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CLCA Splicing Isoform Associated with Adhesion through β_1-Integrin and Its Scaffolding Protein

SPECIFIC EXPRESSION IN UNDIFFERENTIATED EPITHELIAL CELLS[*] [S]

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Background: CLCA family members have been shown to be multifunctional proteins.
Results: A rat splicing isoform was localized to the stratum basale of the epidermis. This isoform was found to alter β_1-integrin expression and cell adhesion.
Conclusion: This isoform may modulate the adhesive potential of undifferentiated epithelial cells.
Significance: Cell-specific splicing might confer a novel function on the epithelium.

We previously found that a rat CLCA homologue (rCLCA-f) modulates Ca^{2+}-dependent Cl^- transport in the ductal cells of the rat submandibular gland. CLCA proteins have been shown to be multifunctional, with roles in, for example, cell adhesion. Here, we describe the mRNA and protein expressions of a splicing isoform of rat rCLCA (rCLCA-t). This isoform is a 514-amino acid protein containing a C-terminal 59-amino acid that is distinct from the rCLCA-f sequence. Immunohistochemistry revealed rCLCA-t to be located in the basal cells of the rat submandibular gland excretory duct and the stratum basale of rat epidermis, whereas rCLCA-f was detected in cells during the process of differentiation. In a heterologous expression system, rCLCA-t was found to be a membrane protein present predominantly in the perinuclear region, and not to be either present on the cell surface or secreted. rCLCA-t failed to enhance ionomycin-induced Cl^- conductance (unlike rCLCA-f). When compared with rCLCA-f, it weakened cell attachment to a greater extent and in a manner that was evidently modulated by intracellular Ca^{2+}, protein kinase C, and β_1-integrin. rCLCA-t was found to associate with RACK1 (receptor for activated C kinase) and to reduce expression of mature β_1-integrin. Treatment of rat skin with rCLCA-t siRNA increased the expression of β_1-integrin in the stratum basale of the epidermis. These results are consistent with cell-specific splicing of rCLCA mRNA playing a role in the modulation of the adhesive potential of undifferentiated epithelial cells.

Ca^{2+}-activated Cl^- channels are found in association with plasma membranes in a variety of tissues, including epithelium, cardiac and smooth muscle, and neurons (1, 2). Various molecular candidates were put forward for Ca^{2+}-activated Cl^- channels, including CLCA (3–6), CLC-3 (7), and bestrophin (8), but it has now been identified as TMEM16A (9). The first reported CLCA clone, from bovine tracheal epithelium, was bCLCA1 (10), and this prototype was followed by the cloning of other members of the CLCA family. We previously identified a rat CLCA homologue (rCLCA),[2] and provided evidence suggesting that it is responsible for modulating Ca^{2+}-dependent Cl^- transport in salivary ductal cells (11). More recently, we presented *in vivo* evidence of a physiological role for rCLCA in transepithelial Cl^- transport in the ductal system of rat submandibular gland (SMG) (12).

Genetic studies have indicated links between the CLCA family and certain secretory disorders (*viz.* cystic fibrosis and asthma) (13, 14). Actually, CLCA proteins have been shown to be multifunctional, with roles in, for example, cell-cell adhesion, apoptosis, and cell-cycle control, and they appear to have a pathophysiological connection with tumor metastases (see Ref. 5 for review). First, a bovine isoform of CLCA (bCLCA2; Lu-ECAM-1) was reported to be an endothelial adhesion molecule (15–17), and then human CLCA2 (hCLCA2) and mouse CLCA1 (mCLCA1), which also possess specific binding motifs, were shown to bind β_4-integrin and to contribute to tumor metastases (18, 19).

Each of these CLCA proteins consists of ~900 amino acids, and there are also reported to be various truncated isoforms, the physiological roles of which are largely unknown. The first truncated CLCA isoform to be described was derived from bCLCA2 (17). Among the four identified clones, the truncated version was predicted to be a secreted protein, and the authors supposed this to be an alternative splicing product from a single gene. In the case of hCLCA3, which is also reported to be secreted (20), the gene encodes the first one-third of the full-

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[2] The abbreviations used are: rCLCA, rat CLCA; mCLCA, mouse CLCA; SMG, submandibular gland; RACK1, receptor for activated C kinase 1; TC, tetracycline; Sup, supernatant; PSS, physiological salt solution; CGM, cationized gelatin microsphere(s); ANOVA, analysis of variance; PDBu, phorbol-12, 13-dibutyrate; PSS, physiological salt solution.

length hCLCA3 protein due to the presence of a stop codon. In mammary adenocarcinoma cell lines, mCLCAs are reportedly down-regulated when their splicing variants are expressed (21). Although the existence of these isoforms has been documented, it remains uncertain whether such splicing isoforms have physiological significance.

To identify another CLCA member present in rat tissue, using total RNA extracted from the ileum, we employed an RT-PCR-based strategy aided by primers based on the cDNA sequence conserved among mouse and rat members. Here, we report a truncated isoform of rCLCA (rCLCA-t) that we found to have characteristics distinct from those of the full-length isoform (rCLCA-f). Our results demonstrate that rCLCA-t exhibits a remarkable distribution among the undifferentiated epithelial cells of SMG and skin. Epidermal homeostasis has been proposed to be mediated by β_1-integrin, which plays regulatory roles in anchorage to the extracellular matrix and the onset of terminal differentiation (22). The present study suggests (*a*) that heterologous rCLCA-t expression may cause an attenuation of β_1-integrin-mediated cell adhesion, with no alteration in Cl^- conductance, in human embryonic kidney 293 (HEK293) cells, and (*b*) that isoform-specific knockdown increases β_1-integrin expression in the basal layer of the skin. These results lead us to hypothesize that this cell-specific CLCA isoform might help to confer novel functions, such as modulation of adhesive potential, on undifferentiated epithelium.

EXPERIMENTAL PROCEDURES

Identification of rCLCA-t and Generation of Stable Expression Cell Lines—Male Wistar rats (6 weeks old) were anesthetized with sodium pentobarbital, permission for the procedures used having been granted by the Animal Research Committee of Fukuoka Dental College. Total RNA was isolated from rat ileum, and then the reverse-transcribed cDNA was subjected to PCR, utilizing *PfuUltra* DNA polymerase (Stratagene, La Jolla, CA) and primers previously employed for the PCR cloning of rCLCA-f (11). To confirm the existence of the contiguous cDNA comprising these PCR products, we amplified the entire ORF for rCLCA-t along with part of its 5′- and 3′-untranslated regions. The PCR product was cloned into a pCR-XL-TOPO vector (Invitrogen).

For the transient expression study, the cDNA was subcloned into mammalian expression vector pIRES-hrGFP-1a (Stratagene). Cell lines exhibiting stable, tetracycline (TC)-inducible expression were also generated for histidine-tagged rCLCA-f and rCLCA-t cDNA using a Flp-In T-REx system together with Flp-In-293 cells (Invitrogen), the parental cell line of which is HEK293 cells. Fifteen nucleotides were inserted 76 bp downstream of the start codon in either construct to produce a histidine tag (one intrinsic His plus five additional His, see Fig. 1). The cells were treated with TC (1 μg/ml) for 24 h prior to the experiments.

Distribution of rCLCA by RT-PCR—Seven-week-old rats provided samples of skin (5 × 5 mm, full thickness) from the back, which had been shaved, and SMG. These tissues were minced and then frozen and crushed in liquid nitrogen. Total RNA (SMG and skin, 2 μg; HEK293 cells, 0.5 μg) was reverse-transcribed using oligo(dT) primers. The cDNA was subjected to PCR for 40 cycles (denaturation, 94 °C, 30 s; annealing, 59 °C for rCLCA-t and 56 °C for rCLCA-f, 30 s; extension, 72 °C, 1 min) using 0.025 unit/ml of *Taq* polymerase (Takara Bio Inc.). Primer pairs for rCLCA-t (P-T) and for rCLCA-f (P-F) were designed, and were as follows: P-T, forward, 5′-GAAGCATGGACAGAAG-3′; reverse, 5′-TGCTCTCCAACTGTCATATC-3′, synthesis being based on the nucleotide sequence connecting the 8th and 10th exons; P-F, forward, 5′-GAAGCATGGACAGAAG-3′; reverse, 5′-GCTGTTCACATCTTTGTTG-3′, as included in the ninth exon (see Fig. 2*A*).

Antibodies—Rabbit polyclonal antibodies (CF1 and CT1) were generated against synthetic peptides based on distinct regions in rCLCA-f and rCLCA-t, respectively (see Fig. 1 and supplemental Fig. S1). An antibody generated against the N-terminal external domain (CN1) common to rCLCA-f and rCLCA-t was also used (11). Others used were: anti-human β_1-integrin (clone 18, BD Transduction Laboratories; TS2/16, BioLegend), anti-rat β_1-integrin (HMβ1-1; BioLegend), anti-cytokeratin 14 (CK14) and anti-CK10 (Novocastra Laboratories), anti-calnexin (StressGen Bioreagents Corp.), anti-RACK1, anti-α_2-integrin (clone 2/CD49b; BD Transduction Laboratories), and anti-β-actin (Santa Cruz Biotechnology) antibodies.

Immunoblotting Analysis—Rat SMG and skin samples were frozen and crushed in liquid nitrogen. The tissues and transfected cells were homogenized in lysis buffer (150 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, and 20 mM Tris-Cl, pH = 7.4) in the presence of protease inhibitor mixture (Sigma). The lysate was centrifuged for 10 min to obtain the 1,000 × *g* supernatant (Sup), and then the Sup was centrifuged for 15 min to collect 6,500 × *g* Sup. Centrifugation at 100,000 or 200,000 × *g* for 2 h separated the 6,500 × *g* Sup into the cytosolic fraction and the membrane pellet. All procedures were performed at 4 °C.

