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Human Keratinocyte ATP2C1 Localizes to the Golgi and Controls Golgi Ca\(^{2+}\) Stores

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Hailey–Hailey disease (MIM6960) is a blistering skin disease caused by mutations in the Ca\(^{2+}\) ATPase ATP2C1. We found that the abnormal Ca\(^{2+}\) signaling seen in Hailey–Hailey disease keratinocytes correlates with decreased protein levels of ATP2C1. Human ATP2C1 protein approximated 115 kDa in size. The ATP2C1 is localized to the Golgi apparatus in human keratinocytes, similar to its localization in yeast and *Caenorhabditis elegans*. To test whether the ATP2C1 controls Golgi Ca\(^{2+}\) stores, we measured intraorganelle Ca\(^{2+}\) concentrations using specifically targeted aequorins. Whereas normal keratinocytes display Golgi Ca\(^{2+}\) levels comparable to other epithelial cells, Hailey–Hailey disease keratinocyte Golgi Ca\(^{2+}\) refill is slower, and the maximum Ca\(^{2+}\) concentration reached is significantly lower. These findings were replicated in *vivo*, because clinically normal Hailey–Hailey disease epidermis contained lower Ca\(^{2+}\) stores and displayed an abnormal Ca\(^{2+}\) gradient. In this report we localize the ATP2C1, demonstrate its physiologic relevance in mammalian cells, and measure intraorganelle Golgi Ca\(^{2+}\) in keratinocytes.


Hailey–Hailey disease (HHD) is an autosomal dominant blistering skin disease, characterized histologically by defects in cell-to-cell adhesion in the suprabasal layers of the epidermis (acantholysis). HHD is a result of mutations in ATP2C1 (Hu et al., 2000; Sudbrak et al., 2000), a Ca\(^{2+}\) ATPase localized to the Golgi in *Caenorhabditis elegans* (Van Baalen et al., 2000) and whose homolog, PMR1, also is localized to the Golgi in yeast (Antebi and Fink, 1992). Histologic and immunofluorescence studies of HHD demonstrate internalization of desmosomal components, whereas adherens junctions and gap junctions are preserved (Hara-da et al., 1994; Hashimoto et al., 1995; Metze et al., 1996). In normal keratinocytes in *vivo*, Ca\(^{2+}\) stimulates the assembly of functional desmosomes, both by causing the formation of actin filopodia and by complexing desmosomal components including E-cadherin, α-, β-, and γ-catenin (Vasioukhin et al., 2000). Raised extracellular Ca\(^{2+}\) acts through a well-defined cascade of signaling events, including binding to a plasma membrane Ca\(^{2+}\) receptor, generation of inositol 1,4,5-trisphosphate (IP\(_3\)), release of intracellular Ca\(^{2+}\), and subsequent influx of Ca\(^{2+}\) through plasma membrane channels. HHD keratinocytes do not release intracellular Ca\(^{2+}\) when stimulated with raised extracellular Ca\(^{2+}\) (Hu et al., 2000), suggesting that ATP2C1 controls a Ca\(^{2+}\) store that is important in the keratinocyte response to raised extracellular Ca\(^{2+}\).

