Defective Epithelial Chloride Transport in a Gene-Targeted Mouse Model of Cystic Fibrosis

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The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes an adenosine 3′,5′-monophosphate (cyclic AMP)–activated chloride channel. In cystic fibrosis (CF) patients, loss of CFTR function because of a genetic mutation results in defective cyclic AMP–mediated chloride secretion across epithelia. Because of their potential role as an animal model for CF, mice with targeted disruption of the murine CFTR gene [CFTR(−/−)] were tested for abnormalities in epithelial chloride transport. In both freshly excised tissue from the intestine and in cultured epithelia from the proximal airways, the cyclic AMP–activated chloride secretory response was absent in CFTR(−/−) mice as compared to littermate controls. Thus, disruption of the murine CFTR gene results in the chloride transport abnormalities predicted from studies of human CF epithelia.

Cystic fibrosis is a potentially lethal genetic disease that affects the function of epithelial cells that line many organs. The clinical manifestations of the disease, which include mucus plugging of the airways and intestinal obstruction (meconium ileus) (*1, 2*), have been attributed to abnormalities in mucus and electrolyte transport that result in intraluminal accumulation of desiccated materials. In both the intestine (*2–10*) and the airways (*1, 11–14*), the pathologic changes characteristic of CF are associated with a generalized electrolyte transport defect in cyclic AMP–mediated activation of a cellular chloride (Cl^-) conductance. This abnormality is a direct consequence of mutations in a CF gene product, the cystic fibrosis transmembrane regulator (CFTR) protein, which appears to be a cyclic AMP–regulated Cl^- channel (*15, 16*).

A mouse model for CF has been generated by targeted disruption [CFTR(−/−)] of the mouse CFTR gene (*17*). We now have investigated the question of whether the epithelia from the CFTR(−/−) mouse model exhibit abnormalities similar to those of patients with CF (*4–14*). First, because intestinal obstruction is often an early clinical sign of CF in newborns (*1*), we studied electrolyte transport in several potentially affected regions of the CFTR(−/−) mouse intestinal tract. Second, because the respiratory system is invariably affected in CF patients (*1*), we investigated whether the CFTR(−/−) mouse expresses abnormal cyclic AMP–mediated Cl^- transport similar to that found in the best characterized regions of CF airways, the nasal (*11, 18–20*) and the tracheal epithelia (*12, 14*).

Individual littermate mice (2 to 3 weeks of age) with genotypes of either homozygous normal CFTR(+/+), heterozygous CFTR(+/−), or homozygous mutant CFTR(−/−), were used in a study of epithelial electrolyte transport (*21*). To investigate intestinal electrolyte transport, we mounted sections from three regions of the intestine (jejunum, cecum, and colon) in Ussing chambers (*22*). Basal ion transport rates [short-circuit current (I_{sc})] and I_{sc} responses to the cyclic AMP–mediated secretagogue forskolin (*23*) were measured. In jejunum, I_{sc} responses to glucose, a stimulus of Na^+-coupled glucose absorption, were also measured (*24–26*). The identity of the stimulated I_{sc} was determined by responses to specific inhibitors of Cl^- transport, bumetanide (*27*), and of Na^+-coupled glucose transport, phloridzin (*24–26*). Because differences in the basal I_{sc} were not apparent in this small sample size (*28*), the data for the CFTR(+/+) and CFTR(+/−) mice were combined into a "control" group for comparison to CFTR(−/−) mice. Typical recordings of jejunal and cecal preparations from a control and a CFTR(−/−) mouse are shown in Fig. 1 and summary data are shown in Fig. 2. The basal ion transport rate, which reflects a component of Cl^- secretion (*29, 30*), was routinely smaller in intestine removed from the CFTR(−/−) mice (Fig. 2A). The smaller basal ion transport rate parallels data reported for intestinal transport in CF patients (*4, 6, 10*). Sections from all three intestinal regions of the control animals responded to the addition of forskolin with a significant increase in I_{sc}, whereas sections of comparable intestinal regions from the CFTR(−/−) mice did not (Fig. 2B). The forskolin-induced change in the I_{sc} of the intestine was identified as a Cl^- secretory current by a series of inhibitor and ion substitution protocols (Fig. 2C). In brief, the forskolin-induced ΔI_{sc} was inhibited by the loop diuretic bumetanide, and also by the removal of Cl^- from the bathing media. Removal of sodium from the luminal solution did not diminish the I_{sc} response, indicating that a Na^+ absorptive current did not contribute to the forskolin response.