In some preparations, the 100,000 × *g* membrane pellet was incubated either with Triton X-100 (1%) or with Triton X-100 (1%) plus sodium deoxycholate (0.1%) for 30 min at 4 °C and separated into detergent-soluble and -insoluble fractions by centrifugation (10,000 × *g*, 1 h). SDS-PAGE (7.5%), immunoprecipitation, and immunoblotting using rCLCA (dilution; 1:8000) and other antibodies were performed as described previously (11). Densitometric analysis of the blots was performed using ImageJ 1.37v (National Institutes of Health).

Immunostaining—For 3,3′-diaminobenzidine visualization using CF1 and CT1 (1:300), anti-CK14 (1:100), and anti-CK10 (1:100) antibodies, transfected cells were seeded onto collagen- and poly-L-lysine-coated coverglasses, whereas 5-μm-thick sections of SMG and skin were mounted on glass slides. For wounding experiments, a full-thickness (2.0–2.5-mm depth) incisional wound (15-mm length) was made in the skin on the back of 7-week-old rats. Five days later, the skin was sampled for immunohistochemistry. For immunofluorescence, the antibodies used were CF1 (1:200), CT1 (1:100), TS2/16 (1:100), HMβ1-1 (1:100), calnexin (1:200), and RACK1 (1:100) antibodies, prior to the appropriate secondary antibody conjugated with Alexa Fluor 488 or 594 (1:800; Molecular Probes Inc.). Fluorescence was observed using a confocal microscope (LSM710; Carl Zeiss MicroImaging GmbH). ZEN 2009 Light Edition (Carl Zeiss) was used for image processing.

Glycosidase Digestion and Isolation of Cell Surface-associated and Secreted Proteins—For glycosidase digestion, the membrane fraction (50 μg of protein) was treated with 0.2% SDS at 95 °C for 10 min. Triton X-100 (1.5%) was added (final volume, 30 μl) prior to incubation at 37 °C with 2.5 units of peptide-*N*-glycosidase F (Sigma) in phosphate buffer (pH = 7.5) for 1 h. The reaction product was denatured by boiling for 5 min.

For isolation of β_1-integrin on the cell surface, cells were labeled with Sulfo-NHS-SS-Biotin (Pierce). The cells were lysed in the presence of 1% Triton X-100, and the cell lysate was applied to NeutrAvidin-agarose beads and incubated for 60 min. The flow-through fraction was collected for the detection of nonsurface proteins. After the column had been washed, the Sulfo-NHS-bound surface protein was eluted by incubation with dithiothreitol (DTT).

To condense the histidine-tagged rCLCA proteins released into the culture medium, cells grown in a 75-cm^2 flask were incubated with 5 ml of serum-free medium for 5 h. The collected medium was concentrated using centrifugal filters (cut-off = 30 kDa; Microcon YM-30; Millipore Corp.) followed by incubation with cobalt-affinity resins (BD TALON; BD Biosciences). The bound protein was eluted with 200 mM imidazole buffer. All procedures were performed at 4 °C.

Whole-cell Patch Clamp Recording—Stably transfected cells were seeded onto collagen- and poly-L-lysine-coated coverslips. After TC treatment, the cells were used for patch clamp recording. The external solution contained (in mM): 140 NaCl, 5 CsCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose, 10 HEPES, 25 mannitol (pH = 7.4). The pipette solution contained (in mM): 145 CsCl, 0.366 $CaCl_2$, 2.2 $MgCl_2$, 1 EGTA, 1 Mg-ATP, 10 HEPES, 10 mannitol (pH = 7.2). Experiments were performed at room temperature. Current amplitudes were measured using a patch clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA), and the currents were analyzed using pCLAMP 8.0 software (Axon Instruments).

Cell Adhesion Assay—Cells were dispersed and incubated in Ca^{2+}-containing HEPES-buffered physiological salt solution (PSS). The cells (0.5–1.0 × 10^6 cells/well) were allowed 20 min to adhere to a collagen-coated plate (24 wells) during incubation at 37 °C in the presence of several reagents followed by a gentle wash three times with low Ca^{2+} (10 μM)-containing HEPES-PSS. To quantify adherence, the attached cells were incubated with tetrazolium salt for 2 h at 37 °C (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). Then, the absorbance of the supernatant at 450 nm was measured. To obtain a control value, the absorbance of the supernatant derived from nontreated cells was measured without the washing procedure.

Epidermal Application of siRNA—A small interference RNA (siRNA) to target the rCLCA-t gene was synthesized in a purified and annealed duplex form (GeneDesign, Inc., Osaka, Japan). The sense sequence of the annealed siRNA duplex form was 5′-CGGAUAUGACAGUUGGAGAdTdT-3′. Scrambled RNA controls were also designed. The specificity of the siRNA was tested in HEK293 cells that stably expressed the CLCA isoform following the use of a transfection reagent (RNAiFect, Qiagen). siRNA or the scrambled RNA (200 μg) was incorporated into 2 mg of biodegradable cationized gelatin microspheres (CGM) (MedGel E50; MedGEL Corp., Kyoto, Japan). Each type of RNA-incorporated microspheres was suspended in saline and injected subcutaneously into both earlobes in 7-week-old rats. Such incorporated compounds have been reported to be released from the hydrogel after its degradation by endogenous enzymes secreted from the surrounding tissues (23). One week after the injection, the rats were sacrificed, both ears were removed, and 7-μm-thick frozen sections were examined in an immunofluorescence study using the same concentrations of antibodies as those shown in the previous section. ImageJ was used for quantifying the intensity of the fluorescence.

Statistics—All values are presented as means ± S.E. (n = number of observations). Statistical analysis was performed using a one-way ANOVA or multivariate ANOVA followed by a post hoc Bonferroni's t test. A grouped t test was employed when two groups were to be compared. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Molecular Cloning of a Truncated Mutant of Rat CLCA—Previously, we identified a rat CLCA member, rCLCA, from rat ileum (903 amino acids) (11) (rCLCA-f in Fig. 1 and in supplemental Fig. S1; named rCLCA2 in the present GenBank™/European Molecular Biology Laboratory (EMBL) nomenclature). The same procedure was employed here to find a new isoform using primers designed for PCR based on the cDNA sequence that is identical among mCLCA1, -2, and -4. Among the nine positive clones obtained from the amplified PCR product, six clones (2.1 kb in length) were almost identical to the central part of rCLCA-f cDNA (GenBank/EMBL accession number AB119249). The remaining clones (named rCLCA-t) were shorter (2.0 kb), and but for a missing 101 bp in the middle of the sequence, they were also identical to rCLCA-f.

We amplified the entire ORF for rCLCA-t along with part of its 5′- and 3′-untranslated regions. rCLCA-t was found to consist of 3,208 bases, of which 20 bases comprise the 5′-untranslated region. Because the absence of 101 bp in the rCLCA-t mRNA sequence (between nucleotides 1,367 and 1,467 of rCLCA-f mRNA) causes a frameshift, a stop codon (UAA) appears in the middle of the entire length of the mRNA (Fig. 1*A*). Because the rCLCA-t isoform was found to possess 1,646 bases in the 3′-untranslated region followed by a poly(A)$^+$ tail, the ORF was predicted to be shorter, encoding a 514-amino acid protein (Fig. 1*B* and supplemental Fig. S1). This protein possessed an N-terminal signal sequence, but lacked the significant C-terminal hydrophobic domain reported to be present in rCLCA-f (11). The amino acid sequence in the C-terminal region downstream of the frameshift (59 amino acids) was entirely different from that of any isoform previously reported for CLCA members (supplemental Fig. S1, *italic letters*). Excluding this region, this isoform was 84, 72, and 56% identical to mCLCA1, Lu-ECAM-1, and hCLCA1, respectively (supplemental Fig. S1).

We performed a BLAST search using a rat genomic sequence (24), with rCLCA-f and rCLCA-t cDNAs as the query sequences (25). The locus of the *rCLCA* gene appears to be 2q44, and the region was found to be identical to the predicted mRNA sequence XM_575054. On the basis of a comparison between the rat genome and rCLCA-f mRNA, the *rCLCA* gene