Although the central role of the PMR1 and Golgi calcium stores is well established in yeast (Sorin et al., 1997; Durr et al., 1998), the discovery that ATP2C1 mutations caused defects in both intracellular Ca\(^{2+}\) sequestration and intracellular Ca\(^{2+}\) signaling, and resulted in human disease, was the first indication of the importance of this Ca\(^{2+}\) ATPase in mammals (Hu et al., 2000; Sudbrak et al., 2000). Mutations found in HHD patients most often predict prematurely truncated products through frameshift mutations or single-base-pair substitutions, consistent with a haploinsufficiency pathogenesis (Hu et al., 2000). Endoplasmic reticulum (ER) calcium stores also are present in keratinocytes, and pharmacologic blockade of the Ca\(^{2+}\)* ATPase ATP2A2 with thapsigargin (TG) prevents Ca\(^{2+}\)-induced redistribution of E-cadherin to the cell periphery (Li et al., 1995). Mutations in the ATP2A2 cause Darier’s disease (Sakuntabhai et al., 1999), a similar blistering skin disease. In contrast, the role of ATP2C1-controlled Golgi Ca\(^{2+}\) stores is clear. In keratinocytes, Ca\(^{2+}\)-sensitive processes such as growth, differentiation, and adhesion have not yet been defined. The yeast homolog to the ATP2C1, PMR1, localizes to the medial Golgi (Sorin et al., 1997) and can raise Golgi Ca\(^{2+}\) concentrations to levels as high as 1 mM (Taylor et al., 1997). In yeast, intraorganelle Ca\(^{2+}\) controls Golgi transport (Carnell and Moore, 1994), N-linked glycosylation, accurate sorting of carboxypeptidase Y, and the appropriate degradation of Cyp Y, a misfolded soluble ER protein (Durr et al., 1998). PMR1 mutants are intolerant to raised extracellular Ca\(^{2+}\) and demonstrate higher cytosolic Ca\(^{2+}\) concentrations (Halachmi and Eilam, 1996), similar to that seen in HHD keratinocytes (Hu et al., 2000).
Experiments demonstrating TG-sensitive and TG-insensitive cellular stores suggested that a Ca\(^{2+}\) ATPase distinct from the ATP2A2 was linked to IP\(_3\)-sensitive Ca\(^{2+}\) pools (Pizzo et al, 1997). These findings were confirmed and expanded by the discovery that the homologous PMRI ortholog expressed in C. elegans controls an IP\(_3\)-sensitive Ca\(^{2+}\) store located in the Golgi (Van Baalen et al, 2001). Human ATP2C1 is homologous but not identical to yeast PMR1 (Rudolph et al, 1989) and encodes two alternatively spliced transcripts, ATP2ClA and ATP2ClB.

Unlike measurements of cytosolic Ca\(^{2+}\), Golgi intraorganelle Ca\(^{2+}\) cannot be measured accurately with Ca\(^{2+}\)-sensitive dyes. Therefore, we used a targeted aequorin approach similar to that used to study the Ca\(^{2+}\) concentrations in mitochondria (Rizzuto et al, 1992), nucleus (Brini et al, 1993), and ER (Montero et al, 1995). We transfected normal and HHD keratinocytes with a chimeric cDNA that encodes an HA1-tagged aequorin (Brini et al, 1995) and the transmembrane portion of sialyltransferase, which targets the aequorin chimera to the Golgi (Pinton et al, 1998). This tool, and its related aequorin mutant (Asp19→ Ala), which converts the aequorin to one with a lower Ca\(^{2+}\) affinity and thus a more long-lasting signal at micromolar Ca\(^{2+}\) concentrations, has been used previously to measure Golgi Ca\(^{2+}\) concentrations in normal and Bcl-2-transfected HeLa cells (Pinton et al, 1998; Pinton et al, 2000). Using this approach, we found that normal human keratinocytes also sequester substantial Ca\(^{2+}\) pools into the Golgi apparatus and that these stores are depleted in keratinocytes and epidermis after mutation of the ATP2C1. Further, we have localized the ATP2C1 protein to the Golgi apparatus in keratinocytes, and that these stores are depleted in keratinocytes and epidermis.

**MATERIALS AND METHODS**

**HHD patient selection** All patients studied were diagnosed with HHD before entry into the study, demonstrating typical clinical findings (blistering and erosions in characteristic locations) and histologic pathology (acantholysis of suprabasal cells without apoptosis). All patients signed consent forms in a study approved by the University of California, San Francisco, Committee on Human Research. Normal human keratinocytes, matched to age, location of skin biopsy (trunk), and pass number were used as controls. Cells from four patients were studied in this report. One patient had been analyzed in a previous report (Hu et al, 2000) and suffered from a nonsense mutation in exon 16, which predicts an abnormal, truncated protein. Cells from this patient were used for some of the aequorin Golgi Ca\(^{2+}\) measurements.

