/-</sup> mice were more severely damaged by ultrasound than those from wild-type mice (Fig. 4), leading to the notion that the nectin-1<sup>-/-</sup> epidermis is likely to be sensitive to mechanical stress.

We then examined whether the cornified layer is functionally intact in nectin-1<sup>-/-</sup> mice. To assess the permeability of the epidermis, we performed the dye penetration assay using Lucifer yellow dye and examined the water loss by measuring their epidermis weight in newborn pups (11, 35). No significant difference was observed between wild-type and nectin-1<sup>-/-</sup> mice (Fig. 4). These results indicate the presence of the cornified cell envelope, in the nectin-1<sup>-/-</sup> mice. To assess the permeability of the epidermis, we performed the dye penetration assay using lucifer yellow dye and examined the water loss by measuring their epidermis weight in newborn pups (11, 35). No significant difference was observed between wild-type and nectin-1<sup>-/-</sup> mice (Fig. 4). These results indicate the presence of the cornified cell envelope, in the nectin-1<sup>-/-</sup> mice.

Compensation for the Reduced Expression of the Loricrin Gene in Nectin-1<sup>-/-</sup> Mice—The loricrin protein contributes to 70–85% of the total protein mass of the cornified layer (36). Since the cornified cell envelope is essential for the barrier function of skin, the reduced expression of loricrin in nectin-1<sup>-/-</sup> mice was suspected to show an impaired epidermal barrier function, but this was not the case. It has been reported that loricrin<sup>-/-</sup> mice show congenital erythroderma with shiny skin and have quasinormal cornified cell envelopes (31, 32), which is similar to the phenotype of nectin-1<sup>-/-</sup> mice. In loricrin<sup>-/-</sup> mice, the expression of other cornified cell envelope components, such as small proline-rich proteins (SPRRPs) and retepin, increases, and this may contribute to the compensatory mechanism preventing barrier dysfunction of the skin. Based on this information, we examined the mRNA level of several cornified cell envelope components. Semiquantitative reverse transcription PCR experiments revealed that the transcription of the loricrin gene was lower in nectin-1<sup>-/-</sup> mice than wild-type mice, whereas the transcription of the retepin and SPRRP2D genes, but not the SPRRP2A gene, was up-regulated in nectin-1<sup>-/-</sup> mice (Fig. 5A). We also performed quantitative real-time PCR to precisely determine the increased mRNA level of retepin; the epidermis of nectin-1<sup>-/-</sup> mice expressed 52 times the mRNA level of retepin than that of wild-type mice (Fig. 5B). These results indicate that nectin-1<sup>-/-</sup> mice appear to compensate the decreased transcription of the loricrin gene by up-regulating other cornified cell envelope components, similar to loricrin<sup>-/-</sup> mice.

Reduction in the Ca<sup>2+</sup>-induced Loricrin Production and ERK Activation in Nectin-1<sup>-/-</sup> Keratinocytes—To elucidate the mechanism of reduced expression of loricrin in the epidermis of nectin-1<sup>-/-</sup> mice, we induced differentiation in the primary keratinocytes from both wild-type and nectin-1<sup>-/-</sup> mice by the Ca<sup>2+</sup> switch. When keratinocytes were cultured at a low Ca<sup>2+</sup> concentration (50 μM), keratinocytes from both wild-type and nectin-1<sup>-/-</sup> mice proliferated similarly (data not shown), and the expression of loricrin remained low in wild-type as well as nectin-1<sup>-/-</sup> mice (Fig. 6A). However, the amount of loricrin increased in wild-type keratinocytes but not in nectin-1<sup>-/-</sup> keratinocytes, at 24 h and later after the concentration of Ca<sup>2+</sup> in the culture medium was switched to 2 mM (Fig. 6A). On the other hand, the expression of other differentiation markers, such as involucrin and keratin 10, was similarly induced in keratinocytes in both mice strains by the Ca<sup>2+</sup> switch (data not shown). During the Ca<sup>2+</sup> switch, the differentiation morphology of wild-type keratinocytes was indistinguishable from that of nectin-1<sup>-/-</sup> keratinocytes (data not shown).