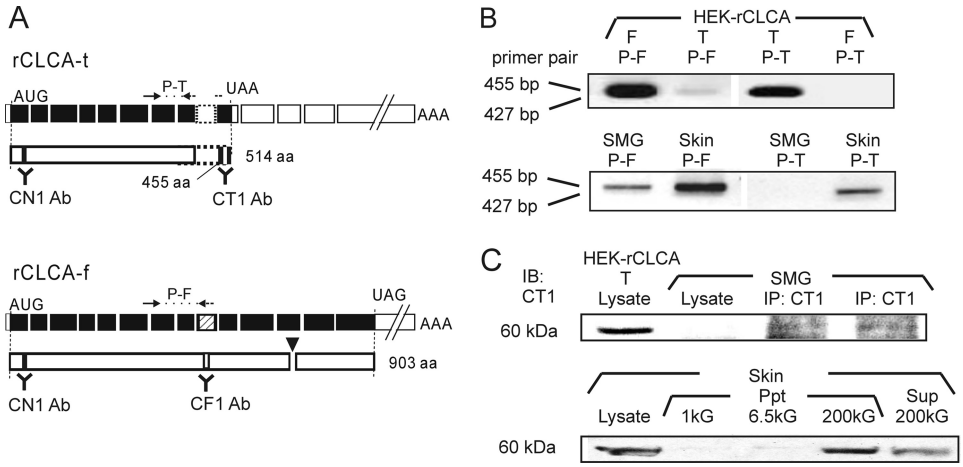

FIGURE 1. **Schematic diagrams of rCLCA-f and the splicing variant (exon 9) of rCLCA (rCLCA-t) and their mRNA and protein expressions in SMG and skin.** *A*, mRNA (*upper*) and the translated proteins (*lower*) for the two rCLCA isoforms. *Closed rectangles* indicate ORF. *Arrows* indicate specific primers for PCR. Recognition sites for the three rCLCA antibodies (*Ab*) are shown (*Y*). The proposed post-translational processing site is indicated by a *closed triangle*. *aa*, amino acids. *B*, mRNA expression was examined in rat SMG and skin and in HEK293 cells transfected with either rCLCA-t (*T*) or rCLCA-f (*F*) cDNA. *P-T* and *P-F* denote primer pairs designed to recognize rCLCA-t and rCLCA-f, respectively (see *panel A*). *C*, expressions of rCLCA proteins from the lysate of rat SMG (100 μg) and rCLCA-t-transfected HEK293 cell (25 μg). The immunoprecipitant (*IP*) was obtained from the SMG lysate (300 μg for the *left lane* and 450 μg for the *right lane*) using CT1 antibody. The expression was also examined for the lysate, 1,000 $\times$ *g*, 6,500 $\times$ *g*, and 200,000 $\times$ *g* pellet (*Ppt*), and 200,000 $\times$ *g* Sup obtained from rat skin. Immunodetection was performed using CT1 antibody. *IB*, immunoblot.

was predicted to consist of at least 14 exons interspersed with 13 introns and to span 22,293 bp from the start to the end of the cDNA. Based on the above argument, the ninth exon appeared to be lost when rCLCA-t mRNA was transcribed and spliced (Fig. 1*A*). A cDNA sequence homologous to rCLCA-t was found in the mouse cDNA database (26; accession number BC010260), although it has not been characterized further.

Localization of Truncated rCLCA Isoform Specifically in Undifferentiated Cells of Rat SMG and Skin—We tested in RT-PCR and immunoblotting experiments whether rCLCA isoforms are expressed in rat SMG and skin, using specific primers and antibodies (Fig. 1*A*). Firstly, we confirmed that the primers recognized each isoform in a heterologous expression system. Amplification of rCLCA-t cDNA at the predicted nucleotide size of 427 bp was detected (using the P-T pair of primers) specifically in transiently rCLCA-t-transfected cells (Fig. 1*B*, *T*). Using these primers, no expression of rCLCA-t mRNA was detected in rat SMG, whereas marked expression was found in rat skin. In contrast, the P-F pair amplified rCLCA-f cDNA at the nucleotide size of 455 bp in the rCLCA-f-transfected cells (Fig. 1*B*, *F*), but produced only a small amount of pseudo-products in T. Using these primers, an abundant expression of rCLCA-f mRNA was observed in both SMG and skin.

Rat SMG and skin were lysed and analyzed by immunoblot using CT1 antibody (Fig. 1*C*). Its specificity between the two rCLCA isoforms is shown in the supplemental text and supplemental Fig. S2. This antibody recognized a 60-kDa-sized band in the rCLCA-t-transfected cells. This corresponded to the size of rCLCA-t protein (Fig. 1*A*). CT1 antibody failed to detect an apparent 60-kDa signal in rat SMG lysate or its immunoprecipitant. This antibody, however, strongly recognized the band in total lysate and in 200,000 $\times$ *g* membrane pellet and Sup fractions obtained from rat skin.

To examine whether the truncated isoform is distributed only in specific regions in rat SMG and skin, immunohistochemistry was performed. Using CF1 antibody, intense staining of rCLCA was observed in the ductal epithelia of the intralobular ducts in SMG (Fig. 2*A*). CF1 antibody that had been preabsorbed with the antigen peptide did not stain the ductal cells (Fig. 2*B*). CT1 antibody failed to stain epithelial cells in the acinar and intralobular ducts (Fig. 2*C*). Interestingly, immunoreactivity to CT1 antibody was found in a few rounded or pyramidal cells at the base of the epithelium in SMG excretory ducts (Fig. 2*D* and *inset*). rCLCA-t was largely limited to the perinuclear region, with little surface distribution. The preabsorbed CF1 antibody did not stain such cells (Fig. 2*E*). CK14 is known to be a specific marker of undifferentiated basal cells (27), and a selective immunoreactivity to an anti-CK14 antibody confirmed that such cells were undifferentiated (Fig. 2*F*).

In skin, CT1 antibody predominantly stained the stratum basale, which is a single layer of undifferentiated epithelial cells (basal cells) (Fig. 3*A*). The preabsorbed CT1 antibody did not stain the stratum basale (Fig. 3*B*). Anti-CK14 antibody recognized basal cells and also suprabasal cells, probably due to their slow turnover (Fig. 3*C*). In contrast, CF1 antibody stained suprabasal layers during the process of differentiation: most intensively in the stratum spinosum and weakly in the stratum granule, which were recognized by anti-CK10 (Fig. 3, *D* and *F*). Little staining was seen when preabsorbed CF1 antibody was used (Fig. 3*E*). Interestingly, CT1 staining was very intense and spread across the basal as well as suprabasal layers of the skin wounds (Fig. 3, *G* and *H*).

Heterologous Expression of rCLCA Isoforms in the Membrane Fraction of HEK293 Cells—We investigated the subcellular distribution and post-translational processing of rCLCA isoforms by immunoblotting using a CN1 antibody (Fig. 4*A*). HEK293 transfected transiently with the empty vector did not exhibit immunoreactivity to CN1 antibody (Fig. 4*A*, *lane V*). In the rCLCA-t-transfected cells (Fig. 4*A*, *lane T*), the antibody recognized a 60-kDa-sized band in 1,000 $\times$ *g* Sup. In the 100,000 $\times$ *g* membrane pellet fractions, substantial immunoreactivity was detected in the Triton X-100-insoluble subfraction, but there

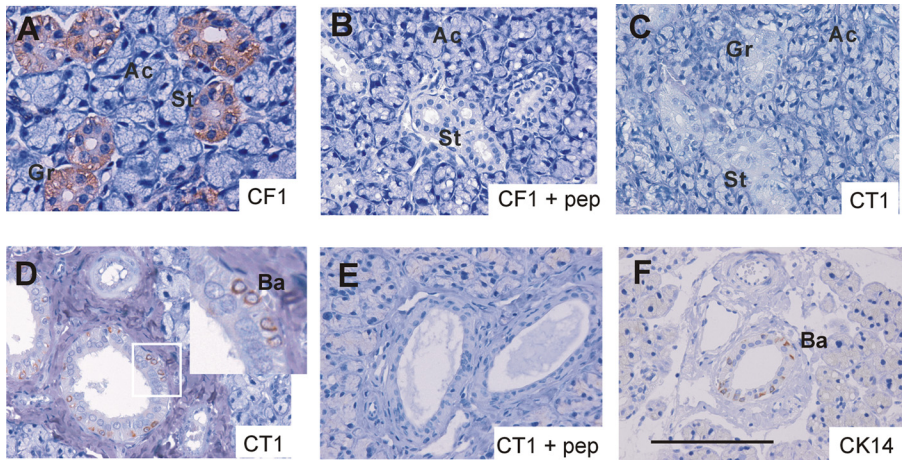

FIGURE 2. **Immunostaining in rat SMG.** *A*, CF1 antibody recognized intralobular ductal cells of SMG. *B*, CF1 antibody that had been preabsorbed with the antigen peptide (*pep*) did not stain the ductal cells. *C*, CT1 antibody did not stain the ductal cells. *D*, immunoreactivity to CT1 antibody is shown in perinuclear region in basal cells of SMG excretory ducts. *Inset*, the *boxed portion* in *D* is shown enlarged. *Bar* = 10 μm. *Ba*, basal cells in excretory ducts. *E*, CT1 antibody that had been preabsorbed with the antigen peptide did not stain the basal cells. *F*, anti-CK14-integrin antibody recognized the basal cells, but not the superficial cells of SMG excretory ducts. CK14 is a marker of undifferentiated cells. *St*, striated ducts; *Gr*, granular convoluted tubule; *Ac*, acini. Counterstaining: hematoxylin. *Bar* = 100 μm.