**Cell culture and transfection** Second-to-fifth passage cultured human keratinocytes from adult normal skin (surgical skin margins) (cultured adult human keratinocytes) or clinically normal HHD skin (punch biopsies) were grown on 60-mm dishes, chamberslides, or glass coverslips in 0.06 mM Ca\(^{2+}\) Eagle's medium (Cascade Biologics, Eugene, OR) until approximately 50% confluence for immunohistochemistry, 100% confluence for aequorin measurements, sucrose gradient studies, and Western immunoblotting. Twenty-four to forty-eight hours before aequorin measurements, 60% to 80% confluent keratinocytes were transfected with 1 µg of GoAEQmut DNA per 2 µl TransIT keratinocyte transfection reagent (PatVera Corp., Madison, WI) per 1 ml of medium. After overnight incubation, the cells were washed with phosphate-buffered saline and returned to the Epilife medium.

**Aequorin measurements** Transfected normal and HHD keratinocytes were depleted of their intracellular Ca\(^{2+}\) stores by incubation in Krebs-Ringer bicarbonate (KR) medium containing 125 mM NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 53 mM glucose, 1 µM ionomycin, and 500 µM EGTA. Cells were incubated for 1 h at 4°C, conditions previously shown not to change the morphology of the Golgi (Pinton et al, 1998). The coverslips were placed in close proximity to a low-noise photomultiplier, in a 4°C chamber, perfused first with KR/B medium containing 2% bovine serum albumin and 1 mM EGTA to wash the cells, then with KR/B plus 500 µM EGTA, and finally with KR/B plus 0.5 mM Ca\(^{2+}\). Calibration was performed at the end of each experiment by lysing the cells with a solution containing 100 µM digitonin and 10 mM CaCl\(_2\) in H\(_2\)O. The photomultiplier output was amplified by a built-in amplifier–discriminator. This signal was captured by a Thor–EMI photon counting board and stored in an IBM-compatible computer for further analysis. The resulting luminescence data were calibrated into Ca\(^{2+}\) values, using a computer algorithm (Brini et al, 1995). Cellular fractionation (sucrose gradient) Normal keratinocytes were cultured as above. The cells were homogenized using a Polytron homogenizer, the nuclei removed by 500 × g centrifugation, and crude membranes were collected by high-speed centrifugation at 70,000 × g. The resulting supernatant was layered on a discontinuous, multilayer sucrose gradient (15%–50%), centrifuged in a Beckman SW28 rotor at 100,000 × g for 16 h, and the membranous materials in the various sucrose layers collected for western blot analysis. Organellar and ATP2C1 localization was accomplished using antibodies to BiP (mouse monoclonal antibody 610978, BD Transduction Laboratories, San Diego, CA) as the ER marker, GM130 as the cis-Golgi marker (mouse monoclonal antibody 610882, BD Transduction Laboratories), p230 (mouse monoclonal antibody 611280, BD Transduction Laboratories), and TGN38 (mouse monoclonal antibody 610898, BD Transduction Laboratories) as the trans-Golgi markers and ATP2C1 (PMRI1, rabbit polyclonal IgG, sc-5548, Santa Cruz Biotechnology, Santa Cruz, CA) as well as ATP2A2 (SERC2A, sc-8094, Santa Cruz Biotechnology).

**Immunohistochemistry** Normal keratinocytes, cultured as described above, were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min. ATP2C1 was labeled using a rabbit polyclonal antibody to human ATP2C1 (sc-5848, Santa Cruz Biotechnology). The same cells also were labeled with the trans-Golgi membrane-associated protein p230 (mouse monoclonal antibody 611280, BD Transduction Laboratories) and TGN38 (mouse monoclonal antibody 610898, BD Transduction Laboratories) as the trans-Golgi markers and ATP2C1 (PMRI1, rabbit polyclonal IgG, sc-5548, Santa Cruz Biotechnology). Secondary antibodies were FITC–goat anti-rabbit (Jackson Immunoresearch, No. 111-096-046) and Texas red–donkey anti-mouse (Jackson Immunoresearch, No. 715–075–151). All primary antibody incubations were performed at 4°C overnight and washed three times. Second antibodies were used at 1:200 for 2 h at room temperature. Digital images were taken on a Zeiss 510 confocal microscope, using a 63 × lens. Negative controls omitting the primary antibodies demonstrated equal to background nonspecific staining.