There were no gross morphologic changes of the CFTR(−/−) mouse intestine to account for the abnormal basal and forskolin-stimulated ion transport rates. Histological sections from the CFTR(−/−) mice used in this study revealed an intact epithelium in both villar (surface) and crypt regions (*17*, see figure 4B). In the jejunum, the bioelectric response to the addition of glucose to the luminal bathing solution, which induces Na^+-coupled glucose transport, was used as a functional test of the viability of tissues (*4, 25*). Responses of the tissues from control and CFTR(−/−) mice to the addition of glucose to the lumen were similar (Fig. 2D), indicating that the failure of CFTR(−/−) tissues to respond to forskolin was not a function of poor tissue viability. The reported magnitude of the I_{sc} response in the CF small intestine to glucose has varied. In some studies of human jejunal biopsies the reported rate of Na^+ linked glucose transport was higher in CF subjects (*26*), whereas in other studies, no differences were found (*4, 6*).

For investigation of airway epithelial electrolyte transport, cell culture approaches were employed to increase the mass of nasal and tracheal epithelia available from young mice for characterization as polarized epithelial monolayers (*31*). Earlier, extensive studies of primary cultures of human normal and CF airway epithelia have confirmed that the cyclic AMP–mediated defects in Cl^- transport are preserved in such culture preparations (*32, 33*). The transepithelial electric potential difference (V_t) was determined and we calculated short circuit current [equivalent short circuit current (I_{eq}) (*33*)] of nasal epithelial preparations from control (*34*) and CFTR(−/−) mice. These epithelial monolayers were bathed in either physiologic media (Fig. 3, A and B) or in low luminal Cl^- solution (Fig. 3, C and D), and the bioelectric responses to the sequential addition of amiloride (*35*), forskolin, adenosine triphosphate (ATP), and the anion transport blocker, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) were measured.

Forskolin stimulated the I_{eq} in both nasal and tracheal epithelia from control mice but not in CFTR(−/−) mice (Fig. 4A). The identity of the forskolin-stimulated current was studied in both nasal (Fig. 4B) and tracheal epithelia (*36*). As was found in the intestine, the forskolin-induced current was identified as a Cl^- current by inhibition with 10^{-4} M bumetanide (baso-

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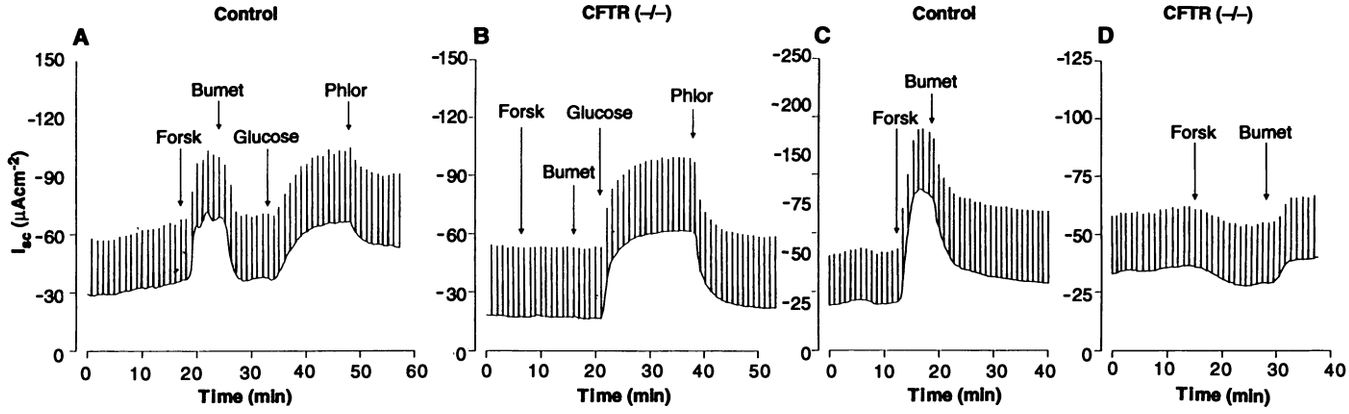

Fig. 1. Short-circuit current recordings from the jejunum and cecum of control and CFTR(−/−) mice. The current deflections in response to constant voltage pulses were used to calculate transepithelial resistance. In the jejunum, basal I_{sc} and the responses to sequential additions of 10^{-5} M forskolin (Forsk, bilateral), 10^{-4} M bumetanide (Bumet, basolateral), 5 mM glucose (luminal), and 2×10^{-4} M phloridzin (Phlor, luminal) are shown for (**A**) a control [CFTR(+/−)] mouse and (**B**) a CFTR(−/−) mouse. In the cecum, basal I_{sc} and the responses to forskolin and bumetanide are shown for (**C**) a control [CFTR(+/−)] mouse and (**D**) a CFTR(−/−) mouse. Intestinal tissues were mounted in Ussing chambers and bathed in standard Ringer solution (*22*).