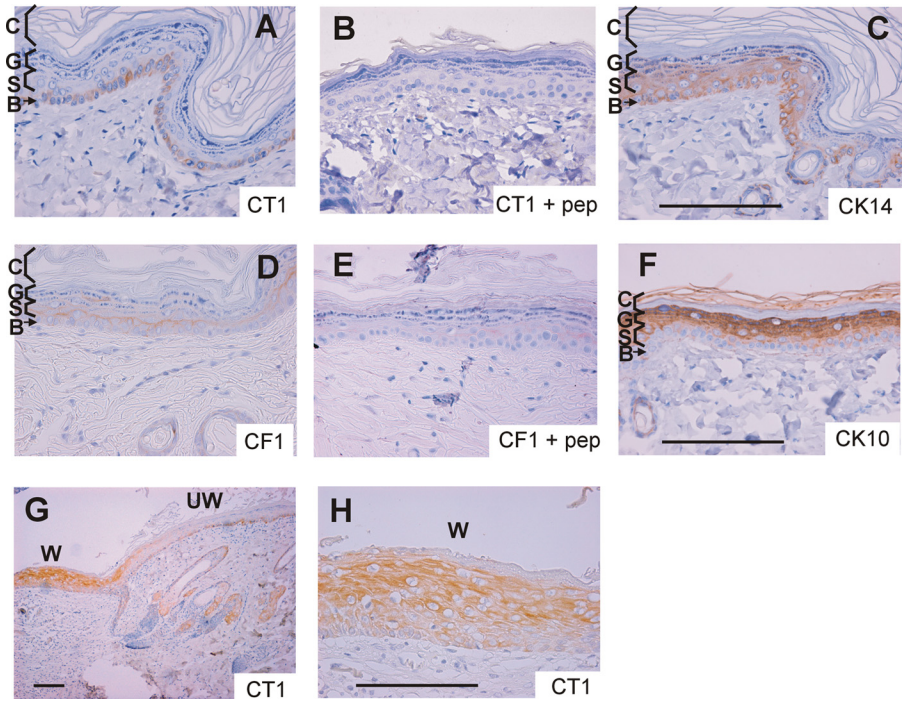

FIGURE 3. **Immunostaining in rat skin.** *A*, immunoreactivity to CT1 antibody is shown in basal cell layers of skin. *B*, CT1 antibody that had been preabsorbed with the antigen peptide (*pep*) did not stain the basal cells. *C*, anti-CK14 antibody mainly recognized undifferentiated layers (see "Results"). *D*, CF1 antibody stained more differentiated cell layers. *E*, CF1 antibody that had been preabsorbed with the antigen peptide did not stain the undifferentiated layers. *F*, anti-CK10 antibody recognized the stratum spinosum and stratum granule. *G* and *H*, rat skin wounds. In *G*, unwounded (*UW*) and wounded (*W*) epithelia can be seen. Hair follicles were also stained. *B*, stratum basale; *S*, stratum spinosum; *G*, stratum granule; *C*, stratum corneum. Counterstaining: hematoxylin. *Bars* = 100 μm.

was much less in the soluble subfraction, suggesting weak solubility of this protein in the detergent. There was no immunoreactivity in the cytosolic fraction.

In contrast, in the rCLCA-f-transfected cells (Fig. 4*A*, *lane F*), the antibody recognized 86- and 120-kDa-sized bands in the 1,000 × *g* Sup, and the immunoreactivities were almost equal between the Triton X-100-soluble and -insoluble membrane fractions, indicating a relatively easy solubilization of this isoform. Immunoreactivity was lowest in the cytosolic fraction. The 86- and 120-kDa-sized rCLCA-f bands corresponded to the N-terminal part and the entire length of the protein, respectively (Fig. 1*A*), the former probably being produced by post-translational proteolysis (*closed triangle*). This modification was not detected for the rCLCA-t protein.

Then, we examined whether rCLCA isoforms might be expressed heterologously on the plasma membrane by investigating the TC-inducible expression of rCLCA-t and -f in stably transfected cells (sT and sF cells). Surface expression was assessed by detecting the biotinylation of membrane proteins on these cells. Streptavidin-HRP failed to recognize the

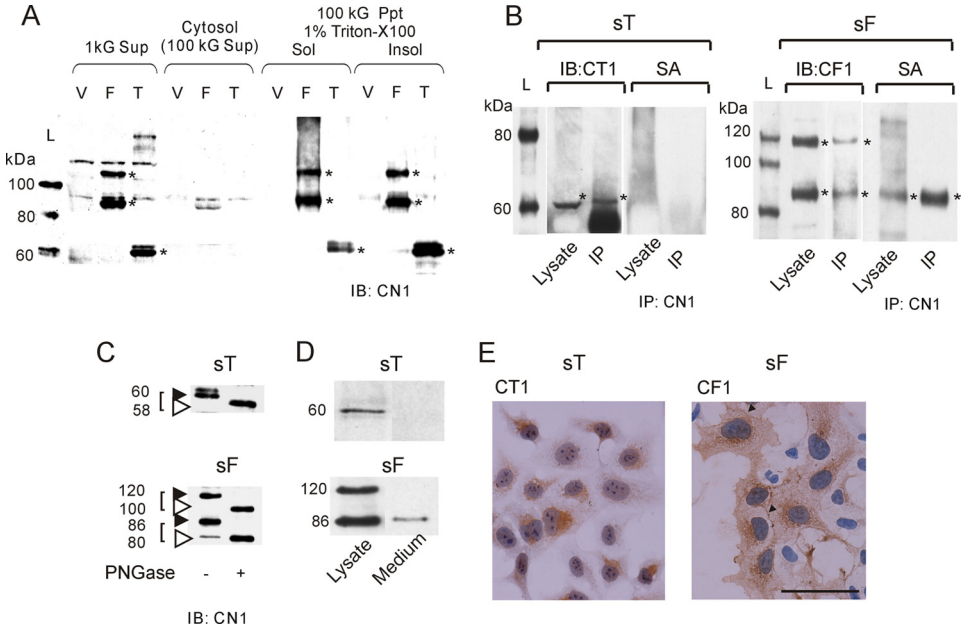

FIGURE 4. **Western blotting and immunocytochemistry using transfected HEK293 cells.** *A*, the 1,000 × *g* and 100,000 × *g* Sup and the 1% Triton X-100-soluble (*Sol*) and -insoluble (*Insol*) membrane pellet (*Ppt*) were obtained from transiently transfected cells (*F*, rCLCA-f; *T*, rCLCA-t; *V*, vehicle). Protein loading was 10 μg per lane for 1,000 × *g* and 100,000 × *g* Sup, and 50 μg per lane for soluble and insoluble fractions. * denotes specific bands at 120 and 86 kDa for rCLCA-f, and 60 kDa for rCLCA-t. *IB*, immunoblotting. *B*, cells subjected to stable transfection with either rCLCA-t (sT) or rCLCA-f (sF) were surface-biotinylated, lysed, and immunoprecipitated using CN1 antibody. The biotinylated proteins in the cell lysate and its immunoprecipitate (*IP*) were detected using streptavidin (*SA*)-HRP (*). Immunoreactive bands were detected using CT1 or CF1 antibody (*). *C*, peptide-*N*-glycosidase F (*PNGase*)-induced migration of rCLCA-f and rCLCA-t bands to lower molecular weights. *Closed triangle*, glycosylated. *Open triangle*, nonglycosylated. *D*, culture medium taken from the transfected cells and total cell lysate were subjected to the affinity resins, and the bound CLCA was eluted and applied to Western blotting using CT1 or CF1 antibody. *E*, immunocytochemistry using anti-CLCA antibodies in permeabilized sT or sF cells. *Arrowheads* indicate intense staining of rCLCA-f on the plasma membrane. Counterstaining: hematoxylin. *Bar* = 50 μm.

60-kDa-sized, CT1 antibody-immunoreactive protein in the lysate, although it did recognize the 86-kDa-sized, CF1 antibody-immunoreactive protein (Fig. 4*B*). Next, rCLCA-t protein was immunoprecipitated from the cell lysate using CN1 antibody. The immunoreactivity of the 60-kDa protein was confirmed by immunoblotting using CT1 antibody. Streptavidin again failed to detect this immunoprecipitated protein, but did recognize the 86-kDa rCLCA-f protein immunoprecipitated using CN1 antibody (Fig. 4*B*). These results suggest that rCLCA-t is unlikely to be a surface protein, whereas the N-terminal 86-kDa portion (modified post-translationally) of rCLCA-f is a surface protein.

Treatment with peptide-*N*-glycosidase F, which can remove *N*-glycan from glycosylated proteins, demonstrated that both isoforms (rCLCA-t, 60 kDa; rCLCA-f, 86 and 120 kDa) are *N*-glycosylated proteins (Fig. 4*C*, *closed triangles*). The corresponding nonglycosylated forms (Fig. 4*C*, *open triangles*) were found to be as follows: rCLCA-t, ~58 kDa; rCLCA-f, 80 and 100 kDa.

Several members of the CLCA protein family have been shown to be secreted into the extracellular environment (28–30). Because the amino acid sequence of rCLCA-t lacks half of the C-terminal part of the full-length CLCA, it seemed possible that this isoform is secreted from cells too. However, in cells stably transfected with histidine-tagged rCLCA-t cDNA, immunoreactivity for a 60-kDa band was found in the cell lysate, but not in the elution from the medium (Fig. 4*D*). This implies that rCLCA-t is unlikely to be a secreted protein. In the histidine-tagged rCLCA-f-transfected cells, 120- and 86-kDa bands were immunoreactive in the cell lysate, but only the 86-kDa band was detectable in the medium (Fig. 4*D*). This suggests that the post-translationally modified N-terminal part, but not the unmodified rCLCA, may be secreted.