**Western immunoblotting** Western immunoblotting was performed using 85% SDS–PAGE, as described previously (Laemmli, 1970). Following transfer of protein to PVDF membranes and incubation overnight with the primary ATP2C1 antibody at a 1:1000 dilution at 4°C (rabbit polyclonal IgG, sc-5548, Santa Cruz Biotechnology) and secondary antibody at room temperature (1:5000 for 2 h), membranes were incubated with alkaline phosphatase (Amersham Pharmacia Biotech Inc., Piscataway, NJ), final detection was performed by chemiluminiscence (Amersham). The cells were thawed and homogenized by sonication, and the protein contents were determined using the BCA kit (No. 23272, Pierce, Rockford, IL), and individual lanes were loaded with volumes containing 30 µg of sample for subsequent SDS–PAGE analysis. Equal loading per sample was controlled via individual protein content analysis and colloidal Coomassie brilliant blue staining (LC6025, Invitrogen Life Technologies, Carlsbad, CA) of the SDS gels following transfer to PVDF membranes. Additionally, densitometry was performed on the final chemiluminiscence images, using the Bio-Rad GS-710 scanner and Quantity One analysis software. Optical density values were first adjusted to average background density of the film, and normalization was achieved by replotting the same PVDF membrane with an anti-B-actin antibody (clone AC-74, Sigma, St. Louis, MO). Expression values were normalized to actin within same samples, and a percentage value for the difference between conditions was calculated. Molecular sizes were calculated by a regression analysis based on the prestained color standards routinely used for PAGE.

**Proton-induced X-ray emission** PIXE analysis was performed using a modification of Bunse et al (1991). Clinically normal trunk skin biopsies from normal and HHD patients were snap-frozen in liquid propane/liquid nitrogen. Thirty-micrometer sections were transferred to a metal-free nylon foil and freeze-dried for 12 h at −80°C. Data were obtained using 3-MeV proton beams, a 0.8-mA current, and a 5-µm spot diameter, with a scan size of 300 × 300 µm and binned into 10-µm segments for analysis. X-rays were detected with a Si(Li) detector, located at an angle of 135° with respect to the incident beam, that subtended a solid angle of ~100 msr. After PIXE analysis, the samples were counterstained with...
hematoxylin and eosin, and epidermal thickness was measured using a lens micrometer. Data were reduced off-line so that X-ray spectra from subregions could be extracted from each irradiated region. X-ray spectra were analyzed with the PIXE spectrum fitting code (Antolak and Bench, 1994). Thin-film calibration standards containing Ca\(^{2+}\) were used to measure the efficiency of the X-ray. Each sample was measured in three separate areas. Data are presented as the mean ± SD.

RESULTS

The ATP2C1 protein localizes to the Golgi in keratinocytes

The C. elegans ATP2C1 and its yeast homolog PMR1 both are found in the Golgi apparatus (Antebi and Fink, 1992; Van Baelen et al., 2001). Using an antibody raised to human ATP2C1 (PMR1, Santa Cruz Biotechnology Inc.), we localized the human ATP2C1 to the keratinocyte Golgi apparatus (Fig 1). Keratinocyte membranes were fractionated with a discontinuous sucrose gradient (15, 25, 35, 45, and 50% sucrose) and analyzed for colocalization of markers of subcellular organelles and ATP2C1. Markers of the ER (BiP) were found primarily in the lighter fractions (15% and 25% sucrose) as well as the cis-Golgi (GM130). The trans–Golgi markers (p230, TGN38) ran in the heaviest fractions (35%–50% sucrose). ATP2C1 was found in the trans–Golgi fractions. As expected, the ER calcium pump, ATP2A2 (SERCA2), was found in the ER fractions. ATP2C1 also was localized to the trans–Golgi using immunostaining, as the localization of the ATP2C1 and the trans–Golgi marker p-230 completely overlapped (Fig 1B). Although there was some overlap of the ATP2C1 and the cis–Golgi marker GM-130, the perinuclear localization of the ATP2C1 seemed to extend beyond the punctate staining of the GM-130 (Fig 1B). Thus, the ATP2C1 is localized to the trans–Golgi both by sucrose gradient and by immunohistochemistry techniques.