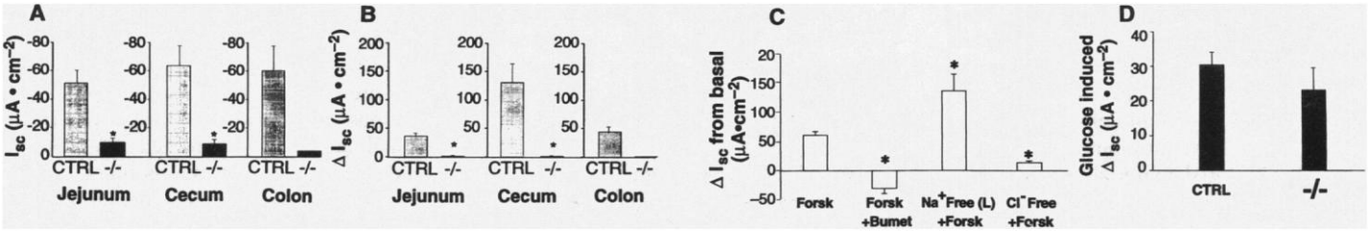

Fig. 2. Summary of I_{sc} studies from control (CTRL) and CFTR(−/−) mice. (**A**) The mean basal I_{sc} of jejunum, cecum, and proximal colon. (**B**) The mean ΔI_{sc} responses of these tissues to 10^{-5} M forskolin (bilateral). (**C**) The mean I_{sc} response to forskolin in jejunum bathed in a standard Ringer solution [Forsk]; inhibition of the Forsk-induced I_{sc} response by basolateral addition of 10^{-4} M bumetanide [Forsk + Bumet]; Forsk-induced I_{sc} response in jejunum after luminal solution Na^+ was replaced with *N*-methyl-D-glucamine [Na^+-free (L) + Forsk] and the Forsk-induced I_{sc} response of jejunum bathed in nominally Cl^--free (gluconate) Ringer [Cl^--free + Forsk]. The animals ($N = 7$) were 3-week-old wild-type (C57 Black 6) mice and not littermates to those used for studies in (A), (B), and (D). In (**D**), the glucose-stimulated I_{sc} responses of jejunal tissues from CTRL and CFTR(−/−) mice are shown. Bars for (A), (B), and (D) indicate the mean ± SEM of nine control and seven CFTR(−/−) mice for jejunum and cecum; and five control and two CFTR(−/−) mice for proximal colon. The individual values calculated for each mouse represent the mean data from three to four jejunal preparations and one or two cecal or proximal colonic preparations. The ΔI_{sc} was calculated by subtracting the stimulated I_{sc} from the I_{sc} measured immediately before the addition of forskolin or glucose. The asterisk indicates that $P < 0.05$ control versus CFTR(−/−), A and B (unpaired t test); $P < 0.05$ Forsk versus Bumet, Na^+-free (L), or Cl^--free, C (paired t test).

lateral solution) and by the removal of Cl^-, but not of luminal Na^+, from the bathing solutions. In the control animals, the magnitudes of the forskolin-induced Cl^- secretory responses in the airways are smaller than those in the gastrointestinal regions. This difference is parallel with the expression of CFTR mRNA, which was shown to be lower in the airways than in the gut (*17*).

Because of the relatively small response to forskolin in the airway preparations, we increased the sensitivity of our system to detect changes in the apical membrane Cl^- conductance by establishing a large outward chemical driving force for Cl^- secretion by removal of Cl^- from the luminal bath (*33*). As was found in the studies of Cl^--replete media, epithelial preparations from control mice responded to forskolin addition with an increase in outward Cl^- flow (ΔI_{eq}) from the cell, whereas airway epithelia from CFTR(−/−) mice did not (Fig. 4C).