Next, we performed immunocytochemistry using permeabilized, stably transfected cells. CT1 antibody predominantly recognized the TC-induced expression of rCLCA-t in the perinuclear area of the transfected cells, with no staining being evident near the cell membrane. In contrast, rCLCA-f appeared to be expressed in more peripheral areas of the cell than rCLCA-t, with intense linear labeling on the cell-to-cell interface, probably indicating its presence on the plasma membrane (Fig. 4*E*, *arrowheads*).

rCLCA-t Does Not Alter Ca^{2+}-induced Cl^- Conductance—We examined whether rCLCA-t might exhibit an electrophysiological function similar to that reported for rCLCA-f: namely, an ability to increase Ca^{2+}-induced Cl^- conductance (11). Fig. 5, *A* and *B*, show typical current traces recorded during step pulses or voltage ramps before and after ionomycin (2 μM) was added (to cause a drastic increase in the intracellular Ca^{2+} concentration). In the Western blot study, there was estimated to be a similar amount of immunoreactivity per sT or sF cell between rCLCA-t and surface rCLCA-f (86 kDa), suggesting that the expression levels of these isoforms are almost the same (Fig. 5*C*). The summarized data (Fig. 5*D*) show that, in the absence of TC (−TC), the background current was negligible in the sT cells, whereas a small increase could be observed in sF cells, suggesting a leaky expression of the rCLCA protein under the present experimental conditions. After TC treatment

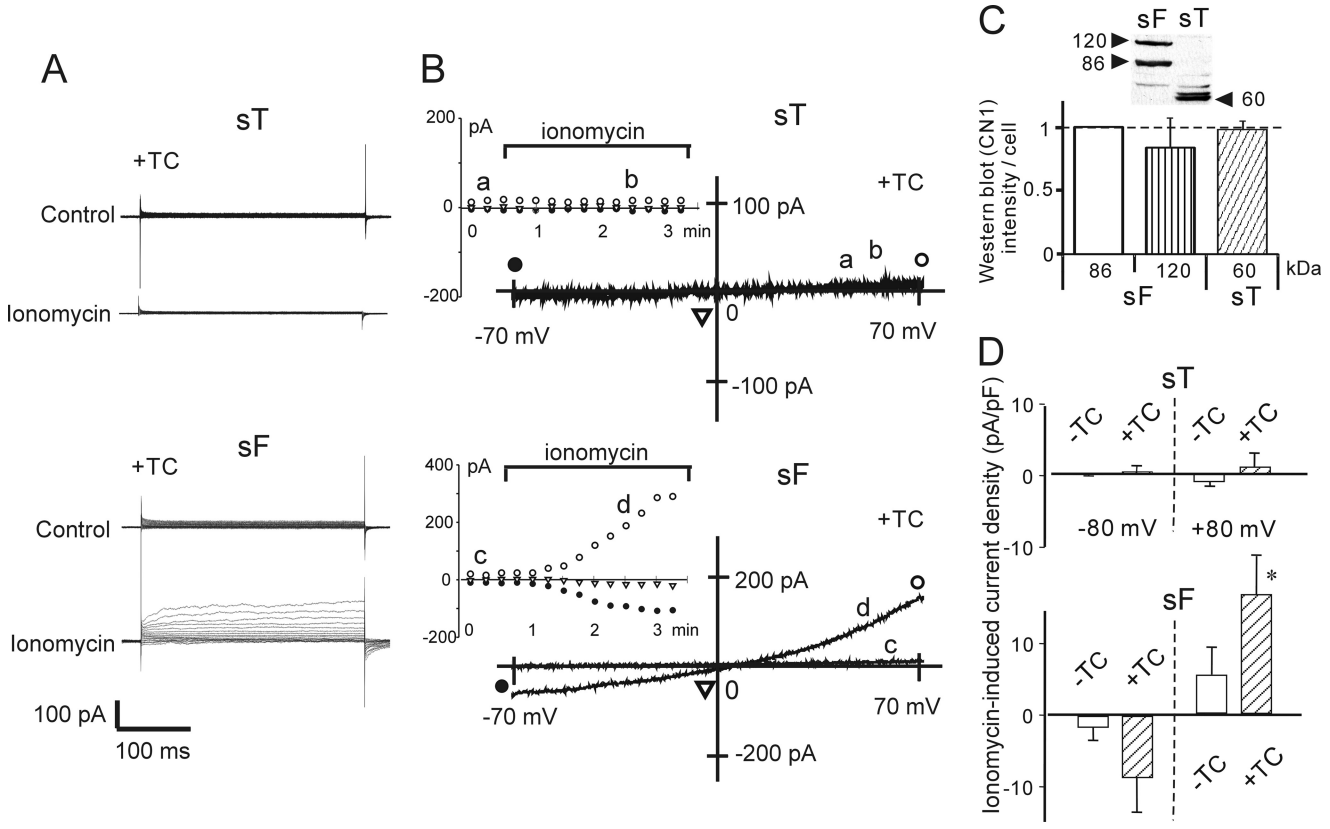

FIGURE 5. **Ionomycin-induced currents recorded from cells transfected stably with either rCLCA-t (sT cells) or rCLCA-f (sF cells).** The whole-cell patch clamp method was applied to cells without (−*TC*) or with (+*TC*) 24-h tetracycline treatment to induce expression of the relevant isoform. Ionomycin (2 μM) was used to stimulate Ca^{2+}-activated Cl^- conductance. *A* and *B*, typical current traces recorded during step pulses (−80 to 80 mV, every 10 mV; 300-ms duration) or during 300-ms voltage ramps (−70 to 70 mV, at time points *a–d* indicated in *inset figures*) before or after the addition of ionomycin to transfected cells treated with TC. *Inset figures* in *B* show time-course data for currents recorded at −70 mV (*open circles*), 0 mV (*reversed triangles*), and 70 mV (*closed circles*). Capacitance, 24.5 pF (sF cells) or 16.1 pF (sT cells). *C*, expression of rCLCA-t was quantitatively similar to that of the rCLCA-f located on the cell surface (86 kDa) in a single sT or sF cell. Cell lysates were obtained from sT and sF cells (+*TC*). Intensity of Western blot obtained using CN1 antibody was quantified (*inset*), and the relative values in a single cell were calculated and normalized to that of the 86-kDa band observed in sF cells. $n = 4$. *D*, summary of difference current density ($n = 4–6$) evoked by voltage steps (300 ms) from −50 mV to −80 mV or 80 mV. Current density was calculated by dividing current amplitude by membrane capacitance. Ionomycin-induced current density was obtained by subtracting the density before ionomycin perfusion from that during the perfusion. *, $p < 0.05$ *versus* corresponding value in sT cells (Student's or Welch's *t* test). *pF*, picofarads.

(+TC), the current was not altered during voltage steps or ramps in sT cells (Fig. 5, *A*, *B*, and *D*, *upper*), but it was substantially enhanced in sF cells (Fig. 5, *A*, *B*, and *D*, *lower*). This lack of such an electrophysiological function for rCLCA-t is supported by the above evidence that there is no surface expression of this isoform.

rCLCA-t Decreases Integrin-dependent Cell Attachment in Response to Increased Intracellular Ca^{2+}—During patch clamp experiments, we noticed that sT cells were easily detached from collagen-coated coverglasses. We made a quantitative comparison between cells transfected stably with one of the rCLCA isoforms and mock-transfected cells for their adherence to collagen-coated plates (24-well) in the presence or absence of either ionomycin or thapsigargin (TG), each of which increases intracellular Ca^{2+} (Fig. 6). In the mock and sF cells, adhesion capability tended to decrease to ~75–85% and ~65–80% of control, respectively, when either ionomycin (2 μM) or thapsigargin (5 μM) was added, depending on the period of drug application. In sT cells, the reduction in adhesion capability induced by either drug was greater than in the above two cell types regardless of the period of drug application (Fig. 6).

We then evaluated cell adherence using another protocol; in this one, the cells were suspended in ionomycin-containing PSS immediately before plating. A 20-min incubation with ionomycin substantially impaired adhesion capability in all groups (Fig. 7*A*; *columns* with *horizontal hatching*), although it was suppressed most strongly in sT cells. This reduction was partially reversed by subsequent additional (for 10 or 20 min) incubation with EGTA to chelate external Ca^{2+} and to avoid Ca^{2+} influx into the cells. In all groups, adhesion capability was also restored by incubation with phorbol-12, 13-dibutyrate (PDBu, 100 nM), which activates protein kinase C (Fig. 7*A*).

HEK293 cells have been shown to express β_1-integrin proteins (31, 32), most of which form receptors for collagen (33). We therefore speculated that rCLCA-t might alter the expression of endogenous β_1-integrin protein. To test whether activation of β_1-integrin can rescue the adherence of sT cells (Fig. 7*B*), we examined the effect of ionomycin in cells pretreated with an activating anti-β_1-integrin antibody (TS2/16) (34). The pretreated cells were exposed to PSS with or without ionomycin (2 μM, 20 min, 37 °C) at 1 to 20 min after the beginning of the adhesion (37 °C). In the absence of the activating antibody,

ionomycin reduced adhesion to 50% of control in sT cells, but only to 90–94% of control in the mock and sF cells. Pretreatment with TS2/16 (2.5 μg/ml, 20 min, room temperature) partially restored adhesion capability (73% of control) in sT cells (Fig. 7*B*).

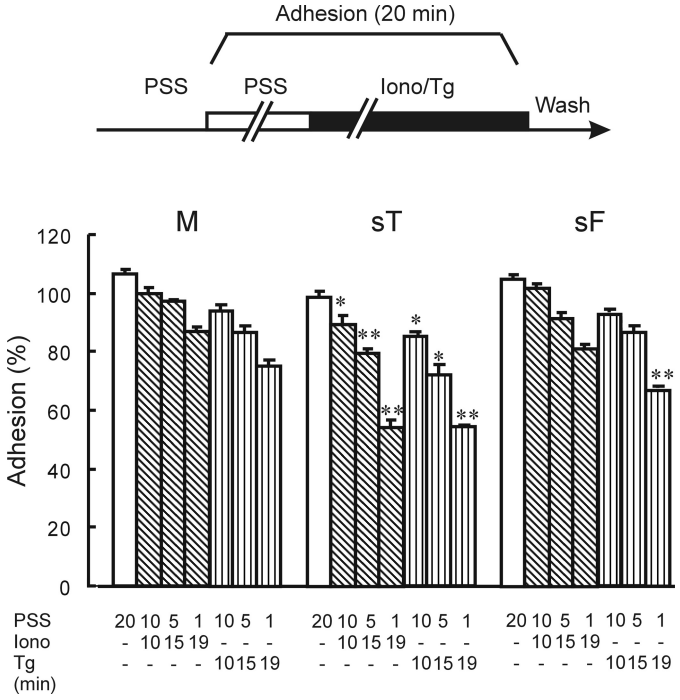

FIGURE 6. **Ionomycin-induced and thapsigargin-induced detachment of mock (*M*), sT, or sF cells.** The *inset* shows the time course of the adhesion assay. Cells were settled on collagen-coated wells for 20 min at 37 °C. The percentage of adhesion was calculated as described under "Experimental Procedures." Ionomycin (*Iono*, 2 μM) or thapsigargin (*Tg*, 5 μM) treatment was given for the time indicated. $n = 4$. *, $p < 0.05$, **, $p < 0.01$ *versus* corresponding value in mock cells (one-way ANOVA followed by a post hoc Bonferroni's *t* test).