The ATP2C1 protein is decreased in HHD keratinocytes

Previous studies described decreased function of the ATP2C1 in HHD keratinocytes (Hu et al., 2000). To test whether impaired function resulted from a decrease in ATP2C1 protein, we compared levels of ATP2C1 protein in normal versus HHD keratinocytes. ATP2C1a is predicted to encode 919 amino acids (Hu et al., 2000), with a predicted weight of 117 kDa, whereas ATP2C1b is predicted to encode 888 amino acids (Hu et al., 2000), with a predicted weight of 113 kDa. ATP2C1 protein measured approximately 115 kDa (Fig 2A,B). Further, ATP2C1 protein was reduced markedly in HHD keratinocytes (Fig 2A,B). Because acantholysis in HHD is found in suprabasal layers (i.e., more differentiated keratinocytes), initial comparisons were performed on a total of six cell preparations from three patients, in cells grown in 1.2 mM Ca\(^{2+}\) (a typical example is shown in Fig 2A). Expression was decreased in all samples and averaged 53.5% of normal (± 19.6 SD, range 25.5%–78%, n = 6, p = 0.001, as assessed in a two-tailed, paired Student’s t test). Moreover, ATP2C1 expression was decreased in all samples and averaged 173 μM (range 95–232 μM, n = 8), a concentration slightly lower than measured in HLD cells (Pinton et al., 1998) and 1000-fold higher than found in cytoplasm.

Next, to test whether the ATP2C1 controls Golgi Ca\(^{2+}\) concentrations in normal versus HHD keratinocytes, Golgi Ca\(^{2+}\) stores were determined in normal and HHD keratinocytes (Fig 3a). HHD keratinocytes for these experiments were derived from three patients, and Golgi Ca\(^{2+}\) refill was variable (range 95–173 μM), probably reflecting differing degrees of ATP2C1 impairment among different subjects, as has been seen in attempts to correlate molecular and clinical findings in HHD patients (Dobson-Stone et al., 2002). These findings, coupled with previously described increased cytosolic Ca\(^{2+}\) in HHD keratinocytes (Hu et al., 2000), are best explained by a defective intracellular Golgi Ca\(^{2+}\) ATPase, leading to both decreased Golgi Ca\(^{2+}\) stores and increased cytosolic Ca\(^{2+}\).

The epidermal Ca\(^{2+}\) gradient is dissipated in HHD

Finally, to determine the location and proportion of ATP2C1-controlled Cytoplasm Ca\(^{2+}\) concentrations to total Ca\(^{2+}\) concentrations, we compared total Ca\(^{2+}\) concentrations in normal versus HHD keratinocytes and epidermis. We had found previously that total Ca\(^{2+}\) stores were decreased by ~40% in HHD cultured keratinocytes, and that total epidermal calcium, measured by PIXE, was decreased in HHD epidermis (Hu et al., 2000). To localize differences between normal and HHD keratinocytes within the epidermis, and to determine whether decreased skin Ca\(^{2+}\) concentrations were limited to the epidermis, we next used PIXE to compare Ca\(^{2+}\) in normal versus HHD skin. We found that total Ca\(^{2+}\) concentrations were decreased substantially in normal HHD epidermis, whereas the dermis (deeper than ~125 μm) showed no difference in Ca\(^{2+}\) concentrations between normal and HHD (Fig 4A). Further, we found that total epidermal Ca\(^{2+}\) did not differ in basal keratinocytes. This was surprising, because we found that undifferentiated keratinocytes in vitro display marked differences in intracellular Ca\(^{2+}\) stores (Hu et al., 2000; and see Fig 3). Because ATP2C1 controls an intracellular Ca\(^{2+}\) store, these data suggest either that intracellular Ca\(^{2+}\) stores represent a relatively small percentage of total epidermal Ca\(^{2+}\) in the basal layer or that other cellular mechanisms successfully compensate for the loss of ATP2C1 in basal layer but not spinous or granular layer keratinocytes. The epidermal Ca\(^{2+}\) gradient might also dissipate if the epidermal permeability barrier is damaged (Mauro et al., 1998). Nevertheless, the HHD biopsy was taken from clinically normal skin. More importantly, gradients in other ions that change with barrier impairment, such as K\(^{+}\) (Mauro et al., 1998), did not change in the HHD epidermis (Fig 4B), confirming that the
decrease in Ca$^{2+}$ seen in HHD epidermis is independent of changes in epidermal permeability barrier integrity.