It has been reported that the transcription of the loricrin gene is regulated by binding of protein factors to an AP-1 consensus
The phosphorylation level of ERK was elevated at 24 h and later after the Ca\(^{2+}\) switch in keratinocytes. The phosphorylation level of ERK1/2, but not JNK or p38 MAPK, was increased in wild-type keratinocytes by the Ca\(^{2+}\) switch, whereas that of Ras was not (Fig. 7A). To clarify the upstream signaling toward the activation of ERK in keratinocytes during the Ca\(^{2+}\) switch, we focused on two pathways: Rap1- and Ras-mediated pathways, both of which are known to induce the activation of ERK through MEK1 (38, 39). By the pull-down assay, the GTP-bound form of Rap1 was observed in nectin-1-knockout (KO) keratinocytes, whereas the phosphorylation levels of JNK and p38 MAPK were increased in wild-type keratinocytes by the Ca\(^{2+}\) switch, the expression of loricrin, and reduced activation of ERK, but not JNK or p38 MAPK, was restored in nectin-1-knockout keratinocytes. The cell extracts from nectin-1-knockout keratinocytes re-expressing human nectin-1 (hNectin-1) as well as wild-type and nectin-1-knockout keratinocytes just before the Ca\(^{2+}\) switch and at 24 h after the Ca\(^{2+}\) switch were used for Western blotting with the anti-nectin-1, anti-phospho-ERK1/2, and anti-ERK1/2 Abs. Since the calculated molecular mass of transiently expressed human nectin-1 is quite similar to that of mouse nectin-1, the different size of these two types of nectin-1 is probably due to the different degree of glycosylation. The results shown in this figure are representative of three independent experiments.

Involvement of Rap1 in the Ca\(^{2+}\)-induced Activation of ERK—To clarify the upstream signaling toward the activation of ERK by the Ca\(^{2+}\) switch in keratinocytes, we focused on two pathways: Rap1- and Ras-mediated pathways, both of which are known to induce the activation of ERK through MEK1 (38, 39). By the pull-down assay, the GTP-bound form of Rap1 was increased in wild-type keratinocytes by the Ca\(^{2+}\) switch for 24 h, whereas that of Ras was not (Fig. 7A). Neither Rap1 nor Ras activation was observed in nectin-1-knockout keratinocytes. The adenovirus-mediated introduction of Rap1GAP, which effec-
Nectin-1 and Loricrin Expression in the Epidermis

Wild-type keratinocytes expressing β-galactosidase (Control) or Rap1GAP by the adenovirus-mediated method were lysed with or without the Ca\(^{2+}\) switch for 24 h. The lysates were used for Western blotting with the anti-phospho-ERK1/2 and anti-ERK1/2 Abs. C, restoration of Rap1 activation by re-expression of nectin-1 in nectin-1\(^{-/-}\) keratinocytes. The lysates from nectin-1\({\text{null}}\) mice were used for the pull-down assay as described in A. The results shown in this figure are representative of three independent experiments.

DISCUSSION

We have recently shown that disruption of each nectin family member, including nectin-1, nectin-2, and nectin-3, exhibits several obvious phenotypes (19, 20, 22–24), although each of the nectin-null mice is viable and has no life-threatening disorder. Functional redundancy may work in each of the nectin-null mice, since many tissues express multiple nectins, and these nectins homophilically or heterophilically trans-interact with each other in a variety of combinations (7, 41). These results may explain the moderate phenotypes in each of nectin\(^{-/-}\) mice.

In this study, we investigated the roles and mechanisms of nectin-1 in the epidermis by using nectin-1\(^{-/-}\) mice. Nectin-1\(^{-/-}\) mice exhibit shiny and slightly reddish skin at birth. Although nectin-2 as well as nectin-1 is expressed in the wild-type epidermis, this phenotype is only observed in nectin-1\(^{-/-}\) mice and not in nectin-2\(^{-/-}\) mice (data not shown). Moreover, ~30% of nectin-1\(^{-/-}\) pups, but not nectin-3\(^{-/-}\) pups, are born with eyes open despite the involvement of both nectins in the apex-apex adhesion between the pigment and nonpigment cell layers of the ciliary epithelia (22). These results demonstrate a specific role of nectin-1 in the epidermis and eyelids. In fact, each nectin is likely to have distinct functions; only nectin-1 and nectin-3 directly bind Par-3, a member of the cell polarity proteins, and integrin αβ, a cell-matrix adhesion molecule (42, 43).