Typically, β_1-integrin protein is first synthesized (87 kDa) and then core-glycosylated to generate the so-called "premature form" (110 kDa), which dimerizes with an α subunit in the endoplasmic reticulum. The dimer is then transported to the Golgi apparatus where it matures to a 130-kDa form that expresses complex *N*-linked glycan (35, 36).

Cell lysate was obtained from mock, sT, and sF cells that had been pretreated with either ionomycin or PDBu for 20 min. Western blots obtained using anti-β_1-integrin antibody revealed that ionomycin decreased the expression of mature β_1-integrin (130 and 110 kDa) in all three types of cells, whereas PDBu tended to increase it. The inhibitory action of ionomycin was most marked in sT cells (Fig. 7*C*).

rCLCA-t Alters the Subcellular Localization of the Mature Form of β_1-Integrin—We examined the localization of endogenous β_1-integrin in immunofluorescence experiments (Fig. 8*A*). In mock and sF cells, β_1-integrin was located near the surface membrane (*arrowheads*) and in the perinuclear region. In contrast, β_1-integrin became localized mainly to the perinuclear site after transfection of rCLCA-t (*arrows*).

Next, mock and sF cells were incubated with ionomycin (2 μM, 5–20 min, 37 °C). The drastic increase in the intracellular Ca^{2+} concentration did not alter the localization of β_1-integrin in these cells (Fig. 8*A*). In sT cells, the β_1-integrin distribution obtained during ionomycin treatment was much less marked in surface membranes, but more marked in perinuclear regions (*arrows*). Some of the other cells exhibited strikingly distinct morphology, the nucleus having become rounded and shrunken.

The activating anti-β_1-integrin antibody TS2/16 (5 μg/ml, 30 min, 4 °C) increased the peripheral ring-like immunostaining of β_1-integrin in sT and sF cells in the presence or absence of ionomycin, indicating a tighter adhesion to the

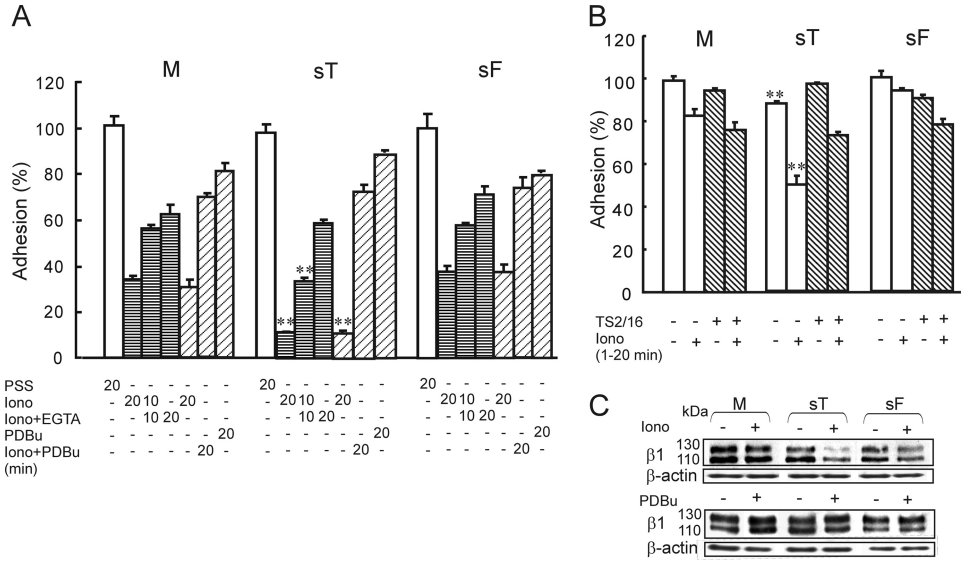

FIGURE 7. **Effects of EGTA, PDBu, and a β_1-integrin-activating antibody on ionomycin (*Iono*, 2 μM)-induced detachment of mock (*M*), sT, or sF cells.** *A*, concomitant incubation with EGTA (1.8 mM; free Ca^{2+} concentration = 10 μM) or PDBu (100 nM) partly restored adhesion capacity. *B*, a β_1-integrin-activating antibody, TS2/16 (5 μg/ml), partly reversed the ionomycin-induced impairment of the adhesion of sT cells. Adhesion capability was assessed using cells exposed to PSS (with or without ionomycin) from 1 to 20 min after the beginning of adhesion of the TS2/16-pretreated or nontreated cells to the collagen-coated wells. $n = 4$. **, $p < 0.01$ *versus* corresponding value in mock cells (one-way ANOVA followed by a post hoc Bonferroni's *t* test). *C*, Western blot experiments using anti-β_1-integrin (clone 18) and anti-β-actin antibodies. Cell lysates were obtained from mock, sT, and sF cells pretreated with ionomycin (2 μM) or PDBu (100 nM) for 20 min. Ionomycin decreased the β_1-integrin expression, particularly in sT cells.

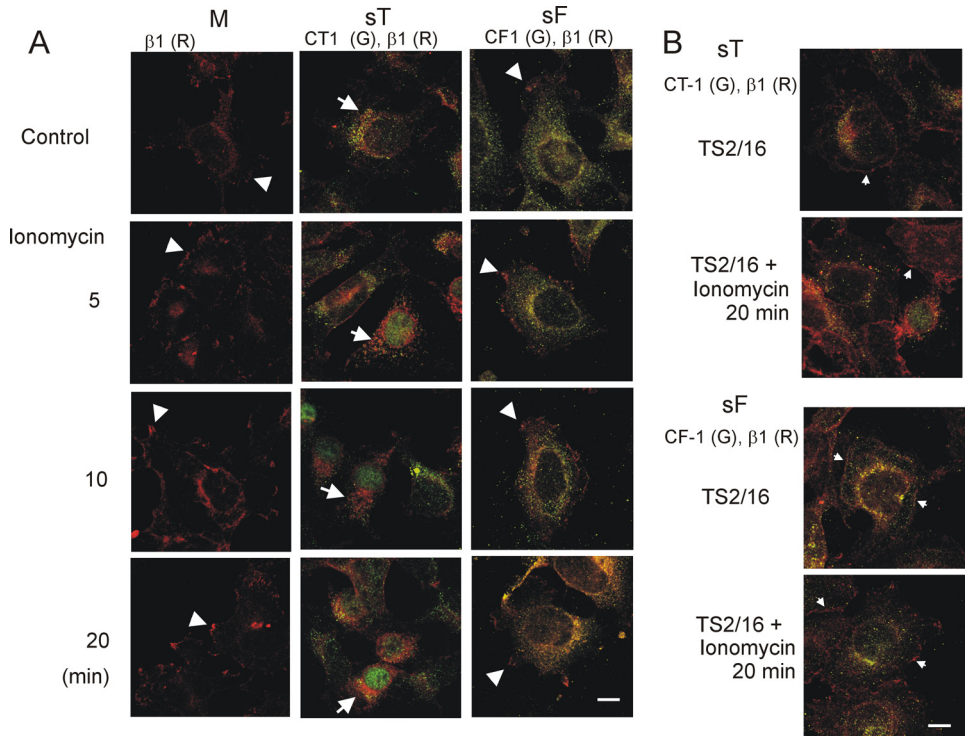

FIGURE 8. **Immunofluorescence labeling for rCLCA-f, rCLCA-t, and β_1-integrin in mock (*M*), sT, or sF cells in the presence of ionomycin (2 μM, 37 °C; for the time indicated) and in its absence.** *A*, localization of β_1-integrin was distinctly different in sT cells *versus* the other types of cells (*arrowheads* and *arrows*). *Bar* = 10 μm. *B*, treatment with a β_1-integrin-activating antibody, TS2/16 (5 μg/ml), partly restored the subcellular localization of β_1-integrin in sT cells. *Arrows* indicate that TS2/16 created a peripheral, ring-like distribution of β_1-integrin. *Green* (*G*), CF-1 antibody was used for rCLCA-f, CT1 antibody was used for rCLCA-t, and no antibody was used for mock cells. *Red* (*R*), anti-β_1-integrin antibody. *Bar* = 10 μm.

collagen-coated coverglass (Fig. 8*B*, *arrows*). Occasionally, ionomycin still caused β_1-integrin to locate around the nucleus of some sT cells.

The surface and subcellular expressions of the glycosylated forms of β_1-integrin were examined by means of surface biotinylation techniques. The 130-kDa β_1-integrin was found to be surface-biotinylated (Fig. 9*A*), indicating that a mature, fully glycosylated type (β_1) is localized on the cell surface. Stable transfection with rCLCA-t tended slightly to attenuate the intensity of the 130-kDa band in the total cell lysate, and it significantly decreased (by 20%) the expression of the surface-biotinylated 130-kDa form (Fig. 9*B*).

In contrast, the 110-kDa premature β_1-integrin was found to be present intracellularly (Fig. 9*A*). The intensity of the 110-kDa band in the total lysate and intracellular unbiotinylated form tended to be increased by transfection with rCLCA-t and to be inhibited by rCLCA-f (Fig. 9*B*). Immunofluorescence obtained using anti-calnexin antibody revealed that β_1-integrin colocalized extensively with calnexin, a marker of endoplasmic reticulum, in sT cells (Fig. 9*C*).