Administration of extracellular triphosphate nucleotides (ATP and uridine triphosphate) activates Cl^- secretion by mechanisms other than cyclic AMP–dependent ones in both CF and normal airway epithelia (*37–39*). When CFTR(−/−) and control mouse airway epithelia were exposed to ATP, Cl^- secretion was activated to a similar degree (Fig. 4D). These data indicate that the failure of CFTR(−/−) epithelia to respond to forskolin is due to an absence of CFTR activity and not to the absence of other cellular processes required to generate Cl^- secretion. These findings also suggest that triphosphate nucleotides activate Cl^- secretion via a DIDS-sensitive (*39*) apical membrane Cl^- channel that is distinct from CFTR, a finding with potentially important therapeutic implications (*38*).

In our analyses of Cl^- transport, we detected a difference between murine and human airway epithelia. In human CF airway epithelia, amiloride inhibits the basal I_{eq} by 95%, whereas in normal subjects, the inhibition is approximately 75% (*11*). The greater efficacy of amiloride in inhibiting the basal I_{eq} in CF airway epithelia reflects, in part, the defect in the apical membrane Cl^- conductance, because Cl^- secretion in CF epithelia cannot be induced by the hyperpolarizing effect of amiloride on the apical membrane (*33*). We observed the same general pattern of response to amiloride in the CFTR(−/−) and control mice [amiloride inhibition of the basal I_{eq} in the airway epithelia from control mice was 52 ± 2% compared to CFTR(−/−) mice where it was 74 ± 5%, $P < 0.05$)]. However, the efficacy of amiloride inhibition of the basal I_{eq} was less in both control and CFTR(−/−) murine airway epithelial preparations than observed in humans. This difference was resolved by finding an amiloride-insensitive, electrogenic Na^+-glucose trans-

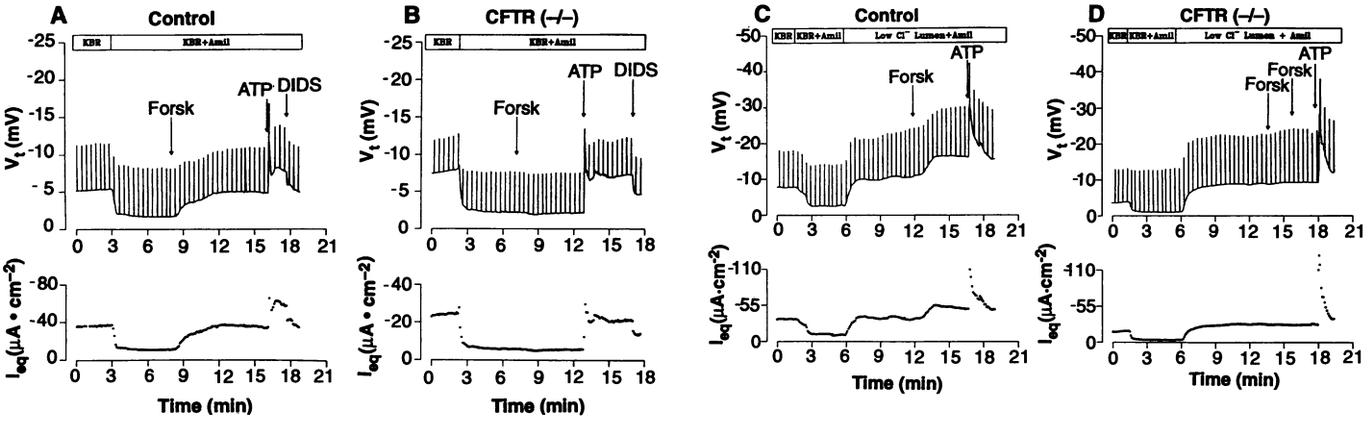

Fig. 3. Bioelectric characterization of cultured nasal epithelia from control and CFTR(−/−) mice. Each shows tracings of the spontaneous transepithelial electric potential difference (V_t) and voltage responses to constant current (1-3 μA) pulses (for the calculation of transepithelial resistance). The calculated equivalent short-circuit current (I_{eq}) is depicted below the V_t tracing. The bioelectric responses of nasal epithelial cultures bathed in a standard Ringer solution (KBR) to cumulative luminal additions of 10^{-4} M amiloride, 10^{-5} M forskolin, 10^{-4} M ATP, and 10^{-4} M DIDS are shown for a control [CFTR(+/−)] (**A**) and a CFTR(−/−) (**B**) mouse. In (**C**) and (**D**), the Cl^- concentration in the lumen was reduced to 4 mM before the addition of Forsk and ATP to nasal epithelial cultures from a control [CFTR(+/−)] and a CFTR(−/−) mouse, respectively.