These studies demonstrate that ATP2Cl-controlled Ca$^{2+}$ forms a significant store of Ca$^{2+}$ in keratinocytes and epidermis. PIXE studies further suggest that the ATP2Cl controls proportionally more Ca$^{2+}$ as keratinocytes differentiate in the upper epidermis and that active mechanisms, such as the ATP2Cl, participate in the formation of the epidermal calcium gradient (Menon et al., 1985).

**DISCUSSION**

HHD, or benign chronic familial pemphigus, is an autosomal dominant blistering skin disease caused by mutations in the ATP2Cl (Hu et al., 2000; Sudbrak et al., 2000). Although the central role of the PMR1 and Golgi calcium stores is well established in yeast (Rudolph et al., 1989; Antebi and Fink, 1992; Sorin et al., 1997; Durr et al., 1998), the finding that mutations of this Ca$^{2+}$ ATPase produced HHD was the first to demonstrate that it was

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**Figure 1.** (A) Localization of the ATP2Cl to the Golgi in subcellular membrane fractions and (B) the ATP2Cl localizes to the Golgi in normal human keratinocytes. (A) Normal keratinocytes were homogenized, the nuclear pellet removed by a low-speed spin, and the remaining membrane fractions were collected by a high-speed spin, resuspended, and layered over a discontinuous sucrose gradient (15%–50% sucrose) for separation by centrifugation. Each fraction was removed and analyzed for colocalization of markers of subcellular membranes (panels 1–4) with ATP2Cl (panel 5) or ATP2A2 (panel 6) by western blot analysis. CM refers to the crude membrane preparation layered onto the sucrose gradient. The Golgi calcium pump ATP2Cl (panel 5) is found mostly in the trans-Golgi fractions, whereas the ER calcium pump ATP2A2 is found in the ER fractions (panel 6). (B) Normal human keratinocytes were immunostained with the trans-Golgi membrane-associated protein p230 or the cis-Golgi matrix protein GM130 (top panels; both shown in red) or immunostained with the ATP2Cl antibody (middle panels; green). Colocalization also is shown by overlapping images (bottom panels) in which colocalization is denoted by yellow. Negative controls omitting the primary antibody demonstrated equal to background nonspecific staining (not shown). In images scanned in the standard (x–y) direction, colocalization in a perinuclear distribution is evident for ATP2Cl and p230, and a z-line scan through cells selected from this image reveals the Golgi-typical nuclear capping in a bipolar fashion. The cis-Golgi matrix protein GM130 localizes to a more restricted perinuclear position and does not completely overlap with ATP2Cl localization. Bar for x–y images, 20 μm; in z-line scan, 5 μm.
important in other organisms (Hu et al., 2000). In this study, we find that the human ATP2C1 not only localizes to the Golgi but also controls a significant Ca\(^{2+}\) store. In the original description of ATP2C1 mutations, more than half of the identified mutations predicted prematurely truncated products through frameshifts or a single-base-pair substitution (Hu et al., 2000), suggesting a haploinsufficiency pathogenesis. We find that protein levels of the ATP2C1 are decreased in HHD keratinocytes, consistent with a haploinsufficiency pathogenesis, although other mechanisms, such as defective processing of the ATP2C1 protein or dominant-negative mechanisms, also may play a role in decreasing ATP2C1 protein levels.