Among proteins known to interact with β_1-integrin and to promote cell adhesion, we focused on a scaffolding intracellular protein, RACK1, a receptor for activated C kinase that is reported to be modified by PKC and intracellular Ca^{2+} (37). RACK1 was mostly localized in intracellular regions, but some was found to be associated with biotinylated proteins on surface membranes (Fig. 9*A*). When compared with that in mock or sF cells, the surface expression of RACK1 was lower in sT cells, in which the surface localization of β_1-integrin was attenuated (Fig. 9*A*).

Combining Triton X-100 (1%) and sodium deoxycholate (0.1%) was found to produce a detergent that dissolved both RACK1 and rCLCA-t together (supplemental Fig. S3). Under the Triton X-100 + sodium deoxycholate condition, RACK1 was successfully co-immunoprecipitated by CT1 antibody, but not by CF1 antibody. Reciprocally, rCLCA-t, but not rCLCA-f, was co-immunoprecipitated by anti-RACK1 antibody (Fig. 9*D*). Any association of β_1-integrin with either rCLCA isoform was barely detectable, although an interaction was confirmed with α_2-integrin (Fig. 9*D*), which is known to associate with a β_1-integrin to form a receptor for collagen (33). These results suggest the existence of a specific interaction between RACK1 and rCLCA-t in the membrane fraction.

Knockdown of rCLCA-t Alters Expression of β_1-Integrin in Skin—To examine the influence of rCLCA-t over the expression of β_1-integrin in skin, an siRNA was generated for isoform-specific knockdown of rCLCA-t (see supplemental Fig. S4). Transfection of an siRNA together with CGM has been reported to have a long-lasting effect on the expression of the target molecule (23). In control (scrambled RNA + CGM-treated) skin, a confocal microscopic study showed that rCLCA-t displayed an intracellular localization. Surface expression of β_1-integrin was detected in cells in the basal layers (Fig. 10*A*). In siRNA + CGM-treated skin, expression of β_1-integrin was significantly augmented in the area within which rCLCA-t localization was depressed (Fig. 10*B*). When compared with the control skin, siRNA + CGM-treated skin exhibited a weaker intensity rCLCA-t band (CT1 antibody) in Western blots, but a greater β_1-integrin intensity (Fig. 10*C*). In rat skin, RACK1 was co-immunoprecipitated by CT1 antibody, and rCLCA-t was

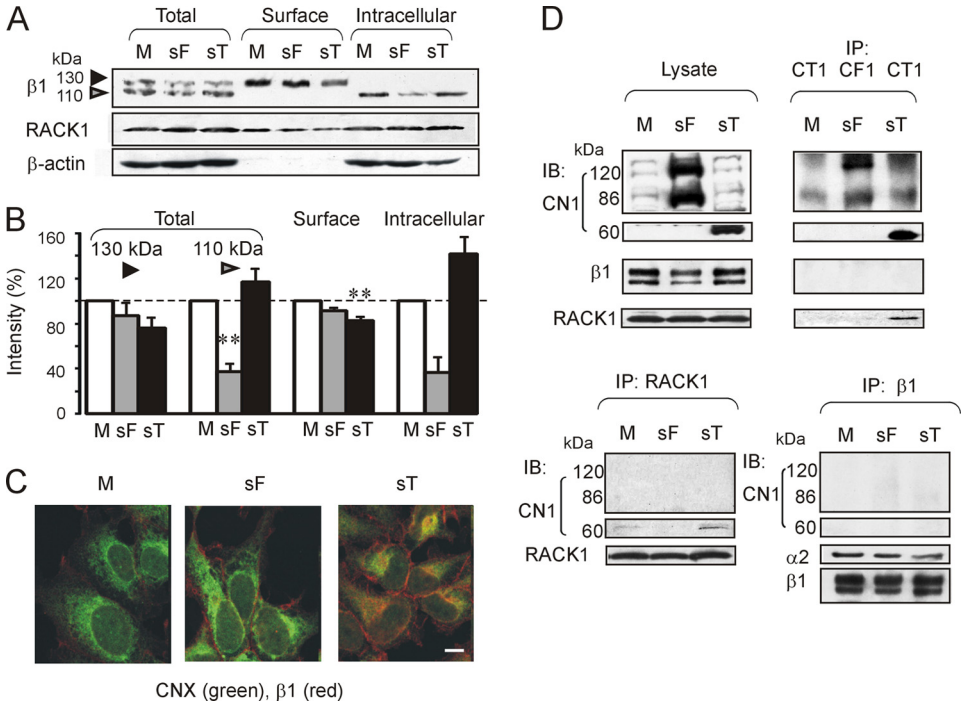

FIGURE 9. **Expression of β_1-integrin in mock (*M*), sT, and sF cells.** *A*, isolation of β_1-integrin on cell surface. Mock, sT, and sF cells were labeled with Sulfo-NHS-SS-Biotin to collect cell-surface proteins. Flow-through fraction was collected for detection of intracellular proteins. The protein samples were used for SDS-PAGE and immunoblotting using anti-β_1-integrin (clone 18), anti-RACK1, and anti-β-actin antibodies. *B*, densitometric analysis of the blots shown in *A* ($n = 4-6$). The intensities of the immunoblot bands obtained from sT and sF cells were normalized to that obtained from mock cells. **, $p < 0.01$ *versus* mock cells (multivariate ANOVA followed by a post hoc Bonferroni's *t* test). *C*, immunofluorescence study showing that β_1-integrin (TS2/16) colocalized with calnexin (*CNX*), a marker of endoplasmic reticulum, in sT cells. *Bar* = 10 μm. *D*, interaction of rCLCA-t with RACK1. rCLCA isoforms, RACK1, and β_1-integrin were immunoprecipitated by the corresponding antibody using the Triton X-100 plus sodium deoxycholate-soluble fractions obtained from mock, sT, or sF cells. The co-immunoprecipitant (*IP*) was subjected to immunoblotting (*IB*).

reciprocally co-immunoprecipitated by anti-RACK1 antibody (Fig. 10*D*). Immunofluorescence reveals that RACK1 extensively colocalized with rCLCA-t, but not with β_1-integrin (Fig. 10*E*).

DISCUSSION

In the present study, we report a novel splicing isoform of rCLCA. Comparison of its cDNA sequence with the rat genome suggests that it is likely to be an exon 9 splicing variant. We initially supposed that rCLCA-t might be a secreted protein because it possessed an N-terminal signal sequence and had no significant hydrophobic domain in the other portion. However, the present results indicate that rCLCA-t is unlikely to be translocated to the plasma membrane or secreted into the culture medium. Therefore, such a limited subcellular localization of the splicing variant suggests that it might perform a cellular function distinct from that of the full-length isoform, rCLCA-f. Indeed, we were unable to detect any electrophysiological activity of this isoform.

An additional striking finding was that the distribution of rCLCA-t differed from that of rCLCA-f in the epithelial cells of rat SMG and skin. Immunohistochemistry clearly demonstrated a predominant perinuclear expression of rCLCA-t in undifferentiated basal cells and a reciprocal expression of rCLCA-f in differentiated epithelial cells. In addition, dense expression of rCLCA-t was found broadly across the epithelial cell layers of wounded skin, where reproduction of the epidermis takes place. These observations raise the possibility that the truncated isoform is localized specifically to subserve a function specific to epithelial cells with an undifferentiation status. Previously, reciprocal patterns of expression of truncated isoforms in epidermis have been reported for other genes closely related to cell differentiation and proliferation. For example, the p63 gene encodes two isoforms, the full TAp63 and a truncatedΔNp63 (38), consistent with p63 performing dual roles (39, 40). Likewise, the reciprocal expression of the rCLCA isoforms reported here could indicate roles in the regulation of cell growth and differentiation in salivary glands and epidermis.

Our results reveal an inhibitory effect of rCLCA-t on cell adhesion, suggesting reduced expression or recruitment of adhesion molecules to the cell membrane. Interestingly, we found that rCLCA-t altered both the maturation of β_1-integrin and its subcellular distribution; specifically, rCLCA-t decreased the surface expression of the fully glycosylated form of β_1-integrin. It is reasonable to speculate that rCLCA-t may associate directly with β_1-integrin. There are published examples of expressions of certain membrane proteins (vacuolar H^+-ATPase subunit and low density lipoprotein receptor-related protein-1) being accompanied by shifts both in the degree of maturation and in the subcellular localization of β_1-integrin (36, 41). Previously, CLCA family members have been reported to possess a von Willebrand A domain, which is known to be involved in the cell adhesion mediated by α- and β-integrins (42). Because we were unable to support this idea by co-immunoprecipitation analysis, it is possible that rCLCA-t affects integrin functions indirectly, through an adaptor protein.