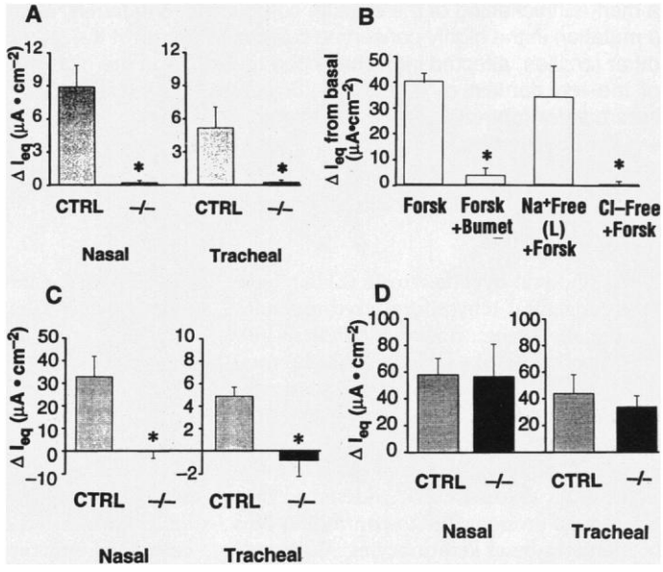

Fig. 4. Equivalent short-circuit current responses of cultured nasal and tracheal epithelia from control (CTRL) and CFTR(−/−) mice. (**A**) The mean I_{eq} response to 10^{-5} M forskolin (luminal) of nasal and tracheal epithelial cultures that had been treated with 10^{-4} M amiloride (luminal) and bathed in physiologic solution [Kreb's bicarbonate Ringer solution (KBR)]. (**B**) The change in I_{eq} with forskolin treatment [Forsk] and the sensitivity of the Forsk-induced I_{eq} response in nasal epithelial cultures to the addition of 10^{-4} M bumetanide (basolateral) [Bumet + Forsk], removal of Na^+ (replacement with *N*-methyl-D-glucamine) from the luminal bathing solution [Na^+-free(L) + Forsk] or removal of Cl^- (replacement with gluconate) from both bathing solutions [Cl^- free + Forsk]. The animals (N = 6) were 3-week-old wild-type (C57 Black 6) mice that were not littermates to the mice used in the studies in (A), (C), or (D). (**C**) The magnitude of the Forsk-induced I_{eq} responses of nasal and tracheal culture preparations bathed with a luminal solution of reduced (4 mM) Cl^- + amiloride for CTRL and CFTR(−/−) mice. (**D**) The mean I_{eq} responses to luminal addition of ATP are shown for nasal and tracheal epithelial cultures from control and CFTR(−/−) mice. Bars for (A), (C), and (D) indicate the mean ± SEM of eight control and 7 CFTR(−/−) mice for the nasal epithelium; and six control and four CFTR(−/−) mice for tracheal epithelium. The individual values for each mouse represent the mean data from one to four preparations for each airway region. The ΔI_{eq} was calculated by subtracting the agonist-stimulated I_{eq} from the basal I_{eq}. The asterisk indicates that $P < 0.05$, control versus CFTR(−/−) for (A) and (C) (unpaired t test); $P < 0.05$ Forsk versus Bumet, Na^+ free (L) or Cl^- free (paired t test).

port system (*40*) in the mouse airway epithelial preparations that is not a feature of human airway epithelial transport. Thus, the current after amiloride treatment in the normal mouse airway is likely a mixture of both an electrogenic Na^+-glucose absorptive current and a Cl^- secretory current. The relative magnitudes of the Na^+-substrate–linked transport systems, as well as the amiloride-sensitive Na^+ conductive pathway, in CFTR(−/−) and control mice, are unknown.

In summary, epithelia from three regions of the gastrointestinal tract and two regions of the airways of CFTR(−/−) mice exhibited abnormal cyclic AMP–mediated Cl^- transport similar to that observed in CF patients. This finding indicates that CFTR functions as a cyclic AMP–regulated Cl^- conductive pathway in murine epithelia, and that mice do not express an alternative cyclic AMP–mediated Cl^- conductance that might protect them from the effects of CFTR gene disruption. The presence of abnormal cyclic AMP–mediated Cl^- transport in CFTR(−/−) mouse epithelia suggests that this defect contributes to the pathological changes in the airways and gastrointestinal tract that resemble human CF disease (*17*). Together, these findings indicate that the CFTR(−/−) mouse will be highly relevant for the study of the relationship between abnormal ion transport and organ-level pathogenesis in CF, and for testing new therapies designed to correct CF epithelial electrolyte disturbances.