Figure 2. The ATP2C1 protein is decreased in HHD keratinocytes, regardless of the state of cell differentiation. Confluent normal human and HHD keratinocytes were probed for relative levels of ATP2C1 protein using the ATP2C1 antibody (Santa Cruz Biotechnology Inc.) and western blotting. (A) Keratinocytes taken from one patient (HHD) and a matched normal control (cultured adult human keratinocytes) were cultured in 1.2 mM Ca\(^{2+}\), a concentration that induces differentiation. Western blotting revealed decreased ATP2C1 expression in differentiated HHD keratinocytes, compared to normal controls. The size of the ATP2C1 protein did not change in HHD keratinocytes. The size of the human ATP2C1 protein in these blots was approximately 115 kDa. (B) Keratinocytes taken from a different patient (HHD) and a matched normal control (cultured adult human keratinocytes) were cultured in 0.06 mM Ca\(^{2+}\), a concentration that induces proliferation, or 1.2 mM Ca\(^{2+}\). Decreased expression of ATP2C1 protein was seen in both undifferentiated and differentiated HHD keratinocytes, compared to normal controls. An equal amount of protein was added to each lane (see Materials and Methods), confirmed by reprobing with an antibody to \(\beta\)-actin (C). Densitometry, normalized to \(\beta\)-actin, revealed reductions in ATP2C1 expression compared to normal human keratinocytes, as depicted in the bar graph (D). ATP2C1 expression is normalized at 100% for normal human keratinocytes, and ATP2C1 expression in HHD keratinocytes is expressed relative to normal human keratinocytes.

Figure 3. Golgi Ca\(^{2+}\) concentrations are decreased in HHD keratinocytes. Normal and HHD keratinocytes were plated on glass coverslips until 60%–80% confluent and then transfected with the specific Golgi aequorin probe GoAEQmut 24 to 48 h before experiments. Keratinocyte Ca\(^{2+}\) stores were depleted by treatment with ionomycin. The cells then were incubated with coelenterazine N, and Golgi Ca\(^{2+}\) stores were refilled by exposure to extracellular Ca\(^{2+}\) (see Materials and Methods). Normal keratinocytes (A) refilled more quickly and reached a higher baseline level than did HHD keratinocytes (B). Each tracing represents data from one experiment. Average values of eight experiments (normal keratinocytes) and nine experiments (HHD keratinocytes) are given under Results.
Figure 4. ATP2C1 controls a significant Ca\(^{2+}\) store in the epidermis.

A

B

normal keratinocytes, raised extracellular Ca\(^{2+}\) signals an increase in intracellular Ca\(^{2+}\) by a mechanism that requires both intracellular Ca\(^{2+}\) release (Tang and Ziboh, 1991; Pillai and Bikle, 1992; Oda et al, 2000) and Ca\(^{2+}\) influx (Kruszewski et al, 1991; Mauro et al, 1998; Csernoch et al, 2000). Increased Ca\(^{2+}\) stimulates actin reorganization and filopodia formation that, together with E-cadherin complexes, form an “adhesion zipper” (Vasioukhin et al, 2000). Both E-cadherin processing (Hakuno et al, 2000) and desmosome formation (Hashimoto et al, 1995) are disturbed in HHD.

Several pathologic mechanisms may link the observed defects in Golgi Ca\(^{2+}\) sequestration with the clinical findings of impaired keratinocyte cell-to-cell adhesion in HHD. HHD keratinocytes demonstrate abnormally high cytoplasmic Ca\(^{2+}\) and a decrease in the cytoplasmic Ca\(^{2+}\) response to stimuli (Hu et al, 2000), and these defects might induce changes in gene expression, or activate protein kinase C, thereby phosphorylating desmoplakin and disrupting desmosomes (Chakravarthy et al, 1995). Alternatively, Golgi intraorganelle Ca\(^{2+}\) depletion might impair post-translational modification of proteins essential for cell-to-cell adhesion (Amagai et al, 1996). Mutations in the yeast PMR1 Golgi Ca\(^{2+}\) ATPase lead to defective folding of proteins within the organelle (Durr et al, 1998), and yeast PMR1 with point mutations in the N-terminal region display both loss of Ca\(^{2+}\) affinity and inability of the PMR1 protein to exit the ER (Wei et al, 1999).