It has been reported that mutation of the nectin-1 gene in humans results in cleft lip/palate-ectodermal dysplasia, which includes Zlotogora-Ogur syndrome and Margarita Island ectodermal dysplasia (26–28). The skin phenotype of cleft lip/palate-ectodermal dysplasia includes Zlotogora-Ogur syndrome and Margarita Island ectodermal dysplasia, which is characterized by thickening of the palm and sole epidermis with a degree of cytolysis that causes proliferation signals to the epidermis (44). However, the epidermis in nectin-1\(^{-/-}\) mice, including their palm and sole epidermis, is histologically similar to that in wild-type mice and does not exhibit abnormal proliferation, although nectin-1\(^{-/-}\) mice have shiny and slightly reddish skin at birth. The reason why there is a different skin phenotype between nectin-1 gene-mutated human and nectin-1\(^{-/-}\) mouse remains to be elucidated.
but distinct compensatory mechanisms between humans and mice may contribute to this difference. Immunofluorescence and Western blotting analyses have revealed that disruption of nectin-1 results in a reduced expression of loricrin in the epidermis. Loricrin is a major component of the cornified cell envelope that serves as a protective barrier against the external environment. However, it has been reported that loricrin−/− mice do not show severe skin phenotypes or impaired epidermal barrier function (31). In addition, the structure of the cornified cell envelope is quasinormal in loricin−/− mice, suggesting the existence of a compensatory mechanism (32). The expression of other components in the cornified cell envelope is actually elevated in these knock-out mice (31, 32), and this is likely to contribute to the maintenance of the epidermal barrier function. Consistent with these observations, we did not detect an impaired barrier function in nectin-1−/− mice and found that transcription of the repetin and SPPRD genes, components of the cornified cell envelope, is up-regulated in the nectin-1−/− epidermis. It is apparent that the transcription of the loricrin gene decreases and that of the repetin and SPPRD genes increases in the nectin-1−/− epidermis, although we cannot decisively determine whether the reduced transcription of the loricrin gene occurs prior to transcription of repetin and SPPRD genes in nectin-1−/− mice, or vice versa.

We then examined whether the loss of nectin-1 affects terminal differentiation, proliferation, or resistance to physical stress in the epidermis. The abrogation of nectin-1 impaired expression of the epidermal differentiation marker loricrin and altered the contents of cornified cell envelope components, suggesting the abnormalities in epidermal differentiation. This is likely to be consistent with nectin-2−/− and nectin-3−/− mice, both of which show abnormal differentiation in spermatogenesis in the testis (19, 24). However, the skin phenotypes in nectin-1−/− are not severe, probably due to the normal expression of other nectin family members, including nectin-2 and nectin-4, and another AJ component, E-cadherin, in the epidermis. In addition, the number of cells positive for Ki-67, a proliferative marker, in the basal layer of nectin-1−/− mice is similar to that of wild-type mice, indicating that nectin-1 is not involved in cell proliferation in the epidermis. On the other hand, cornified cells from nectin-1−/− mice exhibit weak resistance to physical stress induced by sonication. This experiment was performed in suspension where the trans-interaction of nectin does not occur between the cells. Thus, this physically sensitive phenotype of the nectin-1−/− cornified cells seems to be due to the reduction in the expression of a major cornified cell envelope component loricrin.

Finally, we sought to explore the molecular mechanism involved in the reduced expression of loricrin in nectin-1−/− mice. The AP-1 transcription factors enable the regulation of loricrin gene transcription at its promoter region (37); thus, we focused on the MAPK activity for this regulation. The increased phosphorylation of ERK was observed in keratinocytes from wild-type mice, but not nectin-1−/− mice, during the Ca2+ switch, which induces the differentiation of keratinocytes. The similar results were obtained for the frozen section of the skin; the phosphorylation level of ERK was elevated in the epidermis of wild-type mice compared with that of nectin-1−/− mice. It has been reported that the phosphorylation of ERK is involved in the Ca2+ -mediated differentiation of keratinocytes and that the inhibition of the ERK signaling attenuates the expression of differentiation markers, including loricrin, in the Ca2+ switch assay (45). Thus, the suppressed phosphorylation of ERK may partly cause the lower expression of loricin in nectin-1−/− mice. The fact that U0126, which inhibits the phosphorylation of ERK, attenuates the expression of loricin during the Ca2+ switch also reinforces the importance of ERK phosphorylation for the expression of loricin in the keratinocyte differentiation. Thus, the trans-interaction of nectin-1 appears to up-regulate the transcription of the loricrin gene through the phosphorylation of ERK and probably subsequent activation of the AP-1 transcription factors. We also examined the upstream signaling pathway for the activation of ERK and found that the nectin-1-dependent activation of Rap1, but not Ras, was important for the activation of ERK after the Ca2+ switch. We have previously demonstrated that Rap1 is activated by the trans-interaction of nectin and that its activation enhances the nectin-induced formation of AJs (34). Thus, it seems reasonable that Rap1 plays a critical role in the Ca2+ -induced, nectin-enhanced expression of loricin in keratinocytes. Although the molecular mechanisms of Ca2+ in differentiation of the epidermis are largely unclear, various molecules with functions that are controlled by Ca2+, including integrin, are thought to be involved in differentiation. In addition, we show in this study that the trans-interaction of nectin-1 is likely to modulate the Ca2+-induced signaling in differentiation of the epidermis. Further studies are, however, necessary to understand how nectin-1 regulates the Ca2+-induced signaling in differentiation of the epidermis.

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