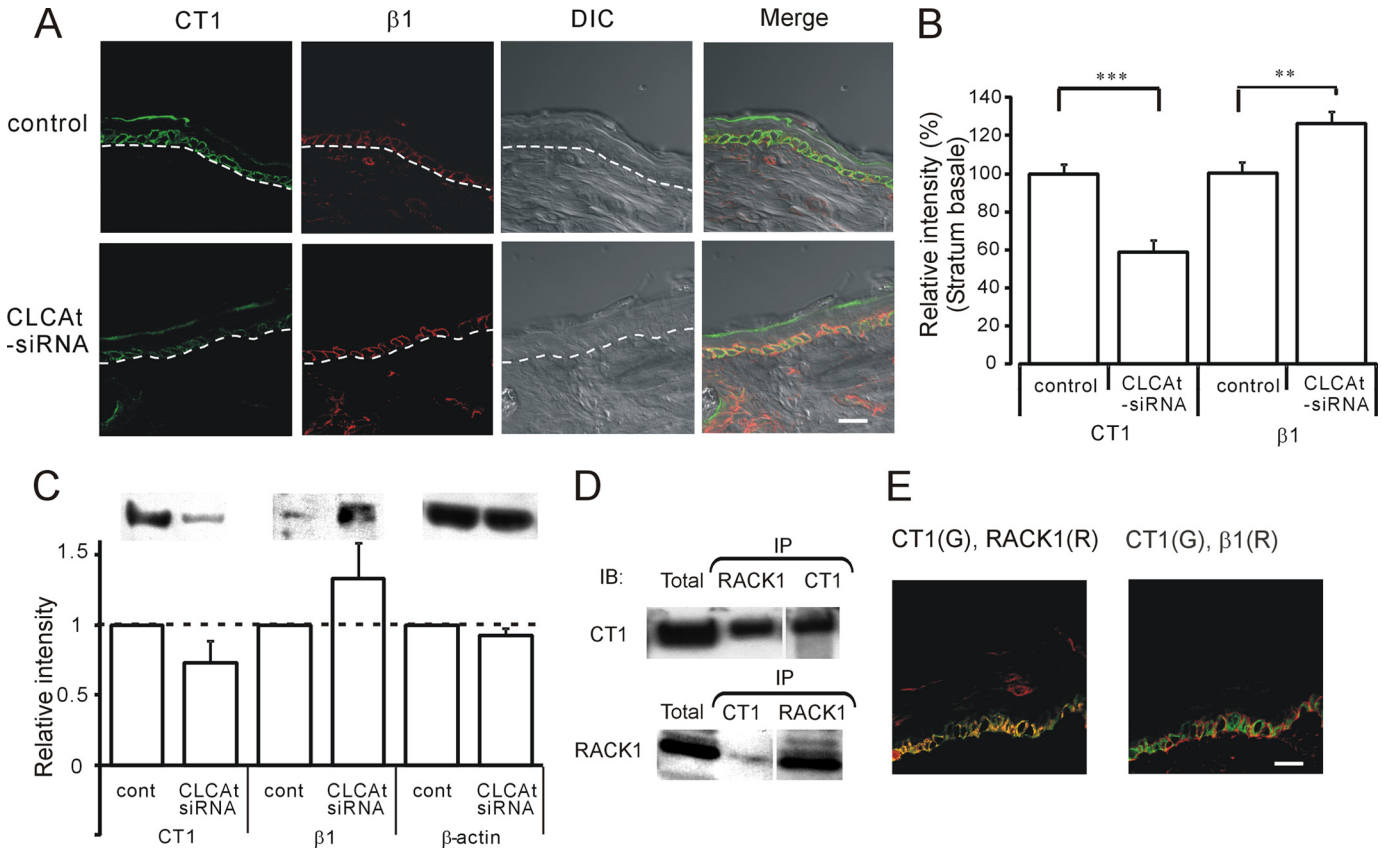

FIGURE 10. **Modulation of β_1-integrin expression by rCLCA-t in rat skin.** *A*, changes in rCLCA-t and β_1-integrin in the stratum basale of rat earlobe following epidermal application of siRNA. Immunofluorescence labeling was detected for rCLCA-t and β_1-integrin in the stratum basale following epidermal application of siRNA. *Green*, CT1 antibody. *Red*, anti-β_1-integrin antibody (HMβ1-1). *Bar* = 20 μm. *DIC*, differential interference contrast. *B*, densitometric analysis of expressions of rCLCA-t and β_1-integrin. The intensities of the immunofluorescence measured within the basal layer at the injection site were normalized to that measured at a noninjected site. $n = 13–14$. **, $p < 0.01$, ***, $p < 0.001$ *versus* the control RNA-injected earlobe (Student's *t* test). *C*, Western blot of 6,500 $\times$ *g* Sup obtained from rat earlobe at 1 week after injection into the earlobe of microspheres incorporating either rCLCA-t siRNA or control (*cont*) RNA. CT1, anti-RACK1, and anti-β-actin antibodies were used. Densitometric analysis of the blot is shown. $n = 4$. *D*, interaction of rCLCA-t with RACK1 in rat skin. rCLCA-t and RACK1 were immunoprecipitated by the corresponding antibodies. The co-immunoprecipitant was subjected to immunoblotting (CT1 and anti-RACK1 antibodies). Representative result of three experiments. *E*, immunofluorescence labeling for rCLCA-t (CT1), RACK1, and β_1-integrin in rat skin, showing that rCLCA-t colocalized with RACK1, but not with β_1-integrin (HMβ1-1). *Bar* = 20 μm.

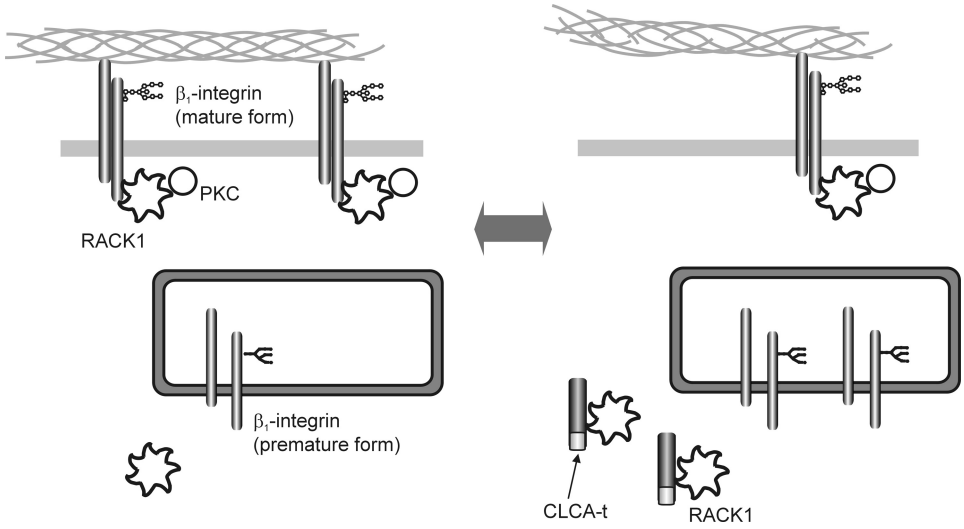

FIGURE 11. **Model illustrating how β_1-integrin expression may be attenuated by expression of rCLCA-t.** The scaffolding protein RACK1 is presumed to facilitate the surface expression of the mature form of β_1-integrin (*left*). rCLCA-t interacts with RACK1 intracellularly (presumably within the Triton X-100-insoluble membrane fraction). This interaction is likely to inhibit recruitment of the mature form to the surface membrane and thereby to decrease the adhesive potential of the cell (*right*).

Proteins that have been shown to interact with β-integrin cytoplasmic domains include actinin, paxillin, talin, focal adhesion kinase, integrin-linked kinase-1, and so on. We found that an increase in intracellular Ca^{2+} (induced by ionomycin or thapsigargin) led to a reduction in the adhesion of rCLCA-t-transfected cells, which PDBu and TS2/16 partly restored. Therefore, phosphorylation/dephosphorylation of some adaptor or scaffolding proteins interacting with rCLCA-t is likely to be important for its regulation of integrins. RACK1, a member of the Gβ superfamily, is known to bind to activated PKC (43), to interact with the cytoplasmic tail of β_1-integrin, and to promote cell adhesion (37, 44). Activation of PKC by phorbol esters and the resultant phosphorylation of RACK1 is reported to be necessary for the association of RACK1 with β_1-integrin (37). In addition, knockdown of RACK1 by siRNA has been shown to attenuate cell adhesion to collagen (45). In contrast, a Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, has been shown to dephosphorylate RACK1 and to block RACK1-mediated functions (46). Previously, it was shown that signaling proteins, such as β_1-integrin and phosphatase 2A, compete for binding to a common site on RACK1 and that the binding preference is switched according to phosphorylation status (47). Our findings of (*a*) a specific association between rCLCA-t and RACK1 and (*b*) a reduced surface expression of both β_1-integrin and its scaffolding protein, RACK1, indicate a possible mechanism by which rCLCA-t might inhibit recruitment of integrins to RACK1 and so impair their adhesive capability (Fig. 11). Through such an interaction between these molecules, an inhibitory effect of rCLCA-t on the expression of β_1-integrin in focal adhesion could result in a reduced resistance to the cell detachment induced by compounds that increase intracellular Ca^{2+}.

We found that an isoform-specific knockdown of rCLCA-t increased the expression of β_1-integrin in the basal layers of rat skin. Because most β_1-integrins adhere to the extracellular matrix, these are likely to provide a physical support within tissues and also to regulate cell function (33). Reportedly, integrins, including β_1-integrin, function not just as direct mediators of adhesion, but also as regulators of epidermal stratification, differentiation, and morphogenesis (48). Signal transduction through β_1-integrin represents an instruction to keratinocytes not to differentiate (22). The above results point to the interesting hypothesis that a causal relationship may exist between rCLCA-t expression and the integrin-mediated cell adhesion and differentiation of native SMG and skin. Intriguingly, a previous study pointed out a close association between CLCA and basal-cell adhesion (a human isoform, hCLCA2, being shown to be colocalized with β_4-integrin in the basal cells of human stratified epithelia) (49).

Overall, the findings made in the present study suggest that the truncated isoform of rCLCA is likely to be expressed preferentially in undifferentiated epithelial cells. We also demonstrated that the isoform may play a role in the modulation of the adhesive potential of undifferentiated epithelial cells. It would be interesting to investigate further the role of the cell-specific rCLCA isoform in regulating the differentiation of epithelial cells.

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