Finally, the human ATP2C1 is capable of high-affinity Mn\(^{2+}\) transport as well and in fact may be more selective for Mn\(^{2+}\) transport than is the yeast PMR1 (Ton et al, 2002). Defective Mn\(^{2+}\) transport, increasing cytoplasmic Mn\(^{2+}\) coupled with decreasing intra-Golgi Mn\(^{2+}\) could interfere with normal cell-to-cell adhesion by increasing Mn\(^{2+}\) toxicity (Ton et al, 2002).

Moreover, Golgi Ca\(^{2+}\) may be an important and previously unrecognized component of keratinocyte Ca\(^{2+}\) signaling. Intracellular Ca\(^{2+}\) release in response to raised extracellular Ca\(^{2+}\) is blunted in HHD keratinocytes (Hu et al, 2000), even though the HHD ER Ca\(^{2+}\) stores do not differ from those of normal keratinocytes (Hu et al, 2000). Decreased Ca\(^{2+}\) release in HHD keratinocytes is not due to decreases in the Ca\(^{2+}\) receptor, which is required for normal Ca\(^{2+}\) signaling in response to raised extracellular Ca\(^{2+}\) (Tu et al, 2001), because mRNA levels and alternative splicing of the plasma membrane Ca\(^{2+}\) receptor are normal in HHD keratinocytes (data not shown). These findings suggest that Golgi Ca\(^{2+}\) mobilization is essential to stimulate normal Ca\(^{2+}\) signaling in keratinocytes.

The identification of IP\(_3\)-sensitive Ca\(^{2+}\) stores controlled by the Golgi ATP2C1 in C. elegans supports this hypothesis (Van Baalen et al, 2001), as does the recent demonstration that a PMR1-controlled Golgi Ca\(^{2+}\) store produces Ca\(^{2+}\) oscillations in PMR-1 transfected COS-1 cells (Missiaen et al, 2001). Because extracellular Ca\(^{2+}\) signals essential processes such as keratinocyte differentiation (Hennings et al, 1983; Pillai et al, 1990; Presland et al, 1995), adhesion (Vasioukhin et al, 2000), motility (Fang et al, 1998), and lipid secretion (Lee et al, 1994), blunting cellular responses to extracellular Ca\(^{2+}\) may have profound effects on keratinocyte and epidermal biology. PIXE measurements of HHD epidermis localize the decrease in epidermal Ca\(^{2+}\) to the upper epidermis, whereas the lower epidermis and dermis do not show differences in total Ca\(^{2+}\). These results correlate well with the clinical, functional impairment seen in HHD, because the acantholysis that characterizes HHD occurs only in suprabasal cells, where the Ca\(^{2+}\) values begin to diverge. In addition, these data also suggest that active mechanisms, such as the ATP2C1, constitute much of the epidermal Ca\(^{2+}\) gradient, which is central to the development and repair of the epidermal permeability barrier (Menon et al, 1985; Mauro et al, 1998).

Mutations in the Golgi Ca\(^{2+}\) ATPase ATP2C1 cause acantholysis (HHD), whereas mutations in the ER, Ca\(^{2+}\) ATPase ATP2A2 cause both acantholysis and apoptosis (Darié’s disease), possibly mediated by stress responses caused by the accumulation of unfolded proteins within the ER (reviewed in Paschen 2001) or generation of the proapoptotic molecule caspase 12 (Nakagawa et al, 2000). This work was supported by NIH AR44341 (T.M.M.) and the San Francisco Veterans Affairs Hospital and was partially performed under the auspices of the U.S. Department of Energy at the University of California Lawrence Livermore National Laboratory, under Contract W-7405-Eng-48.

REFERENCES


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