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21. The individuals doing this experiment (L.C., B.G., R.B.) did not know the genotype of the mice during electrolyte transport experiments on the gastrointestinal and airway preparations. All animals were correctly identified as either CF [CFTR(−/−)] (N = 7) or "non-CF" [CFTR(+/−), or CFTR(+/+)] (N = 9) on the basis of the I_{sc} response to the addition of forskolin.
22. Suckling mice (2 to 3 weeks old) from ten independent litters were studied. This group comprised CFTR(−/−): 10 ± 1 g, range 9 to 14 g; n = 7 (6 females, 1 male); CFTR(+/−) and CFTR(+/−): 11 ± 1 g, range 5 to 18 g, n = 9 (6 females, 3 males). Four to eight sections of gut (jejunum, cecum, proximal colon) from each mouse were mounted full thickness (unstripped) in Ussing chambers (aperture = 0.045 or 0.25 cm²). Tissues were bathed on the luminal and basolateral surfaces with oxygenated Krebs bicarbonate Ringer (KBR) buffer (*33*) containing 5 mM glucose, and bioelectric measurements were made under voltage clamp conditions [R. C. Boucher *et al.*, *J. Clin. Invest.* **78**, 1245 (1986)]. In jejunal preparations, glucose was replaced with 5 mM mannitol in the luminal KBR solution.
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35. To generate Cl^- secretory responses in airway epithelia in physiologic media, all preparations were first treated with the Na^+ channel blocker amiloride to abolish the predominant Na^+ absorptive current (*31*) and provide a favorable electrochemical driving force for Cl^- secretion (*33*).
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Mutations in the Rod Domains of Keratins 1 and 10 in Epidermolytic Hyperkeratosis

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Epidermolytic hyperkeratosis is a hereditary skin disorder characterized by blistering and a marked thickening of the stratum corneum. In one family, affected individuals exhibited a mutation in the highly conserved carboxyl terminal of the rod domain of keratin 1. In two other families, affected individuals had mutations in the highly conserved amino terminal of the rod domain of keratin 10. Structural analysis of these mutations predicts that heterodimer formation would be unaffected, although filament assembly and elongation would be severely compromised. These data imply that an intact keratin intermediate filament network is required for the maintenance of both cellular and tissue integrity.

Epidermolytic hyperkeratosis (EHK) (bullous congenital ichyosiform erythroderma) is clinically distinct from epidermolysis bullosa simplex (EBS) (*1*) and is characterized by an ichthyotic, rippled hyperkeratosis, reminiscent of corrugated cardboard, particularly around joints and folds (*2*, *3*). At birth and during early life, affected individuals exhibit erythroderma, widespread blistering, and erosions due to continuous lysis of the supra-basal keratinocytes. With age, the erythroderma resolves, blistering diminishes, and hyperkeratotic lesions become the predominant feature. Normal epidermis undergoes continuous renewal as keratinocytes move from the proliferative basal layer to the terminally differentiated squames of the stratum corneum. In patients affected with EHK this process is perturbed (*4*) and the normal epidermal barrier function is lost (*2*).

Expression of keratin proteins is specific for each epithelial cell type and its state of differentiation. Keratins consist of a conserved rod domain with four α-helical regions separated by short non-helical linker sequences and flanked by non-helical, globular sequences of varying size and composition (*5*). The smaller, acidic, type I keratins (keratins 9 through 19) are encoded on chromosome 17, and the larger, more basic, type II keratins (keratins 1 through 8) are encoded on chromosome 12 (*6*). One member from each type is required to form the coiled-coil structure of the heterodimer subunits, which then assemble into tonofilaments (*7*). Basal epidermal cells express keratins 5 and 14, but once cells commit to terminal differentiation and begin to migrate into the spinous layer, expression of keratins 5 and 14 is downregulated and expression of keratins 1 and 10 is induced (*8*).

EHK is characterized by a collapsed keratin filament network with clumping of the tonofilaments around the nucleus in the supra-basal spinous and granular cells (*9*, *10*). Although such collapse suggests a defect in a differentiation-specific keratin (*11*), other possible causes for EHK include abnormalities in filaggrin, a keratin filament-associated protein (*10*), involucrin, a cornified envelope precursor (*12*), or a defect in lysosomal metabolism (*13*). However, several families with EHK show linkage to chromosomal 12 near the gene locus for the type II keratins (*14*). We therefore

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